PRUDENT USE OF ANTIMICROBIALS ON DAIRY FARMS: ALTERNATIVE PROTOCOLS FOR THE TREATMENT OR PREVENTION OF MASTITIS AND ASSOCIATED OUTCOMES

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PRUDENT USE OF ANTIMICROBIALS ON DAIRY FARMS: ALTERNATIVE PROTOCOLS FOR THE TREATMENT OR PREVENTION OF MASTITIS AND ASSOCIATED OUTCOMES

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Implementation of protocols for prudent use of antimicrobials on dairies results in economic returns, sustainability of products, and addresses public health concerns such as resistance and residues. Two main areas of application are intramammary (IMM) treatment of clinical mastitis (CM) and dry-cow therapy, which account for 80% of antibiotic use on dairies. The objectives herein were to 1) evaluate if the antibiotic hetacillin fell within a margin of efficacy or "noninferiority" to the reference treatment, a critically important (to human medicine) third-generation cephalosporin, ceftiofur; 2) assess a protocol that selectively treats CM cases based on daily culture results; 3) determine if similar protocols can be effectively executed on moderate-sized dairies without daily access to a professional laboratory; 4) determine if the metagenomic profiles of CM cases highlight a missed opportunity for non-treated cases; and 5) evaluate a culture-independent selective dry-cow therapy (SDCT) protocol. Noninferiority of hetacillin was concluded for bacteriological and clinical cures, as well as for many secondary outcomes. Administration of hetacillin over ceftiofur decreased treatment pen time with no statistical differences in bacteriological, pathogen, or clinical cures; milk yield and linear score (LS) postevent; nor additional risk of culling or quarter-level mastitis after the event. Use of a selective pathogen-based protocol decreased milk withholding time with no statistical differences in days to clinical cure, milk yield, and LS postevent; nor additional odds of culling in the months following. When similar strategies were applied on 8 moderate-sized dairies using on-

farm or 5 day/week diagnostics, irregularity of data recording was drastically improved and no negative changes were reported. Reductions in antimicrobial use ranged from 6.8% to 92.5% and economic savings amounted to $2,200 to $4,600/dairy/yr. 16S rRNA analysis validated that despite dysbacteriosis in the mammary gland, there was no pathogen profile that was associated with negative or positive health and production outcomes; missed opportunities were not apparent. Finally, application of a data-driven, SDCT protocol resulted in no statistical differences in new infection risk, milk production and LS, or additional odds of culling or mastitis over the periparturient period between selectively or blanket-treated low-risk cows. Using SDCT or pathogen-based protocols for the treatment and prevention of CM can reduce antibiotic use by up to 2/3.

BIOGRAPHICAL SKETCH

Amy Kristin Vasquez (nee Rauf) was born and raised in Washington Township, New Jersey. Here she encountered smells of pig, corn, and blueberry farms and often rode members of the equine species, but never came upon cows. Most of her time was spent with family in travels around the US and tropical islands. Small animals were always in attendance.

In the spring of 2001, after surviving Y2K, Amy graduated high-school and matriculated to Cornell University, where her love of Ithaca would begin. As her parents were tired of spending vast amounts of money on an Ivy-league tuition, Amy was encouraged to pay for her own living expenses and she responsibly found employment in the Overton laboratory washing the dishes used in their many experiments. Eventually she was entrusted with retrieving samples from the dairy cattle at the Cornell Teaching and Research Center for several of the research projects, and found her calling. Amy began taking all of the necessary classes to apply to veterinary school. After completing an honors thesis on insulin responses in transition dairy cattle working under Dr. Tom Overton, she graduated *magna cum laude* with double majors in biology and animal science in 2005.

From fall of 2005 to spring of 2009 Amy attended Cornell Veterinary School. During this time she pursued and took classes related to her interest, production medicine. Simultaneously she continued contributing to research by working with Dr. Daryl Nydam on cryptosporidiosis in dairy calves and with Dr. Rodrigo Bicalho on metritis in adult dairy cattle.

In 2009 Amy decided she was finished with Ithaca winters and moved to the northern central valley in California after accepting a position at a mixed animal practice as an associate veterinarian. The veterinarians and mentors she worked with then established a dairy-focused practice in 2010 and she was more than happy to spend all of her days outside. It was during this time that Amy met and married her husband, Joseph Vasquez, a California-born geologist with a love for dogs, rocks, and cacti. In addition to her love for her husband, Amy developed a love for road cycling. Throughout her 5 years spent in California, Amy began to miss her time performing research and found it difficult to make a difference on the dairies that she serviced.

Afternoons spent consulting with clients on mastitis outbreaks, on-farm culture trainings, and discussing milk quality laboratory results inspired her to seek alternative opportunities where she could obtain more knowledge on mastitis pathogens.

It was at this time that she spoke with Dr. Daryl Nydam, the director of the Quality Milk Production Services at Cornell, and a previous mentor of Amy's. As with most of his graduate students, a strong suggestion of completing a PhD was what it took to bring Amy back to the Cornell College of Veterinary Medicine in the summer of 2014.

Amy returned to Ithaca to pursue a degree in Comparative Biomedical Sciences within the Biological and Biomedical Sciences program. Working under Dr. Nydam, Amy was fortunate enough to hone in on surgical skills working for a high volume high quality spay neuter company. She also spent time working at small animal vaccine clinics and provided sabbatical coverage for an ambulatory colleague by working clinics once per week for the last 6 months of her studies. During her time as a PhD student, Amy and Joe welcomed their first child on July 14, 2016, Victor Louis. Amy will defend her thesis in early May 2018 and then remain in her favorite town to continue her contributions to mastitis research as a research associate working under Daryl Nydam and also working closely with Dairy Health Management Services.

To Joe and Lynn who embraced my need for change and who whole-heartedly supported, encouraged, and celebrated this dream and every small accomplishment along the way, and to Tucker, whose unconditional love guided me through almost all of my life challenges thus far.

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I am thankful for the collaborative projects developed by the Bicalho and Van de Walle labs, where I learned skills that I had never performed and pursued topics I would never have followed. I am appreciative of these members of my graduate committee (Gerlinde and Rodrigo) for their mentorship in addition to the wonderful friendships and guidance provided by their laboratory members: Dr. Erika Ganda, Dr. Carla Foditsch, Rebecca Harman, Dr. Gat Rauner, Melissa Ledet, and Matthew Pennington. I am so glad to have asked Dr. Renata Ivanek to be a part of my committee, and I would like to thank her for offering a different prospective and encouraging me to take classes or participate in opportunities that would prepare me for a breadth of careers in the field of epidemiology.

I would like to thank my office mates, mentors, and friends, Dr. Kathryn Bach, Dr. Matthias Wieland, Dr. Sabine Mann, Dr. Vinicius Machado, Dr. Jessica McArt, Dr. Francisco Leal, Dr. Claire Zoellner, Dr. Anja Sipka and Dr. Andre Teixeira, who always offered helpful bits (and sometimes expanses) of information, listened to my complaints, and shared an adult beverage, when needed.

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My very first project would not have run so smoothly without the help and attention to detail provided by Dr. Michael Capel, Dr. Mark Thomas, Dr. Bob Ceglowski, and Lauren Pitman. I am grateful for the great knowledge and skills provided by Dr. Steve Eicker and hope that we can continue traversing the Fingerlakes region on our bikes. Finally, I would like to give a big thank you to the dairy producers and managers, who although we complicated their routines, were excited to be a part of my projects and always asked about my family and my journey. The information you have provided has contributed so much to my learning as well as a positive impact for the dairy community.

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LIST OF ABBREVIATIONS

ABXTS	Antibiotic and teat sealant-treated cattle
ANCOM	Analysis of the composition of microbiome
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
BDCT	Blanket dry-cow therapy
BTSCC	Bulk tank somatic cell count
CI	Confidence Interval
CM	Clinical mastitis
CMT	California mastitis test
CNS	Coagulase-negative Staphylococci
CS	Clinical Score
DC305	Dairy Comp 305
DDD	Defined daily dose
DHIA	Dairy Herd Information Association
DIM	Days in milk
DNA	Deoxyribonucleic acid
dPB	Delayed pathogen based
FDA	Food and Drug Administration
IMI	Intramammary infection
IMM	Intramammary
LPS	Lipopolysaccharide
LS	Linear score
LSM	least squares mean
MALDI-TOF	Matrix assisted laser desorption/ionization -time of flight
MLD	Milk leukocyte differential
NAG-ase	N-acetyl beta-glucosamidase
NMC	National Mastitis Conference
NPV	Negative predictive value
OFC	On farm culture
OR	Odds ratio
OUT	Operational taxonomic unit
PAI	Propionibacterium acnes injection
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
PPV	Positive predictive value
QIIME	Quantitative insights into microbial ecology
QMPS	Quality Milk Production Services
rRNA	Ribosomal ribonucleic acid

SCC	Somatic cell count
SD	Standard deviation
SDCT	Selective dry cow therapy
SE	Standard error
TS	Teat sealant only cows

LIST OF SYMBOLS

Δ	Margin of noninferiority

CHAPTER ONE

LITERATURE REVIEW: DEFINITION AND DIAGNOSIS OF CLINICAL MASTITIS IN DAIRY CATTLE AND CONSIDERATIONS FOR IMPLEMENTATION OF PROTOCOLS THAT PROMOTE PRUDENT USE OF ANTIMICROBIALS FOR TREATMENT AND PREVENTION

A portion of this literature review was published[*] in the proceedings of the annual meeting of the National Mastitis Conference, 2017

[*] Nydam, D., and A. Vasquez. 2017. Selective dry cow therapy: what are the possibilities for North America? Pages 103–113 in Proc. 57th Annu. Mtg. Natl. Mastitis Counc., Tucson, AZ. National Mastitis Council, Madison, WI.

INTRODUCTION

Mastitis is a major cause of economic loss in the dairy industry due to reduced milk yields, increased culling, discarded milk, and decreased fertility (Seegers et al., 2003; Halasa et al., 2007). It is also a cattle welfare concern (Byrd et al., 2017). These reasons make the disease a main focus on dairies for treatment and control measures. In the US, approximately 1.5 million mastitis cases are treated annually (USDA-APHIS, 2008). The following literature review will discuss the epidemiology, treatment, prevention, and economics of clinical mastitis (CM) during lactation and over the dry period. It will highlight recent studies that have shown successful implementation of protocols that promote the prudent use of antimicrobials. Finally, it will outline the need to develop easy and quick methods for determining which cows would benefit most from the administration of antibiotics.

CLINICAL MASTITIS IN LACTATING DAIRY CATTLE

Clinical mastitis is defined as inflammation or infection of the mammary gland and is detected as abnormal consistency or appearance of milk, swelling or redness of the affected quarter(s), and less often as systemic signs such as fever, depression, weakness, and inappetence. The disease is often recorded as mild, moderate, or severe corresponding to, and graduating from, the presence of minimal local signs such as abnormal milk, to signs of udder inflammation, to systemic illness. No one-to-one relationship between clinical severity and causative agent has been established (Klaas and Zadoks, 2017). While Watts (1988) identified 137 contributing organisms including yeasts and algae, CM is caused by a bacterial pathogen more than 90% of the time (Watts, 1988; Olde Riekerink et al., 2008). Traditionally, pathogens are classified as "environmental," those that live in the cow's environment, or "contagious," those that are host-

adapted and transmitted primarily during milking. The most common environmental pathogens are *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., *Streptococcus uberis, Streptococcus dysgalactiae,* and Coagulase-negative *Staphylococcus* spp. (CNS). The most common contagious CM pathogens are *Streptococcus agalactiae, Staphylococcus aureus,* and *Mycoplasma* spp. However, categorization should be based on the mechanistic understanding of pathogen survival and pathogenesis and should be applied at the strain level, as recent research has documented contagious or persistent characteristics of environmental pathogens, such as with *Strep. uberis, Klebsiella,* and *E. coli*, and environmental origins of contagious pathogens such as *Strep. agalactiae* (Bradley and Green, 2001; Zadoks et al., 2003; Munoz et al., 2007; Zadoks et al., 2011; Klaas and Zadoks, 2017).

Mastitis is a commonly encountered disease on North American dairy farms with lactational risks ranging from 20.4% to 51%, dependent upon region, method of data collection, and farms represented by the study or survey performed (Erskine et al., 1988; Olde Riekerink et al., 2008). The USDA-NAHMS survey (2014) indicated that of the 3,500 dairy producers surveyed, 99.7% reported having at least one case of CM during the previous year (USDA, 2016a).

CLINICAL DIAGNOSIS OF MASTITIS

In addition to using clinical signs, there are several methods available for diagnosis of clinical or subclinical mastitis cases; fewer methods are available for etiologic diagnosis. Somatic cell count (**SCC**), or the total number of white blood cells and epithelial cells in milk, becomes elevated during acute or chronic inflammation and has been used as a proxy for the presence of an intramammary infection (IMI) (Schukken et al., 2003). Due to its documented

optimal sensitivity and specificity (73% and 86%, respectively), a cutoff for SCC of 200,000 cells/mL has been used as a diagnostic indicator for the presence of an IMI (Dohoo and Leslie, 1991). Evaluation of SCC can be performed in a diagnostic laboratory using high capacity, accurate cell counters such as the Fossomatic cell counter, or on farm (Miller et al., 1986). A semi-quantitative cow-side tool for measuring SCC is the California Mastitis Test (CMT) which uses a reagent to disrupt cells and form a congealed mass when SCC is elevated. Currently, quantitative on-farm SCC counters are also available. The sensitivities and specificities of these tools are varied, but also depend on factors such as stage of lactation, time of day, or when during the milking procedures the sample was retrieved (Luedecke et al., 1967; Sargeant et al., 2001; Sarikaya and Bruckmaier, 2006). Due to its increasing presence in automatic milking systems, electrical conductivity measurement is another method of CM detection that is recently receiving attention. Conductivity measurements at tested cutoffs have specificities >85%, but have varying sensitivities (66%-85%) that are dependent upon pathogen prevalence in an individual herd (Nielen et al., 1992; Fosgate et al., 2013). It must also be noted that CM is not the only circumstance that causes the ionic content of milk to change, which is a drawback of this method. Measurement of N-acetyl-beta-D-glucosaminidase (NAG-ase) has been shown to be an accurate indicator of subclinical and CM but is also sensitive to the stage of lactation of the animal (Hovinen et al., 2016). Finally, lactate dehydrogenase (LDH) is an enzyme with increased activity during mastitis. Use of LDH measurements had high sensitivities and specificities for detection of CM when used in a model (Chagunda et al., 2006). Additionally, when combined with SCC in a field study, researchers were able to differentiate between gram-positive and gram-negative pathogens (Hernandez-Castellano et al., 2017). Practical application of LDH or NAG-ase in the dairy industry for detecting IMIs has not been implemented.

The current gold-standard for etiologic diagnosis of CM is aerobic culture, which can be performed by diagnostic laboratories, veterinary clinics, or by dairy personnel. Most often, non-selective media and ancillary tests such as potassium hydroxide, catalase, and motility tests are used to characterize and identify CM pathogens. However, culture systems such as the selective Minnesota tri- and bi-plates (University of Minnesota, St. Paul, MN), 3M Petrifilm (3M, St. Paul, MN), and Accumast colorimetric media (FERA Animal Health, Ithaca, NY) have been developed for on-farm diagnostics and some have been tested for successful use with minimal training and guidance (McCarron et al., 2009; Royster et al., 2014; Ganda et al., 2016b). Currently, 57.0% of dairy operations culture milk; 47.2% of operations use commercial and private veterinary labs, and 7.9% use on-farm culture (USDA, 2016a). Culture methods in diagnostic laboratories are often supplemented using proteomic-based testing such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis (MALDI-TOF) (Schabauer et al., 2014). Molecular methods such as polymerase chain reaction (PCR) and sequencing methods such as 16S rRNA or shotgun have been used to define the causative pathogen and to describe the milk microbiome in laboratory situations (Koskinen et al., 2009; Oultram et al., 2017). PCR technology as an on-farm method is currently under development.

EPIDEMIOLOGY OF MASTITIS PATHOGENS

The etiologic distribution of CM pathogens can be diverse, even within the dairy-dense regions of North America, and the current distribution is vastly different from the relative prevalence of pathogens 30 years ago. In the 1980s, the most commonly cultured bacterial isolates were the contagious pathogens *Staph. aureus* and *Strep. agalactiae* (Oliver and Mitchell, 1984; González et al., 1988). The last 30 years' efforts of controlling contagious CM pathogens,

including the advent and use of FDA-approved intramammary (IMM) antimicrobials as well as improvements in parlor procedures have been successful; the predominant pathogens are currently environmental or "opportunistic" and include *E. coli, Streptococcus* spp., *Klebsiella* spp., *Enterobacter* spp., CNS and yeast. The most recent large-scale study characterizing CM was performed by Oliveira et al. (2013) on 50 large dairy herds in Wisconsin. Approximately 1/3 of CM cultures (35.6%) represented gram-negative pathogens such as *E. coli* (22.5%), *Klebsiella* spp. (6.9%), and *Enterobacter* spp. (3.6%). Another 1/3 of cultures were gram-positive pathogens (27.5%) such as environmental streptococci (12.8%), CNS (6.1%), *Enterococcus* spp. (2.0%), and *S. aureus* (2.8%). The final 1/3 of cultures were negative for pathogen growth (27.3%). Yeast, a non-bacterial pathogen, was present in 3.1% of samples. This compares with a Canadian study that included 115 dairy farms (n = 3,033 cultures) where they described 8.4% of cases diagnosed as *E. coli*, 4.3% *Klebsiella* spp., 12.3% environmental streptococci, 5.1% CNS, 2.2% *Enterococcus* spp., and 10.3% *Staph. aureus* (Olde Riekerink et al., 2008). Almost half (43.9%) of the submissions in the Olde Riekerink study were culture-negative. Absent among the listed pathogens are *Mycoplasma* species. *Mycoplasma* spp. are a contagious group of pathogens which, due to unresponsiveness of cases to therapy, are uncommonly included or characterized in mastitis research trials. However, these can be important organisms on dairies; mycoplasma mastitis varies in its prevalence from 2% to 20%, dependent on herd size and geographic location (Fox, 2012).

FACTORS INFLUENCING TREATMENT DECISIONS

Preventative Practices

The consideration of factors such as improved management and housing conditions, use of teat disinfectants, routine maintenance of milking equipment, testing of all new animal introductions, and culling and segregation of contagious mastitis cases can aid in prevention and control of new CM cases (Dodd, 1983; Bramley and Dodd, 1984). Internal teat sealants during the dry period can also effectively reduce incidence of new IMI (Rabiee and Lean, 2013). Finally, several mastitis vaccines have shown success for the reduction of clinical signs or prevalence of mastitis pathogens. The *E. coli* J5 core vaccination can reduce the severity of CM and reduce yield losses if used as labeled (Bradley et al., 2015). This differs from the efficacy of an SRP-technology based *Klebsiella* vaccine, which showed statistically significant protection from *Klebsiella* mastitis in addition to decreases in SCC and increases in milk production for cows that were vaccinated (Gorden et al., 2017).

Cow Level Factors

Cure rates for mastitis pathogens and associated outcomes can be influenced by cow parity, SCC, duration of infection, energy balance, vaccination status, bacterial colony counts in milk, number of quarters infected, and quarter location (Suriyasathaporn et al., 2000; Burvenich et al., 2003; Barkema et al., 2006; Bradley et al., 2015). For example, *Staph. aureus* cure rates decrease with increasing age, increasing SCC, increasing duration of infection, increasing bacterial colony counts, increasing number of quarters and with hind quarter location (Barkema et al., 2006). The probability of cure in animals experiencing *Strep. uberis* mastitis was higher among 1st and 2nd parity animals compared with older cows, and in animals with a single

elevated SCC prior to diagnosis as compared with those with multiple high SCC (Samson et al., 2016). Additionally, those animals with no elevation of SCC before diagnosis were more likely to cure. Because these many factors contribute to cure rates, many should be accounted for when making individual cow treatment decisions.

ANTIBIOTIC USAGE ON DAIRIES

Antibiotic treatment of CM in dairy cattle is an important component of mastitis control programs. In fact, CM has been recognized as the most frequent reason for antimicrobial use on dairy facilities, with the percentage of cows affected and treated reported as 18.2% and 16.4%, respectively (USDA, 2008). Pol and Ruegg (2007) used data from 20 conventional farms in Wisconsin to quantify overall exposure to antimicrobial drugs: 5.43 defined daily doses (**DDD**; the maximum dose that a 680 kg animal would receive if treated with the on-label dose) per year per cow was quantified for mastitis treatments. This compares to foot infections at 0.39 DDD, respiratory infections at 0.14 DDD, and 0.32 DDD for uterine infections. Of the 5.43 DDD attributed to mastitis treatments, 3.58 DDD was for IMM use and 1.85 DDD was given parenterally. Intramammary treatment was further divided into CM treatments (2.02 DDD) and dry cow therapy (1.56 DDD) (Pol and Ruegg, 2007a). Drug use is similar in Canada; a study on 89 farms measured and calculated the overall antimicrobial drug use rate, or the number of animal defined-daily doses per 1,000 cow-days. For IMM treatment of CM, the antimicrobial drug use rate was 3.52 animal defined-daily doses per 1,000 cow-days, with penicillin having the highest rate at 2.20 per 1,000 cow-days (Saini et al., 2012). Antimicrobial drug use rate was not found to be related with BTSCC, herd size, barn type, or geographical region and was positive but weakly correlated with herd level milk production in the study. Finally, a study that used

empty drug containers rather than treatment records to estimate drug use on Canadian dairy farms found that treatment records vastly underestimated drug use; the use for mastitis products was an average daily dose per 100 cow-years of 6,084 (Nobrega et al., 2017).

Concerns of Antibiotic Usage

There is increased public awareness and scientific interest regarding the administration of therapeutic doses or preventative doses of antimicrobials to animals, some of which include classes critical to human medicine. For example, 3rd and 4th generation cephalosporins, which include the very commonly used IMM ceftiofur product, are on the World Health Organizations list of critically important antimicrobials for human medicine (WHO, 2017). Concerns include the development of antimicrobial resistance and the sequelae: zoonotic potential of resistant pathogens and loss of effectiveness of antibiotics for their intended use(s). Several groups have explored the relationships between antimicrobial drug use for the treatment of CM and the development of resistance in North America. In a study of gram-positive pathogens by Pol and Ruegg (2007), a dose-response relationship between exposure to antimicrobials and bacterial susceptibility was observed for some pathogen-antimicrobial combinations (pirlimycin, penicillin, ampicillin, erythromycin, and tetracycline), but other antimicrobials commonly used as IMM preparations for CM (ceftiofur, cephapirin) were not associated with resistance (Pol and Ruegg, 2007b). However, this was a cross sectional study; longitudinal studies have assessed the development of antimicrobial resistance over 6, 7 or 9 years and have found no associations with common use (Erskine et al., 2002; Makovec and Ruegg, 2003; Lindeman et al., 2013). Despite lack of findings for isolates found in diagnostic settings, molecular evidence indicates that some methicillin resistant *Staph. aureus* found in humans may have arisen in cattle (Holmes and

Zadoks, 2011) and extended-spectrum beta-lactamase producing coliforms in mastitic milk have been linked to the use of cephalosporins in the UK and the Netherlands (Gonggrijp et al., 2016). Another public health concern is the presence of antibiotic residues in animal products. The frequency of IMM antibiotic treatments is directly associated with increased risk of antibiotic residues in milk (McEwen et al., 1991). Taking into account concern for overuse, antimicrobials still serve a crucial need for decreasing mortality and morbidity and when used appropriately in agricultural systems they have profound effects on animal health and productivity (Johnston, 1998).

Intramammary Antimicrobial Availability and Efficacy

Few drug classes are approved and currently available in IMM formulations for use in lactating cattle with CM. These classes include the β-lactams that target bacterial peptidoglycan cell walls such as penicillin, ceftiofur, cephapirin, amoxicillin, hetacillin, and cloxacillin; and the lincosamide pirlimycin which interferes with bacterial protein synthesis (US-FDA, 2017). Label indications are similar for each drug formulation, with all achieving approval for at least one staphylococcal organism and one streptococcal organism. The hetacillin and ceftiofur-based products are the only IMM antimicrobials that hold label claims for efficacy against a gram-negative organism (*E. coli*). However, labels claims do not necessarily indicate that efficacy is not achieved against additional non-listed organisms. Research groups have performed field trials to determine if treatment strategies for CM are more effective than others by comparing several different outcomes between treatment groups. These can include subjective measures such as time until clinical cure or objective measures such as whether or not the quarter experienced a microbiological cure. The latter can also be somewhat subjective as the definition

of microbiological cure is dependent on the method used to diagnose and the number of days between sample retrieval. Outcomes that are more visible to the producer and more pertinent to farm economics include post-mastitis milk production and SCC (or linear score; **LS**), number of days that milk is withheld, recurrence of mastitis in the same quarter, and retention in the herd (Lago et al., 2011a; b; Pinzón-Sánchez and Ruegg, 2011). Well-designed field trials can assess the efficacy of IMM antimicrobials for CM. However, most published studies lack an untreated control group and either do not evaluate cure at the microbial species level or do not have a large enough sample size to make claims regarding species-level success as compared with untreated controls. The following sections evaluate the findings of field studies regarding the IMM treatment of specific pathogens or pathogen groups.

PATHOGEN CHARACTERISTICS AND RESPONSE TO ANTIMICROBIALS

Gram-Negative Mastitis Pathogens

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is the virulence factor that is responsible for the pathophysiologic release of inflammatory mediators and subsequent clinical signs detected in gram-negative CM cases (Burvenich et al., 2003). For coliforms such as *E. coli*, current recommendations include the treatment of the sequelae of infection, rather than the infection itself as the presence of bacteria is usually transient and has high spontaneous cure rates. Duration of infection for *E.coli* is less than 10 days (Leininger et al., 2003), with peak bacterial numbers and subsequent rapid drops in counts occurring in less than 24 h, often prior to clinical recognition of the disease (Hill et al., 1979). As spontaneous cure is also high, ranging from 74% to 86.8%, scientists and experts in the industry question whether there is an advantage to antibacterial therapy (Pyörälä and Pyörälä, 1998; Suojala et al., 2010).

There are few peer-reviewed papers with a non-treated control group that evaluate bacteriological cure differences between *E. coli*-positive quarters treated with FDA-approved IMM antimicrobials and those that are not. One such study was performed by Schukken et al. (2011). The group found that a 5 day IMM treatment with ceftiofur resulted in a statistical difference in bacteriological cure for the 48 cows included in the analysis (89% cure in treated animals versus 53% in control animals)(Schukken et al., 2011). However, cows in either group missing follow-up samples were classified as treatment failures, resulting in some bias. Bacteriological and clinical cure did not differ between treatment groups when comparing amoxicillin, cephapirin, or oxytocin (no antibiotics) for CM caused by coliforms in a trial by Guterbock et al. (1993). Similarly, a comparison of IMM amoxicillin, frequent milkout, IMM amoxicillin and frequent milkout, and no treatment by Roberson et al. (2004) showed little effect of treatment on clinical cures, bacteriologic cures, and milk production for *E.coli* positive cows (Roberson et al., 2004). In a longitudinal study by Ganda et al. (2016), natural *E.coli* IMIs receiving treatment with ceftiofur were not different in respect to clinical cure, pathogen clearance rate, or bacterial load when compared to untreated controls. Additionally, the milk microbiome of mastitic quarters was reflective of adjacent healthy quarters within 14 days post-mastitis regardless of treatment group (Ganda et al., 2016a). A challenge trial by the same group (n=12 cows) showed a significant decrease in log of bacterial CFUs in the ceftiofur treated cows but no effect on milk microbiome, rate of pathogen clearance, or SCC (Ganda et al., 2017). These many findings indicate that treatment of *E. coli* mastitis with antimicrobials may not be prudent.

Duration of *Klebsiella* IMI is longer than for *E. coli*, averaging 21 days, and quarters infected with *Klebsiella* have a low chance of spontaneous cure, ranging from 19-37% (Smith et

al., 1985; Roberson et al., 2004; Schukken et al., 2011). Milk production losses are highest for this pathogen as compared with other gram-negative and gram-positive mastitis-causing organisms (Gröhn et al., 2004). Cure for *Klebsiella* cases in Schukken et al. (2011) was low for ceftiofur-treated quarters (57%) as well as for untreated quarters (19%). In the 2004 Roberson trial that assessed no treatment, IMM amoxicillin, and frequent milkout, none of the treatments used provided satisfactory cure rates for *Klebsiella* cases (n=18). This is similar to a Wisconsin study that reported 0/3 cases cured after treatment with intramammary cephapirin, and to another study that reported 50% bacteriologic cure at 3 weeks post-treatment after parenteral antibiotics (Timms and Schultz, 1984; Pyörälä and Pyörälä, 1998).

Increased duration of infection, particularly for non-*E. coli* gram-negative pathogens, might provide reason for IMM treatment. However, beta-lactam antimicrobials target peptidoglycans, and gram-negative bacteria have the complexity and decreased permeability of an outer LPS layer (Schukken et al., 2012). This might explain the lack of differences between antimicrobial and untreated cases as well as the reduced clearance of the organism by the immune system. There are no clinical or challenge trials that evaluate treatment versus non-treatment of less frequently isolated gram-negative pathogens; however, cure rates for treated *Proteus* infections vary between 78 and 86% (Christie et al., 1973; Lee et al., 1980; Ratnakumar et al., 1996). Other gram-negative bacteria such as *Pseudomonas, Pasteurella* or *Serratia* persist into multiple lactations, and are refractory to several different antibiotics (O'Sullivan et al., 1971; Hogan et al., 1989; Anderson et al., 1997)

Gram-Positive Mastitis Pathogens

As beta-lactam antibiotics must enter and deactivate penicillin-binding-proteins responsible for assembly of the peptidoglycan cell wall and the knowledge that gram-positive bacterial cell walls are composed almost entirely by peptidoglycan layers, gram-positive CM infections are often sensitive to IMM antimicrobials. *In vitro* susceptibility of individual Streptococcal pathogens varies among species and is related to herd-level SCC (higher SCC associated with lower susceptibility), stage of lactation (mid-lactation isolates were more susceptible than early lactation isolates), and severity of mastitis (isolates from clinical samples were more susceptible than those from non-clinical quarters)(Cameron et al., 2016). Cure rates of 89-98% have been reported for IMM treatment of the contagious pathogen *Strep. agalactiae* (Weaver et al., 1986; Yamagata et al., 1987; Erskine and Eberhart, 1990). Additionally, post milking teat dipping and antibiotic dry cow treatment reduced the proportion of quarters infected with *Strep. agalactiae* from 21.8% to 1.6% over 2 years in a trial including 120 cows (Eberhart and Buckalew, 1972). High cure rates and adequate post milking teat dipping likely explain why prevalence is low on North American dairies.

The cure rate for streptococci other than *Strep. agalactiae* was 66% overall for untreated quarters when over 1000 quarters were analyzed from dairy herds in New York and Pennsylvania and less than 30% for 50 quarters from a herd in California (Wilson et al., 1999; Pearce et al., 2016). This compares to antimicrobial treated quarters at 90%, 100%, 79%, 100% and 82% for amoxicillin, cephapirin, cloxacillin, hetacillin, and penicillin, respectively (Wilson et al., 1999). A study on the university dairy at Illinois assessed two different treatment groups: antibiotic (IMM ± systemic) and supportive therapy (oxytocin ± anti-inflammatories) or supportive therapy alone. When mastitis was caused by *Streptococcus* spp., clinical cure rate and

bacteriologic cure rate was statistically higher when antibiotics were used (Morin et al., 1998). This differs from a study by Van Eenennaam et al. (2005) who found no differences in clinical cure between *Streptococcus*-positive cows treated with either oxytocin only, IMM amoxicillin, or IMM cephapirin. Cows treated with oxytocin only, however, experienced more CM relapses (Van Eenennaam et al., 1995).

When the group of non-*agalactiae* streptococcal organisms are studied individually, antibiotic therapy continues to show benefits. For example, untreated cure rates for *Strep. uberis* CM range from 0% to 42% (Chamings, 1984; McDougall, 1998; St Rose et al., 2003; Deluyker et al., 2005). *Strep. uberis* is usually susceptible to commonly used antimicrobials such as penicillin, cephalosporins, and lincosamides, achieving cure rates of up to 95% dependent on treatment duration (Erskine et al., 2002; Guérin-Faublée et al., 2002; Deluyker et al., 2005). However, some report low response rates of 50%, and resistance to lincosamides is not uncommon (Erskine et al., 2002; Guérin-Faublée et al., 2002; St Rose et al., 2003; Milne et al., 2005). Many have found increased cure rates with extended therapy for *Strep. uberis* (Gillespie et al., 2002; Deluyker et al., 2005; Loch et al., 2005). Regardless, waiting 48 hours before commencement of treatment led to deterioration of infected quarters (Hillerton and Kliem, 2002). Cure rates for *Strep. dysgalactiae* during the dry period is expected to be close to 100%, and treatment of the organism during lactation is greater than 80% (Østerås et al., 1991; St Rose et al., 2003; Oliver et al., 2004; Scillieri Smith et al., 2017). Erskine et al. (2002) reported that 94.5%, 99.2%, 99.7%, 82.0%, and 89.0% of *Strep. dysgalactiae* strains from bovine mastitis were susceptible to penicillin, ampicillin, cephalothin, erythromycin, and pirlimycin, *in vitro,* respectively (Erskine et al., 2002).

Lactococcus spp. have been recently identified as mastitis pathogens; a majority of isolates from bovine mastitis milk samples were initially phenotypically identified as *Streptococcus* spp. (Werner et al., 2014). In an investigation of NY herds, 13% of farms had at least one case of *Lactococcus lactis* and it contributed to 24% of gram-positive non-*Strep. agalactiae*, coccal infections (Scillieri Smith et al., 2016). Only 59% of cases (n = 66) from 5 New York farms and 41.2% (n = 17) of cases from Wisconsin farms experienced bacteriological cure after IMM antimicrobial therapy (Lima et al., 2014; Scillieri Smith et al., 2017). *In vitro* studies are conflicting. One study including 92 isolates in New York found no resistance in *Lactococcus* isolates for the beta-lactam antibiotics penicillin, ampicillin, and amoxicillin (Plumed-Ferrer et al., 2015). Another study including 32 isolates in Canada reported that only 46.9%, 40.6%, and 0% were sensitive to ampicillin, pirlimycin, and penicillin, respectively (Cameron et al., 2016). *Enterococcus* spp. are also commonly misidentified mastitis pathogens, accounting for 24-30% of all bovine IMIs classified as environmental streptococcal infections (Devriese et al., 1999). Spontaneous cure rate of CM caused by these organisms is 27% (Pearce et al., 2016). *In vitro* analysis of *Enterococcus* spp. is more difficult due to lack of breakpoints as well as intrinsic resistance to many antimicrobials; intrinsic resistance of certain *Enterococcus* spp. might contribute to treatment failure if diagnosis does not go beyond the group level (Rossitto et al., 2002; Cameron et al., 2016). For example, these organisms possess an efflux pump that renders them resistant to lincosamides (Moellering, 2000). However, the susceptibility of isolates to ampicillin and penicillin was 100% (Cameron et al., 2016).

The contagious pathogen *Staph. aureus* behaves very differently from environmental streptococcus and streptococcus-like species and is known to evade the immune system (White et al., 1980). While most management practices including parlor procedures and segregation or

culling of *Staph. aureus* cases contribute to successful control, some producers still elect to treat positive cows. Cure rates vary greatly from 4% to 92% (Barkema et al., 2006). Treatment regimens and pathogen- and cow-related factors impact the probability for cure, with older animals, cows with higher SCC and longer durations of high SCC, and cows with a greater number of infected quarters experiencing lower cure rates (Barkema et al., 2006). Increased duration of treatment is associated with increased chance of cure (Pyörälä and Pyörälä, 1998; Sol et al., 2000).

CNS, unlike *Staph. aureus* are considered minor environmental mastitis pathogens. Species-specific characterization within this group is lacking and differences between species and between strains within species exist (De Vliegher, 2015). Additionally, distribution of CNS species seems to be highly herd-dependent. CNS are able to cause persistent IMI with moderate to no elevations in SCC and rarely affect milk production; *S. chromogenes* is most frequently represented in persistent IMI (Taponen et al., 2006). A meta-analysis on CNS IMI discredited the idea that natural CNS IMIs were protective of infections with major mastitis pathogens. However, challenge studies showed that the organisms have significant protective effects against major pathogens such as *Staph aureus* (Reyher et al., 2012). Spontaneous elimination rates for CNS IMIs are approximately 60 to 70% (McDougall, 1998; Wilson *et al.*, 1999). In regards to antimicrobial treatment, 44.4%, 44.4%, and 22.2% of CNS isolates from milk (n = 9) were sensitive to amoxicillin, ampicillin, and penicillin, respectively (Srinivasan et al., 2006). However, antimicrobial susceptibility differs between the CNS species. 90% of *S. simulans* cases and 2/3 of *S. chromogenes* isolates are pansusceptible to antibiotics (Taponen et al., 2014). On the contrary, *S. haemolyticus* and *S. epidermidis* are often resistant to one or several

antimicrobials (Taponen et al., 2014). In general, one fourth to one third of CNS isolates are resistant to penicillin (Sawant et al., 2009).

Other Mastitis Pathogens

Antimicrobial treatment is not recommended for *Mycoplasma* spp., *Prototheca* spp., and yeast. *Mycoplasma* spp. are bacteria that lack a cell wall around their cell membrane and are able to invade host cells and form biofilms (McAuliffe et al., 2006). As such, they are able to evade antibiotic therapy and are inherently unaffected by beta-lactam antibiotics. Additionally, *in vitro* resistance has been detected to all main antibiotic classes, which might explain lack of effect *in vivo* (Waites et al., 2014). Finally, *Mycoplasma* spp. have the ability to infect different body sites enabling them to maintain a reservoir outside of the mammary gland. Due to their contagious and refractory characteristics, current strategies include testing and subsequent culling of positive animals (Nicholas et al., 2016).

Prototheca spp. are algae for which there are no known effective or approved therapies and current recommendations include segregation and culling as most infections become chronic with periodic shedding of organisms (Moroni et al., 2015). *Prototheca* spp. are environmental, opportunistic pathogens, however, once a critical number of infections is established within the herd, cow to cow transmission during milking becomes the dominant cause of new infections (Moroni et al., 2015).

Yeast or fungal mastitis has been attributed to treatment directed against other pathogens using contaminated syringes or antibiotic applicators, however, infections can occur in untreated glands and teat injuries may predispose to establishment of a yeast infection (Dworecka-Kaszak et al., 2012). There is no clear evidence of the effectiveness of antimicrobial therapies.

Culture-Negative Samples

Culture negative outcomes occur in 10-40% of CM cases (Roberson, 2003; Lago et al., 2011a). These results have been attributed to concentrations of pathogens that are lower than the detection-limit of the diagnostic tool, presence of inhibitory substances in milk, or clearance of the pathogen by the host's immune system (Taponen et al., 2009). Through sequencing of mastitis milk samples, several groups have shown that known mastitis pathogens can be present in culture-negative quarters at low relative abundances. However, these same pathogens can also be present in healthy milk at low levels (Kuehn et al., 2013; Oikonomou et al., 2014). Regardless, when considering prudent use of antimicrobials, treatment of a quarter without a confirmed etiology is not justifiable.

PATHOGEN-BASED TREATMENT

Antimicrobials should only be used for pathogens where there is strong evidence of their benefits. As outlined above, this includes gram-positive, non-contagious pathogens. "Culture-based," "selective," or "pathogen-based" therapy is the use of diagnostic culture results to manage a case of CM. Culture methods using selective media for gram-negative or gram-positive pathogens have been evaluated for on-farm use and have achieved high sensitivities and specificities for their abilities to correctly classify samples into their respective gram-status (McCarron et al., 2009; Keefe et al., 2010; Godden et al., 2007). Additionally groups have shown that the 24 hour delay for those cases that subsequently receive treatment does not contribute to recurrence of CM (Wagner et al., 2007). In a pathogen-based protocol, the number of clinical cases requiring the use of IMM antibiotics can be reduced by up to 80% (Hess et al., 2003). In the study by Hess et al. (2003), statistical comparisons were not made between a treated and non-

treated group, though anecdotally, withholding of treatment did not lead to decrease severity, and cows assigned to treatment in the “no growth” category did not return to normal milk quicker than those that were not treated. Days in the treatment pen was equivalent to the withholding period for the drug used; clinical signs were similar for cows treated and those that were not. Additionally, a study including 54 Canadian dairy farms evaluated the utility of an on-farm culture (OFC) system (Petrifilm) for selective therapy. No differences in clinical or bacteriological cure rates were observed for the culture and blanket treated groups when 631 cases were assessed (Keefe et al., 2010). Pathogen-based protocols can be dairy specific; not all protocols specify treatment of only gram-positive pathogens (Wagner et al., 2007).

Two peer-reviewed manuscripts were published describing the use of culture to drive pathogen-based therapy in 2011 and 2018 (Lago et al., 2011a; b; McDougall et al., 2018). In the earlier study, 422 cows from 8 commercial dairy farms were randomly assigned to blanket therapy with cephapirin sodium or a culture-based treatment program using the Minnesota Easy Culture System in which only cows with cultures positive for gram-positive pathogens were treated with IMM cephapirin sodium (Lago et al., 2011a;b). The group found a reduction in antibiotic use by 50% without differences in days to clinical cure, bacteriological cure risk, new IMI risk, treatment failure risk, recurrence of CM, linear SCC, daily milk production, and risk and days for culling or death events. McDougall et al. evaluated a culture-based system (Check-up; Farm Medix Ltd., Aukland, NZ) versus blanket therapy on 4 herds in New Zealand. Cows in the selective group with no-growth or gram-negative culture results received no treatment. Those cows within the selective group that were treated received IMM cloxacillin (*Staph. aureus)* or IMM penicillin (non-*aureus* gram-positive pathogens), and cows in the blanket group received IMM penicillin. A reduction of 0.66 daily doses (25% lower antimicrobial use) was experienced

for the selective group, with no differences found between blanket and selectively treated cattle in the proportion retreated for CM (McDougall et al., 2018).

In addition to validated culture-systems, success of an on-farm selective protocol requires that herd owners and staff have the time to inoculate and interpret agar plates, are confident in their interpretation, and can tolerate leaving cows untreated for 24 hours (Neeser et al., 2006). For those who are less confident in culture-based diagnostics, an opportunity exists for the submission of mastitis samples to a diagnostic or veterinary laboratory that offers prompt turnaround of results for decision-making. No recent studies have evaluated this opportunity.

Economics of Pathogen-Based Treatment

Cow factors such as days in milk and milk production at the time of CM onset, the number of CM events within the lactation, the number of days of milk withheld from sale, loss of reproductive efficiency, and milk production losses for the remainder of the lactation contribute to the variability of the CM cost (Fetrow, 2000; Hertl et al., 2011; Hertl et al., 2014; Rollin et al., 2015). Using dynamic programing, the average expense per case of CM is highest for gram-negative cases ($211) with the major contribution being costs associated with milk production loss (Cha et al., 2011). This compares with gram-positive and “other” cases where the major contributor to costs was treatment. The findings of Cha et al. indicated that optimal economic profitability occurred when 93.1% of gram-positive CM cases, 93.1% of gram-negative CM cases, and 94.6% of “other” CM cases were treated. However, response of individual pathogens to antimicrobials, which can also factor into treatment costs, was not considered in the model (Cha et al., 2011). The same group estimated the value of information in knowing the causative agent of CM and determined whether obtaining that information was economically justified by

using a dynamic programming model. The primary finding was that the value of information was greatest when identifying CM at the generic level (clinical symptoms only) and treating based on the highest incidence pathogen. However, all information-based treatment decisions involved use of an antimicrobial; no untreated groups were considered in the analysis (Cha et al., 2016). It would be interesting to determine the behavior of selective protocols in these models.

Pinzón-Sánchez et al. used decision tree modeling (which included pathogen, probability of cure, and probability of recurrence, among other variables) to evaluate economics of treatment practices. The group found that when comparing 2, 3, and 5 day protocols, the most cost-effective strategy when implementing OFC was to treat gram-positive pathogens for 2 d while permitting culture-negative and gram-negative results to "self-cure" (Pinzón-Sánchez et al., 2011). In the same year, Monte-Carlo modeling was used to determine the economics of a 3M Petrifilm on-farm system, considering cow factors as well as accurate versus inaccurate diagnoses. The greatest potential for economic return in selective protocols that use on-farm diagnostics is in herds with accurate diagnosis of cases and in herds with higher incidences of coliform and culture-negative cases (MacDonald et al., 2011). Costs in these herds amount to $539 for *Staph. aureus*, $518 for other gram-positive organisms, $346 for coliform cases, and $326 for culture negative cases. This compares to $402, $460, $461, and $452, respectively, when all cases are treated with antimicrobials. Finally, Down et al. (2017) used probabilistic sensitivity analysis to evaluate the cost-effectiveness of using an on-farm culture approach and found that an OFC approach is not cost-effective in many circumstances, in particular not for those in which gram-positive pathogens are responsible for more than 20% of all cases. However, many inputs for the model were based upon findings from other models in the literature rather than findings from clinical trials (Down et al., 2017). Regardless, the cumulative

findings of the economic models outline the need for farm-specific evaluation of pathogen prevalence prior to implementation to determine returns.

DRY COW ANTIMICROBIAL USE

Dry-cow IMM antimicrobials were developed in the 1950s; their use was recommended in the 1960s as part of the National Institute for Research into Dairying's 5-point plan (United Kingdom). Blanket dry cow therapy (BDCT), or the treatment of every quarter of every cow with a long-acting antimicrobial at dry-off, was instituted to combat the high rate of CM observed within the 2-weeks post-calving (Green et al., 2002) as well as to cure existing infections at dry-off. Research groups have demonstrated that a majority of CM cases during this time are contributed by organisms that were present at dry off or newly acquired over the dry period (Todhunter et al., 1991; Bradley and Green, 2000; Green et al., 2002). However, management and control of mastitis pathogens during the dry and lactating periods have been widely successful as indicated by the increase in negative quarter-level culture results at dry-off from 44% in 1985 to between 73% and 95% of quarters within the last decade (du Preez and Greeff, 1985; Pantoja et al., 2009; Rajala-Schultz et al., 2011). The prevalence of contagious pathogens such as *Strep. agalactiae* and *Staph. aureus* and reduction of bulk tank somatic cell counts (BTSCC) from 295,000 cells/mL in 1997 to 193,000 cells/mL in 2014 also suggest that BDCT is currently not a necessity in all herds (Ekman and Østerås, 2003; Robert et al., 2006). Additionally, only 11.1% of overall test days were greater than 400,000 cells/mL in 2016; this compares to 30.3% of test days in 1998 (Miller and Normal, 1999; Norman et al., 2017).

Regardless of these improvements after the implementation of BDCT, over 90% of cows are treated and 90% of operations use antimicrobial products at dry-off in the US according to a

survey by the National Animal Health Monitoring System in 2014 (USDA NAHMS, 2016b). There are several reasons why a producer/management might elect to not use IMM antibiotics at dry-off: 1) economic returns via decreased labor and dry-tube costs; 2) public or government policy: for example, Nordic countries have adopted restrictions that permit only selective use of antimicrobials, leading to reductions of approximately 80% and 40% for dry-cow and CM treatments, respectively (Ekman and Østerås, 2003); 3) the introduction of an applicator into the teat end is not risk-free (Leelahopongsathon et al., 2016); 4) alternative/adjunct products to antibiotics including teat sealants are reported to have success decreasing the risk of new infections (Godden et al., 2003; Rabiee and Lean, 2013); and 5) consequences related to public health such as accidental residues in the bulk tank or the development of antimicrobial resistance. For these reasons, selective treatment of cows at dry-off is an opportunity that is being considered by producers and veterinarians.

SELECTIVE DRY COW THERAPY

Selective dry cow therapy (**SDCT**) is the identification and treatment of only cows/quarters having an infection at dry-off or at high risk for acquiring an infection during the dry period. The clinical, health, and microbiological outcomes resulting from the use of specific strategies have been explored by research groups over the past few decades. Most practitioners and researchers wish to address the following question: “Are cows in selective treatment protocols, or cows that are not treated with dry-cow antimicrobials at higher risk for experiencing negative outcomes?” This question has been interrogated using field trials and subsequent statistical models to compare treatment groups when evaluating outcomes such as

risk of CM, bacteriological cure, new infection risk, early lactation milk production, early lactation SCC/LS, and risk of culling.

Current Strategies for Selective Dry Cow Therapy

Identification of the cows or quarters that would benefit from antimicrobial treatment is the cornerstone of SDCT protocols and can be performed in several manners. The diagnostics elected for use can be performed at the quarter or cow level, and treatment decisions in regards to dry cow therapy can be made similarly. Accuracy, costs, labor, and ease of use/implementation should be considered when selecting tools to identify cows/quarters to be treated within SDCT programs. The following paragraphs describe strategies for SDCT and the respective results of field trials. The selected trials considered all mastitis pathogens in their microbiological and statistical analysis; all trials were published after 1990.

Use of a Bulk Tank SCC or Single Composite SCC Prior to Dry-Off. The first studies on decreased IMM antibiotics at dry-off were performed in herds with low bulk tank somatic cell counts (BTSCC). These studies randomly assigned cows or quarters to either blanket-treatment or to no treatment; cows/quarters were not separated based on risk or infection status. This often resulted in an increased incidence rate of CM or risk of new IMIs in cows/quarters not receiving treatment. For example, Hogan et al. (1995) performed a study on 4 US herds with BTSCC < 250,000 cells/mL where cows were randomized into 4 groups: (i) antibiotic dry cow therapy, (ii) antibiotic dry cow therapy and *Propionibacterium acnes* injections (PAI), (iii) PAI only, and (iv) no treatments. A statistically higher percentage of cured quarters and lower percentage of new IMIs were found in cows that received IMM antibiotic versus those that had not (Hogan et al., 1995). However, statistical comparisons between groups that did not receive PAI were not

provided. Schukken et al. (1993) performed a study on one Dutch herd with a BTCC of 140,000 cells/ml (50 cows) in which 2 quarters were treated and 2 quarters remained untreated within each cow. This resulted in a 10-fold increased risk of CM, or 10 cases of CM in uninfused quarters versus one case in a quarter that was infused with dry-cow antibiotics (Schukken et al., 1993). In the same trial, no statistical differences were found between groups for new IMI or bacteriologic cure of major pathogens. Minor pathogens were reduced in quarters that were treated with antimicrobials. When treatment was performed on the cow level in a California herd (233 cows; 240,000 cells/mL) with a low prevalence of contagious mastitis pathogens, no statistical differences were found between groups for culling, CM risk, or SCC in the first 120 days of the subsequent lactation, even when cows were stratified into high and low (≤500,000 cells/mL) SCC groups (Berry et al., 1997). Scherpenzeel et al. (2014) used a split-udder design (2 quarters received treatment, 2 did not) and evaluated use of an individual-cow SCC threshold rather than use of a bulk-tank threshold. Primiparous animals with SCC <150,000 cells/mL and multiparous animals with SCC <250,000 cells/mL were included; 1,657 cows had 2 quarters that were treated and 2 that were not. In the trial, the incidence of CM was 1.7 times higher in quarters dried without antimicrobials (95% CI: 1.4-2.1). SCC was higher in non-treated quarters and a higher percentage of the quarters were culture positive at calving and at 14 DIM.

Culture Only Method. The current gold standard for diagnosing an IMI is aerobic culture. However, the time, labor, and materials associated with culturing cows can be an added cost, and this diagnostic tool, when used in a SDCT protocol, can identify infected cows/quarters, but will not determine cows at higher risk for acquiring infections during the dry period. The effects of culture-driven antibiotic use at dry-off were determined by Browning et al. (1990) in an Australian field trial including 1044 cows from 12 herds. Culture-negative cows

were randomly allocated to receive blanket therapy or no treatment, while culture-positive cows were randomly allocated to receive blanket therapy or treatment of only infected quarters. No statistical differences were detected in CM risks between the groups within the first 5 months of lactation. No differences were found for new infection risk in the cows that were culture-negative at dry-off; however, new infection risk was 4 times higher in selectively treated culture-positive cows versus blanket treated culture-positive cows (Browning et al., 1990). Only cows infected with a major pathogen (n=608) were enrolled in a Norwegian study that assessed the effects of SDCT on culling, mastitis, milk yield, and SCC (Østerås and Sandvik, 1996). Cows were randomly allocated into a placebo group, control group, or one of two IMM antibiotic treatments. Cows within the antibiotic groups only received treatments in quarters experiencing IMIs. It should be noted that quarters known to have an IMI in the placebo and control groups were not treated with antibiotics. There were 21% less cases of mastitis in antibiotic treated quarters ($P = 0.09$). Treated quarters also had lower SCC and a higher lactational milk yield. There was no effect of therapy on culling rate. Patel et al. (2017) used quarter-level treatment of culture-positive quarters in addition to internal teat sealant (1 herd, 56 cows) and described no statistical differences between BDCT and selective quarter treatment when assessing bacteriological cure and new infection risks (Patel et al., 2017).

Cow Records Only: SCC and/or Mastitis Events. Though not presented as a SDCT trial, Huxley et al. (2002) evaluated the use of a teat sealant in cows with routine composite SCC below 200,000 cells/mL with no previous cases of mastitis and a projected dry period of >51d. Comparisons were made between cows only receiving internal teat sealant and cows receiving only blanket antibiotic therapy. No statistical differences were found for CM cases, new infections with minor pathogens, nor overall bacteriologic cure. While new infection risk for

major pathogens was lower in antibiotic-treated cows, bacteriologic cure rates were only higher for the minor pathogens *Corynebacterium* spp. in antibiotic-treated cows (Huxley et al, 2002). Another trial evaluating internal teat sealant in the UK used a split-udder design with the same cow-level criteria. However, in this trial (Bradley et al., 2010) all quarters received the internal teat sealant product, even those treated with antibiotics. No statistical differences were described between groups of quarters for bacteriologic cure and new infection risks and no differences were found in CM risk.

In a noninferiority study comparison, McDougall (2010) assigned cows (~900 cows from 6 New Zealand herds) with SCC ≤ 150,000 cells/mL and no history of CM in the current lactation to no treatment, a novel antibiotic, or a reference antibiotic at dry-off. When analysis was performed on the quarter level, there were fewer IMIs characterized by any pathogen at freshening in antibiotic treated groups; when analysis was performed on the cow level, there was only a difference seen for major pathogens. SCC was lower in treated groups, and there was a lower hazard of CM during the dry period and in early lactation for treated groups (McDougall, 2010). A study performed in the US by Rajala-Schultz et al. (2011) on 4 herds (~400 cows) also used computer records and mastitis events to determine which cows to enroll. Cows had to have a SCC ≤ 200,000 cells/mL over the last 3 tests with no cases of mastitis in the current lactation. If the cow met the criteria but there was one case of mastitis, the cow had to maintain a SCC < 100,000 cells/mL until dry-off. Cows were then randomly assigned to receive IMM antibiotics or no treatment. No statistical differences were described for new infection risk or early lactation milk production. Overall, there was a lower SCC in the treated cows; however, when evaluated on the herd level, only one herd had a statistical difference between groups for this outcome (Rajala-Schultz et al., 2011).

N-Acetyl Beta-Glucosaminidase (NAG-ase). Hassan et al. (1999) used levels of NAG-ase, a lysosomal enzyme with elevated levels during infection, in a SDCT protocol on 3 dairy farms in Australia (150 cows). Cows were randomized into one of 4 groups: comprehensive treatment (IMM antibiotics in all 4 quarters), selective treatment (IMM antibiotics only in NAG-ase-positive quarters), or no treatment in all 4 quarters. Milk samples retrieved two and six weeks into the dry period showed that the proportion of infected quarters differed significantly between groups (untreated > selectively treated > comprehensively treated); however, after 6 weeks, at calving, and at 3 weeks into lactation there were no significant differences in infection status between groups. Clinical cases of mastitis occurred only in the untreated and selectively treated groups ($P < 0.01$). Using cultures at dry-off as a reference, NAG-ase values did not accurately define infections (Hassan et al., 1999).

Cow-side Tests Only. Though no randomized field trials have assessed the performance of only rapid cow-side tests such as the CMT and milk leukocyte differential (**MLD**) tests in a SDCT protocol, they have been evaluated to determine the infection status of a cow at dry-off. CMT and MLD have fair to good sensitivities and specificities for late lactation animals, but are dependent upon cut-point and interpretation (Godden et al., 2017).

Combination of Culture and Cow-Level Data. A teat-sealant study on 482 low SCC (< 200,000 cells/mL), culture-negative cows was performed using a randomized quarter-level study design and 4 different treatments: control (no treatments), IMM antibiotic, IMM antibiotic and internal teat sealant, and teat sealant only (Woolford et al., 1998). The number of clinical IMIs during the dry period was higher in the control quarters than the infused quarters, but not different between the teat sealant only and control quarters. The same findings were found for new IMIs at calving.

A BTSCC requirement of <250,000 cells/ml (4 herds) was combined with individual culture data at dry-off to evaluate new infection and CM risks (Berry and Hillerton, 2002). The group found a 9% difference in cases of CM between the untreated and treated cows ($P = 0.001$). However, cows randomized into treatment groups were those with negative, CNS-positive, or *Corynebacterium*-positive culture results one week prior to dry-off. The overall new IMI risk was statistically higher in the untreated versus treated cows; the greatest contribution to this finding was for major pathogens (*Strep. uberis)* in quarters already infected with minor pathogens. No statistical differences were found in the prevalence of CNS post-calving between treated and untreated groups (Berry and Hillerton, 2002).

Cameron et al. (2013, 2014) used culture in addition to several other screening tools on 16 Canadian herds: cow level inclusion criteria in the study consisted of a dry period between 30 and 90 days, 3 serial SCC <200,000 cell/mL prior to dry-off, no CM in the 90 days prior to dry-off, and a CMT score of <2 on the day prior to dry-off. Cows were then randomized to BDCT or SDCT. While cows within the BDCT were all treated with antimicrobials, only culture-positive cows within the SDCT group were treated. All cows also received internal teat sealant. No statistical differences were found for bacteriological cure and new IMI risks at calving, normal logarithm of SCC over the first 180 d, CM risk within 120 d, or test day milk production between SDCT and BDCT cows.

Making Sense of Discrepant Data

In trials that did not use a combination of tools, cows at risk due to historically higher SCC or multiple mastitis events, and currently infected cows (if culture was not used) were among the cows included in the non-treated group. Dissimilarities between findings could also

be due to the presence of higher levels of major pathogens on the included dairies, the lack of teat sealant use, or the inclusion of herds with BTSCC >250,000 cells/mL.

In an effort to generate an overall outcome for trials that evaluated treatment protocols at dry-off, two groups performed meta-analysis on previously published research. One analysis by Halasa et al. (2009a) was performed on 4 SDCT trials (SDCT protocol versus BDCT protocol) and 13 BDCT trials (BDCT versus no-treatment). In it, the meta-analytic pooled relative risks for bacteriological cure were 1.76 and 1.78, respectively. For new infection, pooled relative risks of 0.58 and 0.55 were described for BDCT versus SDCT in the 2 meta-analyses (Robert et al., 2006a; Halasa et al., 2009b). While statistical differences in relative risk were seen for protection against new quarter IMI, no statistical differences were calculated when the selection unit was the cow (Halasa et al. 2009b). In the Robert et al. (2006a) meta-analysis, pooled differences in new IMI risk were statistically significant for streptococcal and *Staph. aureus* IMIs and not for IMIs caused by CNS or coliforms. Statistically different findings for BDCT versus SDCT in 15 of the 25 studies could be due to the fact that contributions of streptococcal species and *Staph. aureus* represented more than 35% of IMIs in 50% of the studies included.

Readers will note that the main objective of many of the studies described here was not to compare a selectively treated or untreated group of cows/quarters to a blanket-treated group of cows or quarters, rather the data comparing these groups could be extracted from the results presented in each manuscript. Overall, there are limited studies that adequately capture the best comparisons in regards to sample size, study design and statistical evaluations. These include Sherpenzeel et al., 2016 (Netherlands), Cameron et al., 2013 and 2014(Canada), and Rajala-Schultz et al., 2007 (US). The differing findings in each of these trials dictate the need for more

research on the subject. However, we do know that selection of farms for SDCT should be dependent on pathogen prevalence and SDCT protocols should be implemented on the cow level.

Economics of Selective Dry Cow Therapy

An economic analysis comparing BDCT to no dry-cow therapy of all cows within a herd concluded that dry-cow therapy was advantageous. However, the modeled costs of not using dry-cow antibiotics always included lower milk production and higher SCC for these cows, with values retrieved from regression analyses with suboptimal R^2 values (McNab and Meek, 1991). More recently, stochastic modeling was used to evaluate the economics of SDCT by Huijps and Hogeveen (2007). The economic parameters associated with the greatest influence on costs were antibiotics, milk losses, and the hourly rate for labor. The infection parameters that produced the most influence on costs were CM, the probability of culling, and infection rate over the dry period. When infection rate and antibiotic costs are low, no DCT might be best, but variation is high; in scenarios where selection criteria has high sensitivity, there will be lower average costs. Default values of the input variables and probabilities in this Dutch model showed that SDCT economically is the best option (Huijps and Hogeveen, 2007). In studies where "economic" outcomes were similar between groups (milk production, infection risk, CM risks, and culling risks) partial budget analysis can easily be performed. A net benefit of $2.62 per cow was calculated for the pilot study performed at the University of Minnesota by Patel et al. (2014). This accounted for the cost of labor and supplies to segregate, sample, and culture all cows at the quarter-level (Patel, 2014). As cure and infection risks were similar between groups, the authors did not account for additional cases of mastitis experienced by one group over another. The economic analysis performed by Scherpenzeel et al. (2016) used computer modeling to predict

economic outcomes using 7 different SDCT scenarios. These models assumed higher subclinical and CM prevalence and decreasing total antimicrobial usage for each scenario of decreasing sequential SCC thresholds. Two of 7 programs produced an economic advantage of SDCT over BDCT: 1. using 50,000 cells/mL for 1st lactation and higher animals and 2. using 150,000 cells/mL for first lactation animals and 50,000 cells/mL for >1st lactation animals (Sherpenzeel et al., 2016). Subsequent to this analysis, the same group (Sherpenzeel et al., 2018) used mathematical modeling to determine the effect of individual-farm BTSCC and CM incidences on economic values (costs associated with CM or subclinical mastitis in early lactation). BDCT was compared to a sliding scale of SDCT (100% to 0% antibiotic use) on farms with permutations of low, high, and average BTSCC and low, high and average CM incidences. The authors concluded that for all evaluated BTSCC levels, SDCT was more economically beneficial than BDCT, with greater profits occurring in herds with lower incidences of CM; all types of herds can reduce dry-cow antimicrobial use without negative economic consequences (Sherpenzeel et al., 2018).

Application of Selective Dry Cow Therapy

Herds with a bulk tank SCC ≤250,000 cells/mL, hygienic dry-off procedures, and very low prevalence of contagious pathogens could be considered for SDCT (Ekman and Østerås, 2003; Cameron et al., 2014). Treatments should be on the cow level: many groups have also shown that due to interdependence of quarters, split-udder or quarter-level treatment design might contribute to negative outcomes, and quarters do not act independently when considering infection risk (Robert et al., 2006b; Paixão et al., 2017).

Cow-side or record-based tests such as CMT, CM history, and Dairy Herd Information Association (DHIA) SCC offer the convenience of readily accessible data, but if using microbiological culture as a reference gold-standard, these methods will result in more misclassifications. High sensitivity will minimize the potential risk of not treating a cow that might benefit from treatment. Sensitivity can be increased by using lower SCC thresholds to define "at risk" cows. With a more sensitive test, more cows will be treated. Regardless, lower thresholds will result in more prudent use of antimicrobials than in a BDCT system. Sensitivities and specificities of using monthly SCC and CM events as treatment criteria for SDCT range from 58.4% to 69.4% and 62.7% to 71.5%, respectively (Torres et al., 2008; McDougall, 2010; Rajala-Schultz et al., 2011). As described by the referenced field trials, aerobic culture can be used on all cows or a subpopulation of cows (e.g. cows with SCC below a certain threshold) to screen cows or quarters for treatment. This generates additional costs and the need for reliable and conscientious sampling as well as trained personnel or external laboratory staff to define an infected quarter. Additionally, cows need to be segregated for sampling at least one day prior to dry-off and again when animals to be treated are identified.

SUMMARY

Public health concerns, the risk for the development of resistant pathogens, and the many trials that indicate that particular cow and/or pathogen traits contribute to treatment failure all support the need to use antimicrobials responsibly and prudently. Producers are aware of this need: over 70% of dairy farmers in the UK said that reducing antibiotic usage would be "a good thing to do" (Jones et al., 2015). BDCT is a means to reduce new IMI and increase bacteriologic cure in subclinical and clinically infected cows at and during dry-off. IMM antimicrobials for

lactating cows also have their place in treating susceptible infections; however, selective use of IMM antibiotics for those cows that will likely benefit can produce similar results when applied in appropriate herds. Selective antimicrobial use at dry-off and pathogen-based therapy protocols create opportunities to practice good drug stewardship and in some situations these programs have shown to offer economic benefits. Research indicates that success is farm specific. Veterinarians should remain abreast of current research findings and consider farm management and pathogen presence as they work with producers to develop the best strategies. Further research on pathogen-based treatment can create an armory of information to help veterinarians and producers guide treatment decisions, particularly when more than half of cultures from CM cases yield no growth or gram-negative organisms and up to 90% of dry cow cultures yield no growth.

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CHAPTER TWO

RANDOMIZED NONINFERIORITY TRIAL COMPARING TWO COMMERCIAL INTRAMAMMARY ANTIBIOTICS FOR THE TREATMENT OF NONSEVERE CLINICAL MASTITIS IN DAIRY COWS

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ABSTRACT

The purpose was to evaluate two intramammary treatments for mild-to-moderate cases of clinical mastitis in a noninferiority comparison. Noninferiority trials are intended to show whether a given treatment, hetacillin potassium, has at least comparable efficacy as the reference treatment, ceftiofur hydrochloride. Treatments can be deemed inferior to the reference treatment by an amount less than the margin of noninferiority, or inconclusive if the confidence interval crosses the margin of noninferiority. Cows with clinical mastitis from 6 farms were considered for enrollment. Using a randomized design, cows with mild or moderate mastitis in one quarter were assigned to on-label treatment with either ceftiofur or hetacillin. A total of 596 cows met the criteria needed for continued enrollment. Treatment distribution resulted in 309 cows in the ceftiofur group and 287 cows in the hetacillin group. Mixed regression analysis was performed for the following outcomes: bacteriological cure, pathogen cure, clinical cure, postevent milk production and linear score, and survival to day 30 and 60. Cox proportional hazards analysis was used to describe treatment effect on survival and mastitis risks. Bacteriological cure, defined as absence of causative organism in samples retrieved at days 14 and 21 post-mastitis, was similar between groups. No significant statistical differences were found in cure risk, and noninferiority of hetacillin relative to ceftiofur for bacteriological cure was conclusive (hetacillin = 67%, ceftiofur = 72%). Absence of a pathogen on both follow-up samples designated a cow as a pathogen cure. Pathogen cure was similar between treatment groups and noninferiority of hetacillin relative to ceftiofur was shown (hetacillin = 35%, ceftiofur = 32%). Clinical cure (hetacillin = 68%, ceftiofur = 64%), postevent milk production (hetacillin = 37.0 kg, ceftiofur = 38.2 kg), and linear scores (hetacillin= 3.4, ceftiofur =3.1) were also not statistically different between treatment groups. Noninferiority of hetacillin relative to ceftiofur was shown for

survival to d 30 and survival to d 60, while hetacillin was more likely to have a clinical cure than ceftiofur by day 4. No differences were seen between groups when Cox proportional hazards were performed: for exit from the herd in the 60 d following the event or in the risk for a subsequent mastitis event. These findings can be used to develop farm-specific protocols for clinical mastitis treatment.

Key words: clinical mastitis, hetacillin, noninferiority, ceftiofur

INTRODUCTION

The antimicrobial treatment options available to dairy producers for clinical mastitis (**CM**) are required by United States agencies to exhibit efficacy relative to a non-treated negative control. Very few studies have rigorously compared available treatments to positive controls using appropriate on-farm trials. It would be of benefit to perform controlled trials comparing two existing treatments.

In the US, only two classes of antimicrobials are commercially available for intramammary (**IMM**) treatment of CM; beta-lactams and lincosamides (Baynes and Riviere, 2014). In this study, we compared two beta-lactams. Hetacillin potassium (Hetacin K®, Boehringer Ingelheim, St Joseph, MO) is a broad-spectrum IMM antibiotic approved to treat gram-negative and gram-positive mastitis-causing pathogens such as *Streptococcus agalactiae, Streptococcus dysgalactiae, Staphylococcus aureus,* and *Escherichia coli* (Food and Drug Administration; **FDA**, 2010). It is chemically related to ampicillin and was shown to maintain necessary minimum inhibitory concentrations in a three times per day milking regime (Lindquist et al., 2015). Ceftiofur hydrochloride (Spectramast® LC, Zoetis, Kalamazoo, MI) is a broad spectrum third generation cephalosporin indicated to treat mastitis associated with CNS, *Strep.*

dysgalactiae, and *E. coli* (FDA, 2005). While both drugs have an established milk withdrawal time of 72 h, average length of recommended treatment varies. While the label indication for ceftiofur is administration every 24 h for 2-8 days, the hetacillin label states a three day duration at the same daily interval. The average duration for the use of ceftiofur for mild, moderate, and severe CM was 4.0, 4.4, and 4.5 d in a recent Wisconsin survey, respectively (Oliveira and Ruegg, 2014).

A two-sided comparative study must prove a higher defined efficacy for the proposed treatment over the current standard-of-care treatment in a clinical setting with the purpose to reject the null hypothesis that the therapies are not different; if the evidence is not strong enough to favor the tested product, the researcher cannot rule out nor conclude equality. Noninferiority of a therapy is established when the data provide evidence to conclude that its efficacy is within a certain amount of the equivalence margin of the comparison therapy (Walker and Nowacki, 2011). If a researcher can hypothesize and confirm that a therapy is equivalent or superior to the reference, benefits to the patients can include convenience, lower costs, fewer side effects, improved delivery systems, and better integration into a current protocol (Piaggio et al., 2006; Walker and Nowacki, 2011).

In regards to mastitis research, three major studies involving FDA-approved IMM preparations have been previously performed as noninferiority trials. Schukken et al. (2013), concluded noninferiority between a first-generation cephalosporin and a third-generation cephalosporin when considering both clinical cure of all non-severe CM cases and bacteriological cure of gram-positive cases. The specific interest was to determine if there was a difference in efficacy given that first generation cephalosporins have limited gram-negative activity while third-generation products have broader activity claims (Hornish and Kotarski,

2002; Guérin-Faublée et al., 2003; Pfeifer et al., 2010). The second and third noninferiority trials performed by Arruda et al. (2013) and Johnson et al. (2016) on dry-cow therapy treatments determined noninferiority when considering various indices including post-calving new infection risk. No similar comparison has been made between a third generation cephalosporin, ceftiofur, and a semi-synthetic penicillin, hetacillin, in the treatment of mild to moderate CM. A Northeast survey study comparing bacteriological cure rates for 7 mastitis drugs was performed by Wilson et al. (1999). While cure rates for cephapirin rather than ceftiofur were analyzed, hetacillin cure rate was comparable to the cephalosporin product (62%, n = 35/56 versus 68%, n = 152/222, respectively). However, given its comparable cure rate, a 2014 study on 51 large dairy herds in Wisconsin indicated that only 3.3% (n = 21/645) of CM cases were treated with IMM hetacillin while 71.6% (n = 462/645) of cases were treated with ceftiofur (Oliveira and Ruegg, 2014).

This study compared the treatment efficacy of a 3 day administration of intramammary hetacillin to a 5 day administration of the reference treatment ceftiofur. The objective of the trial was to evaluate whether noninferiority of the test product against the reference product can be established.

MATERIALS AND METHODS

Study Design

This was a randomized noninferiority study. The concept of a noninferiority trial is formalized in the term equivalence margin, which defines a range of values for which the efficacies or margins between differences in clinical outcome are close enough to be considered equivalent (Walker and Nowacki, 2011). A pre-stated margin of noninferiority is chosen as the smallest value that would show a clinically important effect. This margin is also used in a

statement of hypothesis: in this study, the null hypothesis was that a 3 d treatment with hetacillin is inferior compared with an extended 5 d treatment with ceftiofur when considering bacteriological cure, clinical cure, pathogen cure, and survival indices. The alternative hypothesis was that a 3 d treatment with hetacillin is noninferior compared with an extended treatment with ceftiofur by more than the equivalence margin of 15%. The choice of equivalence margins is often less than half of the expected effect in a traditional study. In a trial comparing treatment of gram-negative CM cows, bacteriological cure was ~35% greater for cows treated with ceftiofur than for cows receiving no treatment (ceftiofur n = 41/56; control n = 18/48; Schukken et al., 2011). Additional assessment of differences in postevent milk production, postevent linear score, and days in the treatment pen (hospital days) was evaluated using traditional hypotheses rather than noninferiority comparisons.

Study Farms

This trial was conducted between September 2014 and June 2015 at 6 commercial dairy farms in major dairy-producing regions of New York State. Farms eligible for inclusion in the study met the following criteria: herd size >500 lactating cows; participation in monthly Dairy Herd Information Association (**DHIA)** testing; accurate recording of CM cases; and willingness to participate and follow standard operating procedures, which included not wavering from treatment plans for 7 days. A known presence of streptococcal CM was also required to ensure appropriate representation of gram-positive and gram-negative etiologies. Medians for this sample of herds were: 1057 lactating cows (range: 524 to 1466), 12,150 kg per cow per year of milk (range: 11,773 to 13,091 kg), and a bulk tank somatic cell count (**SCC**) of 140,500 cells/mL (range: 100,000 to 267,000 cells/mL).

Case Definition

All cases of CM at each of the 6 Holstein dairies were assessed for enrollment at the time of occurrence. A cow was defined as having CM if milk was abnormal from one or more quarters. A clinical scoring system (**CS**) adapted from Wenz et al. (2001) was used to classify CM cases based on severity and only cows with clinical scores of 1 or 2 were included. Severity of clinical signs was scored as mild (CS = 1), moderate (CS = 2), or severe (CS = 3). A score of 1 was assigned if the milk was visually abnormal; a score of 2 was assigned if the milk was visually abnormal and there was firmness or swelling of the affected quarter; and a score of 3 was assigned if the milk was grossly abnormal, there was firmness or swelling of the affected quarter, and there were signs of systemic disease (e.g. fever, dehydration, or depression).

In addition to a CS < 3, a cow needed to meet the following criteria: less than 300 days in milk (**DIM**), neither CM nor antimicrobial administration in the last 30 d, parity < 6, only one quarter exhibiting signs of CM, and no previous enrollment in the trial.

Treatment Groups

At each farm location, lactating cows with CM that met the inclusion criteria were randomly allocated to one treatment group via Dairy Comp 305 (**DC305**, Tulare, CA; four farms) or via randomized envelopes containing treatment assignment (two farms). Treatment groups were as follows: the test treatment group consisting of cows receiving one treatment of hetacillin once per day for 3 d into the affected quarter and a reference treatment group consisting of cows receiving one treatment of ceftiofur once per day for 5 d into the affected quarter. Intramammary treatments were administered using partial insertion technique by trained farm personnel.

Timing of Events

The day of enrollment, also the first day of treatment, was defined as day 1 and commenced upon randomized allocation of treatment group. Before treating a cow, the teat of the affected quarter was thoroughly disinfected and 3 streams of milk from the affected quarter were discarded. A milk sample was taken using aseptic technique and immediately placed in the on-farm freezer. After the sample was taken, the teat end was disinfected again and treated with the allocated treatment. The cow was marked as treated using a leg band, per standard on-farm protocol. For each treatment, a dated and signed treatment confirmation was completed. A CS for cows in both groups was also obtained on days 2-5 following the initial score on day 1. Approximately 14 d (±4 d) and 21 d (± 5 d) after the onset of treatment, a milk sample was collected from the enrolled quarter and a CS was determined. Resamples were obtained at least 7 d apart for each cow. If at any time an animal became systemically ill following randomization and treatment, farm personnel was advised to remove the animal from the study and initiate additional treatments. In these cases, a milk sample and CS were collected before additional treatments were provided. If a cow sustained mastitis in another quarter, it was removed from the trial with the same requirements.

Bacteriological Culture

Standard culture technique was performed on all samples according to National Mastitis Council guidelines for identification of aerobic organisms (National Mastitis Council, 1999). Briefly, 0.01 mL of milk was streaked on trypticase soy agar containing 5% sheep blood and 1% esculin (PML Microbiologicals, Mississauga, ON, Canada), and plates were incubated aerobically at 37°C for 18-24 h. After observations of colony morphology and hemolytic

patterns, isolates were examined further by means of 3% potassium hydroxide, gram staining, catalase and indole testing, and additional biochemical and metabolic evaluations as needed. Gram-negative organisms were identified by colony morphology on MacConkey's agar (Hardy Diagnostics, Santa Maria, CA). Diagnosis and confirmation of IMM infection by culture was based on standard published definitions and assessed at the quarter level. Briefly, an intramammary infection was defined as one or more colonies isolated from the ten microliter milk sample for all pathogens except for CNS. For CNS, 2 or more colonies isolated from the ten microliter milk sample were needed to establish presence of an infection (Dohoo et al., 2011). When two different types of bacteria were identified, the result was classified as a mixed infection. When at least one colony each of 3 or more of types was isolated, the sample was considered contaminated.

Data Capture

Sampling and clinical data collection was performed by farm staff (5 farms) or a support technician (one farm). Farm staff was trained by Quality Milk Production Services (**QMPS**, Ithaca, NY) personnel and veterinarians to identify mastitis, assign severity scores, enroll cases, collect samples, and complete paper records. Quality assurance audits were performed by QMPS and herd veterinarians and regular visits were performed by the first author.

Sample Size Determination

The required sample size was determined using the confidence interval approach, considering where the confidence interval for the treatment effect lies with respect to both the margin of noninferiority (**Δ**), and a null effect (treatments are equal). Sample size depends on the

level of confidence chosen, the risk of Type II error (or desired power), and Δ. Here we specified Δ as the difference in cure risk ratios between the two treatments. A binomial distribution was performed assuming a ceftiofur cure risk of 65% based on a randomized clinical efficacy trial (Schukken et al., 2011), plotting Δ as the independent variable and power and sample size as dependent variables. This was used to find the sample size (n = 250 per group) with a chosen value for power (0.9) and a chosen value of Δ (0.15), which is less than half of the expected effect of ceftiofur in a traditional clinical trial (Schukken et al., 2011). The total study size is then twice this number, or 500. We enrolled 600 cows to ensure adequate study power while accounting for loss to follow-up and a Type I error risk of 5%.

Primary and Secondary Outcomes

Primary outcomes were bacteriological cure, clinical cure, and pathogen cure. A quarter was defined as a bacteriological cure when there was a pathogen present in the pretreatment sample and this pathogen was absent from both post-treatment samples (days 14 and 21). Cows that initially cultured with no growth or contamination were not included in analysis for bacteriological cure. If one of the follow-up samples contained the same organism as the pretreatment sample, the quarter was defined as a non-cure. If a cow left the trial due to mastitis reasons and her exit sample revealed the same organism as the pretreatment sample, the quarter was defined as a non-cure. If none of the present samples cultured with the enrollment organism, but a cow was missing one or both follow-up samples or a removal sample, the cow was deemed lost to follow-up for bacteriological cure. If none of the present samples cultured with the enrollment organism and one of the samples was contaminated, the cow was also lost to follow-up for this outcome. Samples were missed due to treatment for other ailments, input error, or

early dry/sale. If no exit sample was present before the 21 d sample collection period, the cow was lost to follow-up for bacteriological cure. Differences in frequencies of lost to follow-up between treatment groups were noted for each outcome.

A quarter was defined as a clinical cure within the first 5 days and for the follow-up records when its CS became and remained zero. Clinical cure was dichotomized to include two groups: those cows that cured by day 4 and those that did not. Day 1-5 trial records were considered sufficient to determine clinical cure.

When both follow up samples contained no growth, the cow was defined as a pathogen cure. A pathogen did not need to be present in the pretreatment sample to be defined as a pathogen cure. Cows with contaminated samples were lost to follow-up and not considered for pathogen cure analysis. If a cow had one of two follow-up samples with no growth, but was missing a second sample, the cow was also lost to follow-up.

Secondary outcomes were extended clinical cure, number of clinical days, post treatment milk production and linear score, survival of the cow in the herd, occurrence of another mastitis event in the same quarter, and hospital days. A quarter was defined as an extended clinical cure when its CS was 0 on d 14 and 21 after the event. The clinical days outcome was defined as the time to clinical cure and was calculated as the number of days from entrance into the treatment pen until clinical signs were undetectable. Hospital days were defined as the number of days (in 24 h intervals), between entrance dates and exit dates into or from the treatment pen. Previous milk production and linear scores (**LS**) were obtained from the test day data prior to the event (range: -8 d to -43 d pre-event). Post event milk production and LS were obtained from the test day after the event (range: +8 d to +43 d postevent). LS is a transformation of SCC, calculated as

[Ln(SCC / 100) / Ln(2)] + 3 (Ali and Shook, 1980). Survival analysis was evaluated by following the cow to dry, cull, or 60 days post-enrollment, whichever came first.

Statistical Analysis

Noninferiority analysis of binary outcomes was completed using PROC FREQ in SAS® version 9.4 (SAS Institute, 2014) with one-sided 95% confidence intervals and noninferiority margins. Statistical analysis of the primary and secondary outcomes was performed using regression models, taking into account the following covariates: parity, DIM, hospital days, previous milk yield, LS, and CM etiology. To select the best model, all possible confounding variables were subjected to bivariate analysis by means of Chi-squared, t-tests, or analysis of variance tests using PROC FREQ, PROC TTEST, PROC REG, or PROC ANOVA. The primary explanatory variable of interest was treatment group; all interactions with this variable were tested in a similar manner. Any terms with a *P*-value < 0.2 were offered into the models. Each variable was then examined by manual backwards stepwise elimination in the regression until all possible explanatory variables with $P < 0.1$ were included. In many of the models, the treatment variable was forced. Farm was first tested as a fixed effect and if determined not to be significant to the level of $P < 0.10$, it was then included as a random effect. This corrected for any within-farm clustering that violated the assumptions of independence. Additionally, basic techniques were used to assess normality, homoscedasticity, and linearity of variables. Transformations were performed and reported as necessary. Not all included animals had complete LS or milk yield data as some cows experienced the event in early lactation with no prior test day or late in lactation with no post-CM test day. Cows were excluded from hospital days or clinical days

analyses if pen moves were not complete in DC305 records. Where analyses were performed on a subgroup of animals, the number of animals with complete data is indicated in the tables.

Mixed logistic regression models were performed for the binary outcomes bacteriological cure, pathogen cure, clinical cure by day 4, survival to day 30, and survival to day 60. Mixed linear regression models were performed for postevent milk yield and postevent LS. Time in the herd from day 0 to day 60 postevent was evaluated using Cox proportional hazards regression (PROC PHREG). Similar analysis was performed to describe the effect of treatment on the survival distribution of quarters experiencing another case of mastitis before 60 days post-enrollment.

RESULTS

Descriptive Results

Enrollment characteristics for each of the 6 farms are shown in Table 2.1. In total, the farms experienced 1168 cases of mastitis, 161 of which were severe (13.8%). The number of mastitic cows meeting eligibility criteria was 627. Thirty-one cows did not remain in the study due to secondary treatment of the quarter or cow within the first 7 d of enrollment (n = 15; hetacillin = 12, ceftiofur = 3), mastitis in another quarter within 7 days (n = 8; hetacillin = 4, ceftiofur = 4), non-survival of cow or quarter (n = 7; hetacillin = 3, ceftiofur = 4), and input errors (n = 1, ceftiofur). The mean time of first follow up sample was 14.1 d postevent (standard deviation, **SD** ± 1.9 d) and second follow-up sample was 21.2 d postevent (SD ± 2.2 d). Eight cows did not have enrollment samples, but could be assessed for the following outcomes: clinical days, hospital days, postevent milk production and LS, and survival indices. Of the cultures, 63% produced diagnostic growth (n = 369/588), 31% resulted in no organism (n = 183/588), and the

remaining 6% resulted in no significant growth or contamination (n = 36/588). Of all etiologies, the most frequently cultured gram-positive organisms found were *Strep. dysgalactiae* (14.0%; n = 84/588) followed by *Staph. aureus* (8.0%; n = 47/588) and *Strep. uberis* (7.8%; n = 46/588). *E. coli* (13.8%; n = 81/588) was the most commonly isolated gram-negative organism followed by *Klebsiella* spp. (5.4%; n = 32/588) and *Pasteurella* spp. (1.0%; n = 6/588). All etiologies were evenly distributed ($P > 0.05$) except for *Strep. uberis,* for which 72% (n = 33/46) of cases were randomly allocated to the ceftiofur group while 28% (n = 13/46) were allocated to the hetacillin group ($P < 0.01$). Distribution of organisms within each treatment group can be seen in Table 2.2.

The distribution of treatments was nearly equivalent; 48% of cows were assigned to the hetacillin group (n = 287/596) and 52% of cows were assigned to the ceftiofur group (n = 309/596). Distribution of treatment group by farm was also not statistically different ($P = 0.68$). We found no statistical differences in the means of DIM (hetacillin = 130 d, ceftiofur = 129 d; $P = 0.78$), lactation number (hetacillin and ceftiofur = 2.7; $P = 0.46$), previous test-day linear scores (hetacillin = 2.3, ceftiofur = 2.5; $P = 0.37$), and milk yields (hetacillin = 42.5 kg, ceftiofur = 42.7 kg; $P = 0.80$). The quarter affected did not differ between groups ($P = 0.70$).

Bacteriological Cure

Cases were not eligible for bacteriological cure when the treated quarter had no growth (n = 183) or contaminated growth (n = 25) on initial culture. If a case did not meet the appropriate collection times for follow-up samples, it was not included in analysis (n = 1). If a cow had any of the criteria defined in materials and methods as lost to follow-up, the cow was not included in analysis (n = 35). The number of cases meeting inclusion criteria was 353; 164 hetacillin-treated

cows and 189 ceftiofur-treated cows had complete data. Bacteriological cure was seen in 250 cases (71%); 112 (68%) of the hetacillin cows and 138 (73%) of the ceftiofur cows. Differences in bacteriological cure percentages based on etiology and treatment group can be seen in Figures 2.1 and 2.2.

When stratified by gram-stain classification, 74 of 106 (70%) ceftiofur-treated cows with gram-positive mastitis cured while 57 of 90 (63%) of hetacillin cows with gram-positive cultures experienced bacteriological cure. Of the cows with gram-negative mastitis, 82% of the ceftiofur group cured (n = 49/60) while a similar percentage (79%) of the hetacillin group cured (n = 42/53).

Mixed model logistic regression of bacteriological cure produced a model that included etiology and treatment group as explanatory variables (Table 2.3). Interactions between treatment group and etiology were assessed but any observed interactions were likely due to chance assuming the null hypothesis was true. When controlling for treatment, odds of bacteriological cure for all CM organisms, except for *Staph aureus* were higher than the grouping of other organisms that included *Pseudomonas,* Yeast, *Prototheca, Corynebacterium,* and *Enterococcus*. These increased odds were statistically significant for *E. coli* and *Strep. dysgalactiae.* The odds ratio (**OR**) for bacteriological cure between treatment groups was approximately 1.3 times higher for ceftiofur-treated cows compared with hetacillin-treated cows (95% confidence interval, **CI**: 0.77-2.1, $P = 0.32$). Least squares means (**LSM**) for bacteriological cure for hetacillin-treated cows was 67% (95% CI: 59%-75%), while it was 72% for ceftiofur-treated cows (95% CI: 65%-79%).

Calculation of point estimates for cure risk differences can be seen in Figure 2.3. The risk difference between the groups was -0.047, with a 95% confidence interval of -0.14 to 0.048.

When stratified by gram staining classification (n = 309; gram-positives n = 196, gram-negatives n = 113), a larger risk difference was seen in the gram-positive organisms (-0.065; 95% CI:-0.197-0.068). The risk difference was least negative for gram-negative etiologies at -0.024 (95% CI: -0.171-0.122).

Clinical Cure

A total of 572 cows met the criteria necessary to assess this outcome (hetacillin = 268, ceftiofur = 304). Of the initial 596 cows, 24 cows did not have scores recorded for the required 5 sequential days and were not included in analysis. Overall, 64% of cows experienced ≤ 4 clinical days (n = 366/572). Of the hetacillin-treated cows, 70% experienced ≤ 4 clinical days (n = 187/268) while 59% of ceftiofur-treated cows experienced ≤ 4 clinical days (n=179/304; P = 0.007). A noninferiority analysis calculated a proportion difference of 0.12 (95% CI: 0.031-0.19; Figure 2.3.).

A mixed binomial logistic regression model including treatment group and log of hospital days resulted in similar LSM of clinical cure for each group (68% for hetacillin, 64% for ceftiofur). A total of 352 cows with full data were included in regression analysis. The effects of treatment group were not statistically significant in the model (P = 0.65). When hospital days are included, the odds ratio of having ≤ 4 clinical days for hetacillin-treated cows versus ceftiofur-treated cows, although higher (1.2), is not significantly different from 1 (95% CI: 0.62-2.2; P = 0.57). As interpreted, an increase in hospital days leads to a decrease in the log-odds for the outcome of clinical days ≤ 4.

Pathogen Cure

Absence of a pathogen on both follow-up samples designated a cow as a pathogen cure. To be considered a pathogen cure, both follow-up samples as well as the enrollment sample had to be present and not contaminated (missing, n = 23; contaminated, n = 39). If a cow had any of the criteria defined in materials and methods as lost to follow up, it was not included in analysis (n = 11). If a case did not meet the appropriate collection times for follow-up samples, the case was not included in analysis (n = 2). A total of 521 cows were used in the analysis (ceftiofur = 271, hetacillin = 250) and 462 cows had full data for modeling. A cow with a no-growth or growth result upon enrollment had to maintain a no-growth result in both of its follow-up samples to be considered a pathogen cure. Overall, 44% of cows experienced a pathogen cure, 45% of hetacillin-treated cows and 43% of ceftiofur-treated cows. Mixed model logistic regression resulted in a numerically better pathogen cure (OR = 1.1) for hetacillin-treated cases as compared with the ceftiofur cases, but the observed difference was likely due to chance (P = 0.57). Least square means for hetacillin-treated cows was 35% in the model while 32% cured for ceftiofur (95% CI: hetacillin, 22%-50%; ceftiofur, 20%-47%). Variables significant in the model were etiology, previous LS, and previous milk yield (Table 2.4).

No-growth results were exempt from the analysis described by Table 2.3. However, the effects of this etiology were reflected in the logistic model for pathogen cure: when all other variables were held constant, odds of pathogen cure for all etiologies except for *E. coli* were trending lower or significantly lower than no-growth results, indicating that cows experiencing a no-growth result were likely to remain no-growth at both resample dates. While *E. coli* CM had an increased odds of pathogen cure, it was not statistically significant (P = 0.93). Interactions

between treatment group and etiology were assessed but not statistically significant to the level of $P < 0.05$. As milk yield or LS increased, the odds of pathogen cure decreased.

Point estimates for pathogen cure risk differences with confidence intervals can be seen in Figure 2.3. The risk difference between the groups was 0.020, with a 95% confidence interval of -0.065 to 0.11.

Extended Clinical Cure and Clinical Days

Using resample CS at days 14 ± 4 and 21 ± 5 (n = 520), bivariate analysis shows 92% (n = 221/241) of hetacillin-treated cows with clinical cure and 91% (n = 253/279) of ceftiofur-treated cows with clinical cure. A Chi-square comparison indicates no differences between treatment groups ($P = 0.68$). Noninferiority analysis confirms that the treatments are noninferior when assessing this outcome (risk difference = 0.010, 95% CI: -0.039-0.059).

Any cow experiencing 5 or more clinical days was given a value of 5. Bivariate analysis indicates a difference in clinical days between treatment groups ($P = 0.0026$), with the hetacillin-treated cows experiencing overall less days (3.4 d; 95% CI: 1.3-1.5) versus ceftiofur-treated cows (3.7 d; 95% CI: 1.3-1.5). Lack of normality of clinical days did not permit linear regression modeling.

Postevent Milk Yield

All cows with milk yield values at 8-43 days post-mastitis event were included in the analysis. The mean number of days between mastitis event and test date for data retrieval was 23.6 d with a median of 24 d. The total number of included cows was 542, with 261 in the hetacillin group and 281 in the ceftiofur group. Average postevent milk yield between the groups

was similar; the average test-day milk for hetacillin-treated cows was 38.2 kg while the average milk for ceftiofur-treated cows was 38.7 kg (bivariate analysis, $P = 0.54$). A mixed linear regression model used 484 observations and included DIM category, previous test-day milk yield, and etiology. The LSM for hetacillin-treated and ceftiofur-treated cows was 37.0 kg and 38.2 kg, respectively ($P = 0.09$). In the model, as expected, as previous milk yield increased, postevent milk yield increased. Cows lower in DIM had significantly greater milk production than those > 200 DIM, and the LSM for milk yield decreased with increasing DIM category. All etiology groups had lower milk production than those with no-growth results (Table 2.5).

Postevent LS

All cows with LS values at 8-43 days post-mastitis event were assessed for analysis (n = 531; hetacillin = 251, ceftiofur = 280). The mean number of days between mastitis event and test date for data retrieval was 23.6 d with a median of 24 d. Average postevent LS for hetacillin-treated cows and ceftiofur-treated cows was 3.4 and 3.1, respectively (bivariate analysis, $P = 0.66$). A mixed linear regression model included previous LS, parity, and etiology. The LSM for hetacillin-treated and ceftiofur-treated cows was 3.4 and 3.1, respectively ($P = 0.086$). A total of 466 cows were included in the model. As expected, previous LS was significant in the model; as previous LS increased, postevent LS increased. In the model, each earlier parity had a lower LS than the third and greater parity when other variables were accounted for ($P = 0.0032$). All culture results had higher LS than those cows culturing with no-growth results. *P*-values and LSM for each etiology can be found in Table 2.6.

Survival to d 30

A cow not surviving was defined as having experienced culling or death. A total of 585 cows were included in the analysis, 284 hetacillin-treated cows and 301 ceftiofur-treated cows. Overall, 95% of cows survived to day 30. Of the hetacillin-treated cows, 12 (4.2%) did not survive to 30 d compared with 15 (5.0%) of ceftiofur-treated cows. In a mixed logistic regression model with 521 observations, the odds of survival were higher in the hetacillin group (OR = 1.2) but treatment group was not statistically significant (P = 0.73). The only significant variable was previous milk yield (P = 0.022), which showed an increased odds of survival with increasing previous milk yield (OR = 1.05; 95% CI: 1.01-1.1). Noninferiority analysis resulted in a risk difference of 0.0076 (95% CI:-0.026-0.042; Figure 2.3).

Survival to d 60

The same cow inclusions were made as for survival to day 30 (n = 585; hetacillin = 284; ceftiofur = 301). Overall, 91% of cows survived to day 60. Of the hetacillin-treated cows, 22 (7.8%) did not survive to 60 d compared with 30 (10.0%) of the ceftiofur-treated cows. In a mixed logistic regression model, the odds of survival were higher in the hetacillin group (OR = 1.4) but the P-value = 0.26. In the model, the odds of survival increased as previous milk yield increased (OR = 1.04; 95% CI: 1.01-1.07; P = 0.012). The risk difference was 0.022 (95% CI:-0.024-0.068; Figure 2.3).

Time-to-Event: Survival Analysis and Mastitis Recurrence

The same cows included in logistic analysis of survival to 30 and 60 d were included in the time-to-event analysis for overall survival (n = 585; hetacillin = 284; ceftiofur = 301). A total

of 521 observations were included in Cox proportional hazards regression. Modeling for overall survival in time-to-event analysis provided a hazard ratio of 0.74 when comparing hetacillin-treated cows to ceftiofur-treated cows, indicating a reduction in risk of 26% for that group. The treatment group *P*-value was 0.27. The only variables that remained in the model were parity ($P = 0.086$) and previous milk yield ($P = 0.0010$).

Modeling for time-to-mastitis event in the same quarter provided a hazard ratio for the hetacillin group over the ceftiofur group of 1.1 ($P = 0.73$) when controlling for milk yield. One farm did not reliably record mastitis at the quarter level, leading to removal of 104 cows from analysis. Three cows were not included because their quarter ceased production within 7 d of enrollment. The total number of cows eligible for analysis was 478 (hetacillin = 229; ceftiofur = 249). A total of 421 observations were included in a Cox proportional hazards regression model. Only previous milk yield was significant in the model ($P = 0.0004$).

Hospital Days

A cow was eligible for hospital days analysis when there was a pen move indicating exit from the treatment pen in both DC305 and the written record (n = 351). Four of six of the dairies reliably recorded pen moves; cows enrolled from two of the dairies did not contribute to analysis of this outcome. The number of cows meeting criteria was 344 (hetacillin = 164; ceftiofur = 180). Data was normalized by using the log of hospital days as the outcome. Bivariate analysis revealed a greater number of hospital days for the ceftiofur-treated group (8.0 d) versus the hetacillin-treated group (6.2 d; $P < 0.001$). A mixed linear regression model included etiology as the only significant covariate, but values for LSM remained constant (ceftiofur = 8.0 d, hetacillin = 6.2 d; $P < 0.001$). Table 2.7 describes the model of log of hospital days. On average, all

etiologies aside from *Klebsiella* spp. and other experienced more hospital days than no-growth cows. Using no-growth as the base in the model, only *Staph. aureus* differed statistically in hospital days.

Table 2.1. Basic description (numbers) of clinical mastitis (CM) cases and enrolled cases on 6 commercial dairy farms (A-F) in New York during the trial period

Item	A	B	C	D	E	F	Total
Total CM cases	110	335	138	198	168	219	1,168
Total nonsevere CM cases	94	226	132	183	161	211	1,007
Number meeting eligibility criteria	74	130	84	105	129	105	627
Number of cases maintaining enrollment	71	119	78	99	125	104	596
Hetacillin	28	55	40	50	62	52	287
Ceftiofur	43	64	38	49	63	52	309
Culture results (pretreatment)							
No growth	30	53	11	18	40	31	183
Gram-positive	17	29	48	44	43	41	222
Gram-negative	8	17	8	35	29	16	113
Other[1]	16	20	11	2	13	16	78

[1]Culture results falling into this category are defined in Table 2.2.

Table 2.2. Distribution of organisms between treatment groups for pre-treatment samples

Organism	Hetacillin, no. (n = 282)	Ceftiofur, no. (n = 306)
No growth	95	88
No significant growth[1]	17	11
Escherichia coli	36	45
Klebsiella spp.	17	15
Streptococcus dysgalactiae	47	37
Streptococcus uberis	13	33
CNS	5	6
Staphylococcus aureus	21	26
Other cocci[2]	20	26
Other *Enterobacteriaceae*	6	2
Other organisms	21	29
Pseudomonas spp.[1]	0	1
Pasteurella	1	5
Proteus spp.	0	1
Yeast[1]	4	8
Prototheca spp.[1]	1	0
Trueperella pyogenes[1]	3	5
Gram-positive bacillus	1	1
Contamination[1]	0	2
Missing sample[1]	5	3

[1]Included in other category of Table 2.1.

[2]Includes non-*Strep. uberis*/non-*Strep. dysgalactiae Streptococcus* spp., *Enterococcus* spp., and *Lactococcus* spp.

Table 2.3. Parameter estimates, SE, statistics, and odds ratios (OR) for fixed effects of a mixed logistic regression model evaluating bacteriological cure = true[1] (n = 353)

Parameter	Description	Estimate	SE	*P*-value	OR	95% CI
Intercept		0.79	0.28	0.037		
Treatment	Hetacillin	-0.25	0.26	0.32	0.78	0.47-1.3
	Ceftiofur	Referent				
Etiology	*Streptococcus uberis*	0.28	0.42	0.50	1.3	0.58-3.0
	Streptococcus spp.	0.0065	0.48	0.99	1.0	0.39-2.6
	Streptococcus dysgalactiae	0.92	0.41	0.024	2.5	1.1-5.6
	Staphylococcus aureus	-1.48	0.42	0.0005	0.23	0.10-0.52
	Klebsiella spp.	0.11	0.48	0.82	1.1	0.44-2.9
	Escherichia coli	1.4	0.45	0.0023	4.0	1.6-9.7
	Other[2]	Referent				

[1]Parameters evaluated for potential confounding included cow parity, DIM at clinical mastitis event, etiology, and linear score and milk production at test day previous to event. Farm was included as a random effect but had no contribution to variance.

[2]Included nonsignificant growth, *Pseudomonas* spp., *Proteus* spp., gram-negative bacillus, yeast, *Prototheca* spp., *Trueperella pyogenes, Corynebacterium* spp., *Enterobacter* spp., and *Enterococcus* spp.

Table 2.4. Parameter estimates, SE, statistics, and odds ratios (OR) for fixed effects of a mixed logistic regression model evaluating pathogen cure = true[1] (n = 462)

Parameter	Description	Estimate	SE	*P*-value	OR	95% CI
Intercept		1.5	0.61	0.054		
Treatment	Hetacillin	0.12	0.21	0.57	1.1	0.75-1.7
	Ceftiofur	Referent				
Milk[2]		-0.027	0.01 1	0.014	0.97	0.95-1.0
Linear Score[3]		-0.10	0.05	0.037	0.90	0.82-0.99
Etiology	*Streptococcus uberis*	-0.80	0.40	0.047	0.46	0.20-1.0
	Streptococcus spp.	-1.76	0.54	0.0011	0.18	0.063-0.51
	Streptococcus dysgalactiae	-0.75	0.33	0.026	0.49	0.25-0.94
	Staphylococcus aureus	-2.37	0.55	<0.0001	0.094	0.032-0.28
	Klebsiella spp.	-0.86	0.48	0.075	0.44	0.17-1.1
	Escherichia coli	0.029	0.34	0.93	1.1	0.54-2.1
	Other[4]	-0.61	0.33	0.066	0.56	0.29-1.1
	No Growth	Referent				

[1]Parameters evaluated for potential confounding included cow parity, DIM at clinical mastitis event, etiology, and linear score and milk production at test day previous to event. Farm was included as a random effect.

[2]Milk production (kg) on test d 8 to 43 d before clinical mastitis event.

[3]Linear score on test day d 8 to 43 d before clinical mastitis event.

[4]Included nonsignificant growth, *Pseudomonas* spp., *Proteus* spp., gram-negative bacillus, yeast, *Prototheca* spp., *Trueperella pyogenes, Corynebacterium* spp., *Enterobacter* spp., and *Enterococcus* spp.

Table 2.5. Parameter estimates, SE, statistics, and LSM for fixed effects of a mixed linear regression model evaluating postevent milk production (kg)[1] (n = 484)

Parameter	Description	Estimate	SE	*P*-value	LSM (kg)	95% CI
Intercept		11.3	1.7	0.0011		
Treatment	Hetacillin	-1.2	0.68	0.091	37.0	35.9-38.2
	Ceftiofur	Referent			38.2	37.1-39.3
Previous Milk[2] (kg)		0.56	0.035	<0.0001		
Days in Milk	1-100	10.2	0.92	<0.0001	43.0	41.8-44.3
	101-200	4.2	0.89	<0.0001	37.0	35.8-38.2
	>200	Referent			32.8	31.3-34.3
Etiology	*Streptococcus uberis*	-1.7	1.4	0.20	37.9	35.5-40.4
	Streptococcus spp.	-0.85	1.6	0.59	38.8	35.9-41.7
	Streptococcus dysgalactiae	-3.2	1.1	0.0033	36.5	34.6-38.3
	Staphylococcus aureus	-1.8	1.4	0.18	37.8	35.4-40.3
	Klebsiella spp.	-1.4	1.6	0.39	38.3	35.4-41.2
	Escherichia coli	-4.2	1.1	0.0002	35.4	33.6-37.3
	Other[3]	-3.3	1.1	0.0026	36.4	34.5-38.2
	No Growth	Referent			39.7	38.4-40.9

[1]Within 8 to 43 d postevent. Parameters evaluated for potential confounding included cow parity, DIM at clinical mastitis event, etiology, and linear score and milk production at test day previous to event. Farm was included as a random effect.

[2]Milk production (kg) at previous test-date (8-43 d premastitis event).

[3]Included nonsignificant growth, *Pseudomonas* spp., *Proteus* spp., gram-negative bacillus, yeast, *Prototheca* spp., *Trueperella pyogenes*, *Corynebacterium* spp., *Enterobacter* spp., and *Enterococcus* spp.

Table 2.6. Parameter estimates, SE, statistics, LSM for fixed effects of a mixed linear regression model evaluating postevent linear score (LS)[1] (n = 466)

Parameter	Description	Estimate	SE	*P*-value	LSM	95% CI
Intercept		2.41	0.28	0.0004		
Treatment	Hetacillin	0.36	0.21	0.086	3.4	3.0-3.8
	Ceftiofur	Referent			3.1	2.7-3.5
Previous LS[2]		0.21	0.049	<0.0001		
Parity	1	-0.85	0.31	0.0064	2.9	2.3-3.5
	2	-0.64	0.23	0.0065	3.1	2.7-3.6
	≥3	Referent			3.8	3.4-4.1
Etiology	*Streptococcus uberis*	0.92	0.41	0.024	3.5	2.7-4.3
	Streptococcus spp.	0.082	0.48	0.87	2.7	1.7-3.6
	Streptococcus dysgalactiae	0.76	0.33	0.024	3.3	2.8-3.9
	Staphylococcus aureus	1.8	0.44	<0.0001	4.3	3.5-5.1
	Klebsiella spp.	0.69	0.48	0.15	3.3	2.4-4.2
	Escherichia coli	0.60	0.35	0.085	3.2	2.6-3.8
	Other[3]	0.68	0.33	0.039	3.3	2.7-3.8
	No Growth	Referent			2.6	2.1-3.0

[1]Within 8 to 43 d postevent. Parameters evaluated for potential confounding included cow parity, DIM at clinical mastitis event, etiology and LS and milk production at test day previous to event. Farm was included as a random effect.

[2]Linear score at previous test-date (8-43 d premastitis event)

[3]Included nonsignificant growth, *Pseudomonas* spp., *Proteus* spp., gram-negative bacillus, yeast, *Prototheca* spp., *Trueperella pyogenes*, *Corynebacterium* spp., *Enterobacter* spp., and *Enterococcus* spp.

Table 2.7. Parameter estimates, SE, statistics, and LSM of a mixed linear regression model evaluating log(hospital days)[1] (n = 344)

Parameter	Description	Estimate	SE	*P*-value	LSM (d)	95% CI
Intercept		2.0	0.14	0.0007		
Treatment	Hetacillin	-0.26	0.03	<0.0001	6.2	4.7-8.1
	Ceftiofur	Referent			8.0	6.1-10.4
Etiology	*Streptococcus uberis*	0.05	0.08	0.50	7.0	5.1-9.4
	Streptococcus spp.	0.11	0.072	0.11	7.4	5.6-10.0
	Streptococcus dysgalactiae	0.087	0.058	0.14	7.23	5.5-9.6
	Staphylococcus aureus	0.22	0.062	0.0004	8.3	6.2-11.0
	Klebsiella spp.	-0.055	0.068	0.42	6.3	4.7-8.4
	Escherichia coli	0.017	0.050	0.74	6.8	5.2-8.9
	Other[2]	-0.0025	0.054	0.96	6.6	5.0-8.7
	No Growth	Referent			6.6	5.1-8.7

[1]Parameters evaluated for potential confounding included cow parity, DIM at clinical mastitis event, etiology, and linear score and milk production at test day previous to event. Farm was included as a random effect.

[2]Included nonsignificant growth, *Pseudomonas* spp., *Proteus* spp., gram-negative bacillus, Yeast, *Prototheca* spp., *Trueperella pyogenes*, *Corynebacterium* spp., *Enterobacter* spp., and *Enterococcus* spp.

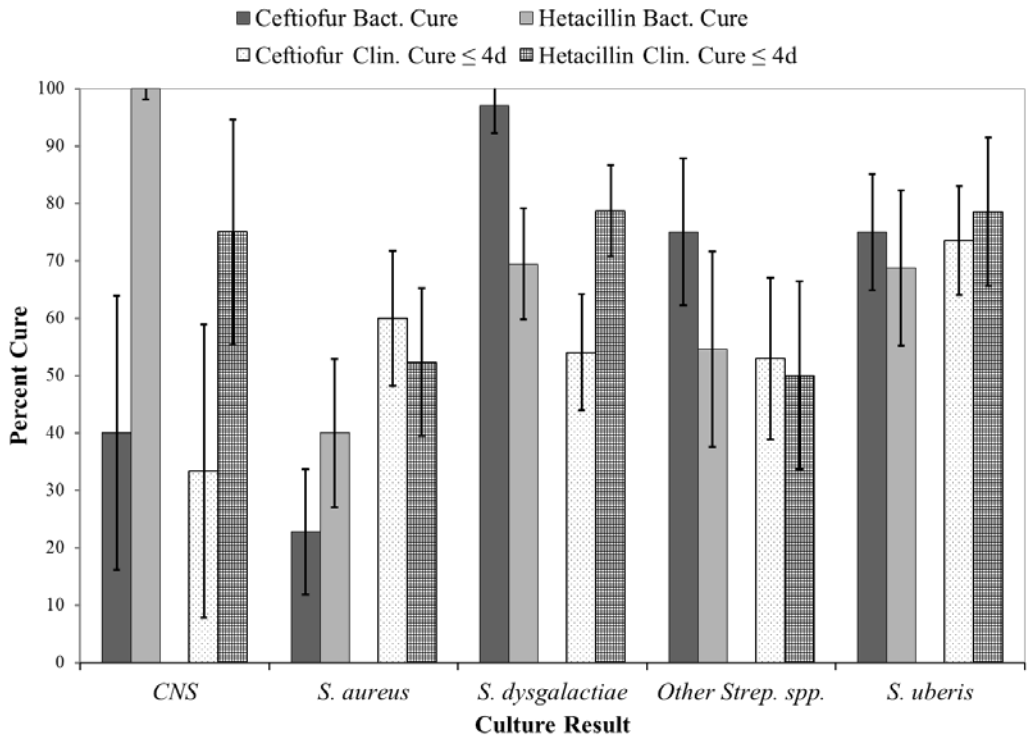

Figure 2.1. Bacteriological (Bact.) and clinical (Clin.) cure percentages for the following mastitis etiologies on aerobic culture: CNS, *Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis*, and other streptococcal organisms. Error bars indicate 95% CI based on SE of the mean proportion.

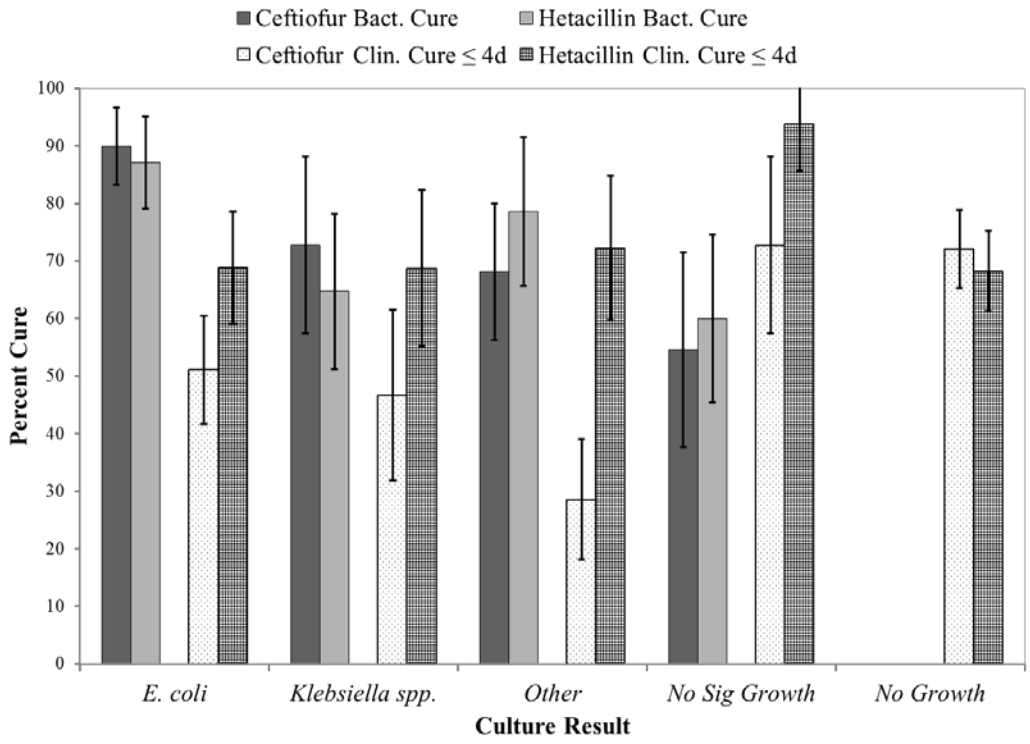

Figure 2.2. Bacteriological (Bact.) and clinical (Clin) cure percentages for the following mastitis etiologies on aerobic culture: *Escherichia coli*, *Klebsiella* spp., and other defined as *Pseudomonas* spp., *Proteus* spp., gram-negative bacillus, yeast, *Prototheca* spp., *Trueperella pyogenes*, *Corynebacterium* spp., *Enterobacter* spp., and *Enterococcus* spp. No significant (Sig) growth and no growth also included. Cows with no growth results were not included in bacteriological cure analysis. Error bars indicate 95% CI based on SE of the mean proportion.

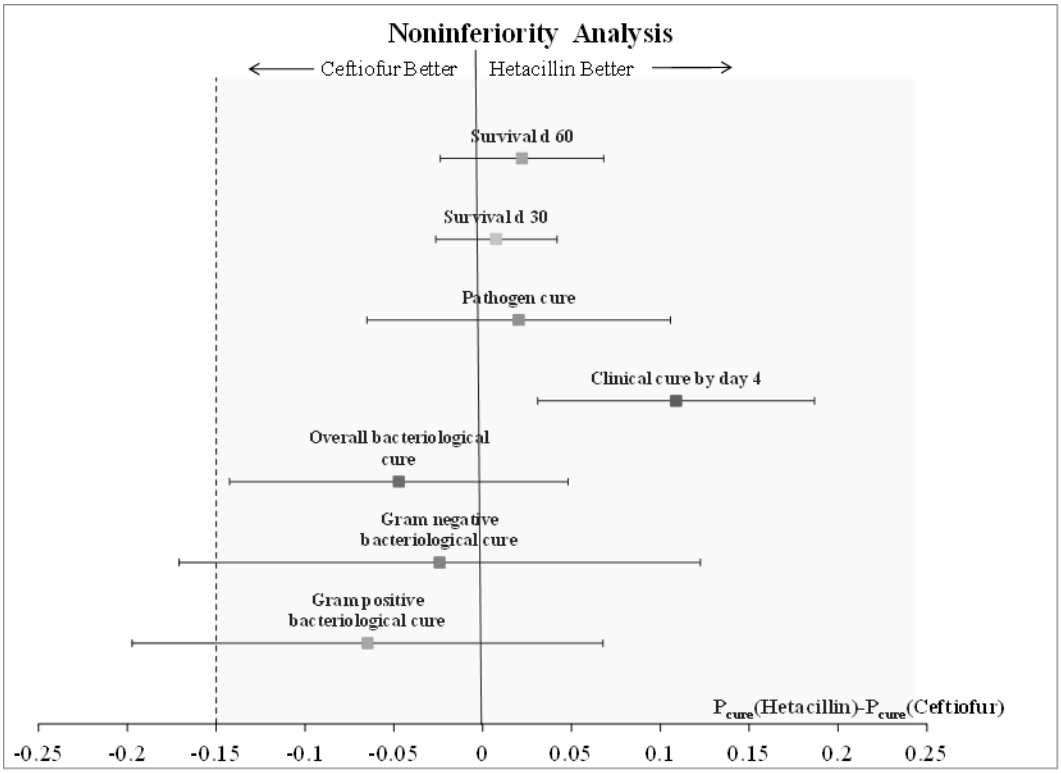

Figure 2.3. Representation of outcome probability differences for hetacillin versus ceftiofur in a noninferiority comparison. The gray boxes represent the point estimates for each difference. Arrowheads indicate a 95% CI. Shaded area is representative of noninferiority. Intervals falling in the area >0 conclude superiority of hetacillin to ceftiofur. Values crossing -0.15 (the noninferiority margin) are inconclusive. Overall bacteriological cure = all etiologies. Pathogen cure is defined as absence of a pathogen on both follow-up samples (d 14 and 21). P_{cure} = Probability of cure.

DISCUSSION

Using a noninferiority comparison, this study evaluated the efficacy of two IMM antibiotic preparations for the treatment of mild to moderate CM. The most important findings are summarized in Figure 2.3; the current study found that hetacillin was noninferior to ceftiofur in the primary outcomes of bacteriological, pathogen, and clinical cures and secondary outcomes describing survival to 30 and 60 d. Hetacillin was superior to ceftiofur when clinical days was dichotomized to less than or equal to 4 d.

The overall bacteriological cure risk difference between the groups was -0.047. Noninferiority could be stated as the 95% confidence interval, -0.143 to 0.048, falls above the noninferiority margin of -0.15. When comparing gram-positive to gram-negative etiologies, a larger risk difference than for an overall comparison was seen in CM caused by gram-positive organisms. Upon further examination of the risk difference for gram-positive etiologies, it was noted that although numerically higher cure rates for hetacillin-treated CNS and hetacillin-treated *Staph. aureus* cows than the respective ceftiofur-treated cows, ceftiofur had higher cure rates in the more represented culture outcomes such as *Strep. dysgalactiae* (Figure 2.1). The bacteriological cure risk difference was lowest (least negative) for gram-negative etiologies, resulting from a numerically higher but non-significant cure rate for ceftiofur-treated *E. coli* and *Klebsiella* cows versus the respective hetacillin-treated cows. The confidence intervals of the risk differences for gram-positive and gram-negative etiologies cross both the noninferiority limit and zero, suggesting that we lacked the power to identify an observed difference smaller than our declared margin. This may have been due to the smaller sample size when culture results were separated by gram-negative and gram-positive status.

Despite the large difference seen in bacteriological cure of *Strep. dysgalactiae* CM between hetacillin and ceftiofur-treated cows (ceftiofur having a greater cure), the rate of clinical cure by day 4 for this organism was greater for hetacillin-treated cows. The same could be seen for the gram-negative organisms *E. coli* and *Klebsiella* spp.: despite a numerically greater bacteriological cure for ceftiofur, this drug had a lower percentage of clinical cures for these organisms as compared to hetacillin. Analysis of the binary outcome "clinical cure in less than 4 days" resulted in a positive risk difference of 0.119; a confidence interval beyond both the noninferiority limit, -0.15, and zero, indicates superiority of hetacillin over ceftiofur for this outcome (Figure 2.3). One could speculate that clinical cure rates may be higher for hetacillin cows due to bias: CS is a subjective measure and having to treat ceftiofur cows two additional days may have led observers to be more critical of symptoms before administration of medication. Blinding in regards to treatment was not performed in this trial, but producers and researchers were unaware of objective primary outcomes, including bacteriologic and pathogen cures and the quantitative secondary outcomes LS and milk production.

In order to obtain the power and numbers needed to evaluate CM treatment efficacy, trials that use bacteriological cure as a primary outcome must restrict treatment assignment to cases in which bacteria are isolated. This then determines the need to wait for culture results prior to administration of the product (Schukken and Deluyker, 1995). In this study, we used a pathogen cure outcome, following and modifying the ideas outlined in Pinzón-Sánchez and Ruegg (2011) and Oliveira et al. (2013) to facilitate the inclusion of no-growth results. This allows researchers to analyze the risk of remaining pathogen free. Our values of 43% and 45% for pathogen cure of ceftiofur and hetacillin-treated cows, respectively, were lower than the 65% (n = 276/427) found in the Oliviera et al. study (IMM treatment with any antimicrobial). Low

values for pathogen cure may be due to the large number (~25%) of *Strep.* spp. positive cows in the trial. While the cure rate for CM caused by environmental streptococci may exceed 50%, cows can suffer frequent relapses (Guterbock et al., 1993; Van Eenennaam et al., 1995; Morin et al, 1998). The delay of treatment may have impacted these cows as aggressive IMM treatment of *Strep. uberis* CM has been shown to result in cure rates that exceed 90% (Hillerton and Kleim, 2002).

Our average clinical days, 3.4 and 3.7 for hetacillin and ceftiofur-treated cows, respectively, are lower than the ceftiofur-treated cows (4.5 d, n = 262) and cephapirin treated cows (4.9 d, n = 49) in a 2014 CM study performed by Oliveira and Ruegg. Our results were also less than cephalosporin treated cows in an earlier study (2011) by Pinzón-Sánchez and Ruegg (5.4 d, n = 143). This is likely due to our assignment of 5 clinical days to those cows that exhibited clinical signs beyond the initial observation period. An additional study detected lower values than found in the present study for clinical days, at 2.7 d and 3.2 d for blanket-treated cephapirin and selectively treated cephapirin cows, respectively (Lago et al., 2011a). Despite these deviations from our findings, all authors referenced concluded no statistical differences between treatment groups in days to clinical cure.

For extended clinical outcome comparisons, 92% of hetacillin-treated cows and 91% of ceftiofur-treated cows retained their cured statuses on days 14 and 21 post-enrollment. This differs from the Schukken et al. (2013) study that found an overall clinical cure of 62% (n = 184/296) at days 10 and 17. This may be due to the clinical scoring system in that study, which included rectal temperature, hydration scoring, and attitude assessment.

The model for postevent milk production included etiology as a significant variable. When the causative microorganism was absent on initial culture (a no-growth result), cows

responded with a significantly higher milk production than those with growth. Mastitis is associated with losses in milk production, particularly for coliform cases (Gröhn et al., 2004). Our data indicates that cows with *E. coli* average the greatest loss in milk production at next test day relative to other etiologies. Values were similar to a change of -4.62 kg found in a 2004 study by Gröhn et al. for cows 15-21DIM. In the latter study, however, *Klebsiella* affected cows suffered the greatest loss with an average change of -6.24 kg of milk per day during this time period. Our differences may be reflective of the few numbers of *Klebsiella* cases or the various genetic strains found on six dairies (Gröhn et al., 2004).

Parity, previous LS, and etiology were significant in a model for postevent LS. Inclusion of these variables agreed with known risk factors associated with short-term post-treatment outcomes (Pinzón-Sánchez and Ruegg, 2011). In regards to etiology, *Staph. aureus* cows experienced the highest postevent LS. This is supported by the fact that *Staph. aureus* is commonly cultured in dairies with high bulk tank SCCs (Barkema et al., 1997). Treatment was not significant in the model. No comparisons have been described in recent literature between hetacillin and ceftiofur for post-treatment milk quality and quantity indices. However, a 2011 trial performed by Schukken et al. concluded no significant differences in SCC nor milk yield for the remainder of the lactation when considering ceftiofur-treated gram-negative cases and untreated gram-negative cases.

Noninferiority was described for hetacillin in both survival indices (Figure 2.3). Historically, the culling rate for CM cows was estimated to be more than twice that of non-CM cows; the risk of culling is highest in the period immediate to the mastitis event (Barkema et al., 1997; Gröhn et al., 2004). For this reason, we used a 60 d interval for event analysis, but expected to find similar results had we followed the cows for a longer period of time. Though

this is the first trial comparing IMM hetacillin to ceftiofur, recent selective treatment trials have reported no statistical differences in removal from the herd within the weeks after CM when cows were treated with different cephalosporin products or protocols (Lago et al., 2011a; Lago et al., 2011b). The costs of culling contribute to the total costs of CM and also influence the benefits returned from intensive antimicrobial treatments; knowledge of noninferiority of one product to another for survival indices is economically important (Steeneveld et al., 2011).

Milk yield was similarly significant in Cox proportional hazards analysis when assessing survival in the herd and time-to-next-mastitis in the same quarter. However, the recurrence risk of CM was not dependent on etiology in our analysis. While this model agreed with a previous study performed by Lago et al. (2011b) in which the effect of organism was not significant, etiology was a sensitive indicator for recurrence risk in a streptococcal CM study (Van Eenennaam et al., 1995). The uneven distribution of *Strep. uberis* may have precluded the detection of an effect of etiology in survival analysis. Additionally, the larger percentage of these cows in the ceftiofur-treated group may introduce bias when assessing etiological analysis for each of the treatment outcomes, particularly if ceftiofur cows generally experience higher cure rates for this etiology.

The only significant difference between treatment groups was for hospital days, with a difference in LSM of 1.8 d, favoring hetacillin. This difference results from two less treatment days for hetacillin cows while maintaining the same 72-hour milk withhold for each product. Within the design of the study, although milk withhold times were the same, treatment duration protocols for the drugs were not identical. Based on a recent Wisconsin study, which characterized an average duration for IMM ceftiofur use between 4 and 5 days, 5 days was selected for treatment length in this trial (Oliveira and Ruegg, 2014). The authors felt that

selection of this duration 1) reflected current farm use, and 2) permitted ceftiofur the best chance to display better efficacy over hetacillin. The hetacillin label indication dictated the 3 day treatment period for this drug. While the difference may be evident to readers, as producers are simply following the pasteurized milk ordinance, we were unable to assume that hetacillin-treated nor ceftiofur-treated cows would experience unresolved clinical signs beyond the withhold date without actually testing it. Cows in the study remained in the hospital pen until their milk was no longer violative and symptoms of CM were resolved. Initial study design considered that clinical days may have been a more important factor in hospital day analysis than both the withhold and treatment durations. An economic analysis for hetacillin treatment in regards to decreased labor costs and increased days of saleable milk was not performed.

When assessing severity of CM, approximately 14% of cows did not qualify due to systemic signs. This was similar to the 15.3% value found in a survey of 50 Wisconsin dairy farms (Oliveira et al., 2013), but higher than values found on a smaller survey (8%, n=266) and in a recent Canadian survey (10%, n=2,311; Olde Riekerink et al., 2008; Pinzón-Sánchez and Ruegg, 2011). Exclusion of severe CM cows limits the generalizability of our results to cows with nonsevere mastitis. Due to individualized farm-specific treatment for severe mastitis, additional trials would need to be performed to assess differences in the two antibiotic products.

CONCLUSIONS

A CM trial comparing a 5 d treatment with ceftiofur to a 3 d treatment with hetacillin showed noninferiority of the latter in regards to bacteriological cure, clinical cure, pathogen cure, and survival to 30 and 60 days. When noninferiority analysis is applied to gram-positive and gram-negative etiological groups, the data is inconclusive. However, results are consistent with

the fact that these two IMM products are effective against one or more gram-positive organisms as well as the gram-negative *E. coli*. No statistically significant differences in continuous outcomes such as clinical days, postevent milk yields, and postevent linear scores were found between treatment groups. Hospital days differed favorably for hetacillin versus ceftiofur, allowing milk from hetacillin-treated cows to contribute to the bulk tank 1.8 days earlier than the respective ceftiofur-treated cows. No effect of treatment was observed on risk of leaving the herd in 60 d or on risk of experiencing a new mastitis event in the same quarter. Differences in bacteriological cure profiles, however, determine the importance of pre-treatment diagnostics. Farms with access to culture results may benefit most from our results. Our findings can aid in herd-specific decisions, specifically in developing treatment protocols for nonsevere CM.

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CHAPTER THREE

CLINICAL OUTCOME COMPARISON OF IMMEDIATE BLANKET TREATMENT VERSUS A DELAYED PATHOGEN-BASED TREATMENT PROTOCOL FOR CLINICAL MASTITIS IN A NEW YORK DAIRY HERD

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ABSTRACT

The purpose was to compare immediate intramammary antimicrobial treatment of all cases of clinical mastitis to a selective treatment protocol based on 24 h culture results. The study was conducted at a 3500 cow commercial farm in NY. Using a randomized design, mild to moderate clinical mastitis cases were assigned to either the blanket therapy or pathogen-based therapy group. Cows in the blanket therapy group received immediate on-label intramammary treatment with ceftiofur hydrochloride for 5 d. Upon receipt of 24 h culture results, cows in the pathogen-based group followed a protocol automatically assigned via Dairy Comp 305: *Staphylococcus* spp., *Streptococcus* spp., or *Enterococcus* spp. were administered on-label intramammary treatment with cephapirin sodium for 1 d. Others, including cows with no-growth or gram-negative results, received no treatment. A total of 725 cases of clinical mastitis were observed; 114 cows were not enrolled due to severity. An additional 122 cases did not meet inclusion criteria. Distribution of treatments for the 489 qualifying events was equal between groups (pathogen-based, n = 246; blanket, n = 243). The proportion of cases assigned to the blanket and pathogen-based groups that received intramammary therapy was 100% and 32%, respectively. No significant differences existed between blanket therapy and pathogen-based therapy in days to clinical cure; means were 4.8 d and 4.5 d, respectively. The difference in post-event milk production between groups was not statistically significant (blanket therapy = 34.7 kg; pathogen-based = 35.4 kg). No differences were observed in test-day linear scores between groups; least squares means of linear scores was 4.3 for pathogen-based cows and 4.2 for blanket therapy cows. Odds of survival 30 d post-enrollment was similar between groups (odds ratio of pathogen-based as reference = 1.6; 95% confidence interval: 0.7-3.7) as were odds of survival to 60 d (odds ratio = 1.4; 95% confidence interval: 0.7-2.6). The one significant difference found

for the effect of treatment was in hospital days; pathogen-based cows experienced, on average, 3 fewer days than blanket therapy cows. A majority (68.5%) of moderate and mild clinical cases would not have been treated if all cows on this trial were enrolled in a pathogen-based protocol. The use of a strategic treatment protocol based on 24 h post-mastitis pathogen results has potential to efficiently reduce antimicrobial use.

Key words: clinical mastitis, cephapirin, ceftiofur, no treatment

INTRODUCTION

Clinical mastitis (**CM**) is defined by visible signs of inflammation in an affected mammary gland such as redness, swelling, pain or heat, and alterations such as clots, flakes, discoloration, or abnormal consistency of secretions. Clinical mastitis has a high incidence on North American dairy farms, ranging from 20% to 51% of cows (Sargeant et al., 1998; Olde Riekerink et al., 2008). This disease can create severe economic losses due to discarded milk, reduced production, decreased conception, premature culling, transmission to other cattle, and treatment costs (Fetrow, 2000; Hertl et al., 2014). Current practice on many farms is treatment of all CM cases or "blanket treatment" with intramammary (**IMM**) antimicrobials. In a previous Wisconsin study, 80% of all antimicrobial drugs used on dairies were used for treatment or prevention of mastitis (Pol and Ruegg, 2007a). Problems attributed to the use of antimicrobials in animals include potential drug residues in the food supply, possible development of antimicrobial resistance, and monetary losses associated with treatment and discarded milk (Owens et al., 1997; Barton, 2000).

A recent economic deterministic approach estimated financial losses for CM during the first 30 days of lactation at $444 per case, accounting for diagnostics, antimicrobial costs, non-

saleable milk, veterinary costs, milk and reproductive losses, and replacement costs (Rollin et al., 2015). Pathogen-specific treatment may be preferential in an economic model as it has the ability to decrease the use of antimicrobials, leading to reduced risks of residues and lower treatment and milk-discard costs (Schukken et al., 2011; MacDonald et al., 2011).

Targeted therapy of CM using on-farm culture (**OFC**) results or other accurate diagnostic tools can replace the routine use of broad-spectrum antimicrobials, replacing the dated practice of treating without diagnosis (Hogeveen et al., 2011). Selective treatment of CM is defined as the use of antimicrobials only for cases that may benefit from them; outcomes regarding antimicrobial usage in specific pathogen groups have been studied in depth. Differences in cure rates between etiological bacteria may be attributed to the targeting of components of bacterial cell walls (*Pyörälä* et al., 1994) . This proves difficult in gram-negative bacteria due to their complex additional lipopolysaccharide layer, likely reflected in the failure of efficacy for antimicrobial products in induced coliform mastitis trials (Lago et al., 2014). Where antimicrobials are not used, 85% spontaneous bacteriological cure rates for experimentally induced gram-negative *Escherichia coli* have been observed by day seven (Leininger et al., 2003). Furthermore, 30% or greater of CM cases exhibit culture-negative outcomes when sampled, for which the use of antimicrobials can be difficult to justify (Lago et al., 2011a; Oliveira and Ruegg, 2014).

Contrary to gram-negative CM, many IMM products are labeled for the treatment of gram-positive bacteria. Aggressive intramammary treatment of clinical and subclinical cows infected with coagulase-negative *Staphylococcus* spp. (**CNS**) and experimentally induced cases of environmental *Streptococcus uberis*, for example, is often successful with some cure rates exceeding 90% (Oliver et al., 2004; Hillerton and Kleim, 2002). Alternatively, the spontaneous

cure rate for CM caused by environmental *Streptococcus* spp. may exceed 50% but these cows can suffer frequent relapses (Morin et al., 1998).

Subsequent to the publication of studies analyzing "gram-specific" treatment response, farms gravitated toward a "treat or no-treat" system: gram-positive environmental pathogens were treated with IMM antimicrobials and gram-negative and no-growth culture cows remained untreated. No significant differences were seen in probability of bacteriological cure of a culture-based system versus blanket therapy when such a protocol was employed (Keefe et al., 2010). Likewise, Lago et al. (2011a,b) showed no significant differences between blanket treatment and selective treatment groups in CM recurrence, days to clinical cure, bacteriological cure risk, treatment failure risk, somatic cell count (**SCC**), culling, or milk production when 24-hour culture results were used to make treatment decisions on eight commercial farms.

Antimicrobial products currently available for IMM use not only have varying label claims that include effectiveness against gram-negative organisms, but also have diverse durations of use, formulations, and withdrawal periods, making it difficult to determine whether a treat or no-treat regimen is beneficial. Cephapirin sodium, a first generation cephalosporin, was recently described as noninferior to ceftiofur hydrochloride, a third generation cephalosporin, when considering bacteriological cure of gram-positive etiologies and clinical cure of all CM cases (Schukken et al., 2013). Results of this study led to speculation of how cephapirin would perform in a treat or no-treat pathogen-based system, as treatment time would result in three less antimicrobial treatments and four less treatment days per cow as compared to the more widely used ceftiofur: a Wisconsin survey of 51 dairies found of the cows receiving only IMM treatment for CM, 74.9% received ceftiofur and 13.7% received cephapirin (Oliviera and Ruegg, 2014). Economic benefits could be realized with reduction in treatment time, duration, and product cost.

The current trial is not a comparison of two different antimicrobials. Our objective, rather, was to determine if a protocol based on culture results, specifically treating gram-positives with cephapirin, offered similar outcomes to treating all cows with ceftiofur. To compare, we investigated differences in days to clinical cure, milk production, linear score (**LS**), risk of culling post-CM event, and hospital days. Our hypothesis is that a pathogen-driven treatment protocol as used in the current trial, may not only decrease the use of antimicrobials and protect aspects of public health, but also create economic benefits for the farm.

MATERIALS AND METHODS

Study Animals

Clinical mastitis cases were assessed for inclusion at a 3500 Holstein-cow commercial dairy in central New York between December 2014 and April 2015 under Institutional Animal Care and Use Committee approval. This farm was chosen due to its large herd size, a monthly incidence of five to six percent CM, availability of reliable health records, consultation opportunity with management and veterinarians, and access to microbiological diagnosis of milk samples within 24 h of collection. This farm used Dairy Herd Information Association (**DHIA)** services, which included monthly SCC and milk weights. Health records included treatment, treatment pen moves, time to resolution of clinical signs, and culling data.

Case Definition

Each CM case was detected by trained on-farm employees by observing abnormalities in milk such as changes in consistency and color or “udder” signs including hard, swollen, or red quarters. Cows exhibiting severe symptoms such as depression, anorexia, dehydration, or fever

received systemic antimicrobials and anti-inflammatories and were excluded from the study. Other exclusion criteria were treatment with antimicrobials or anti-inflammatories in the previous 15 d or impending sale of the animal. Multiple quarters and subsequent cases from an individual cow were included. A cow was not excluded if it had multiple cases of mastitis before enrollment.

Sample Collection and Treatment Assignment

Using sterile technique, a milk sample was collected from each affected quarter into a milk culture tube. The tube was labeled and promptly placed in a 5°C refrigerator. Severity, quarter, and date entering hospital were recorded in Dairy Comp 305 (**DC305**). Culture results within 24 h of sample collection were achieved through the use of daily sample pick-up via the Quality Milk Production Services (**QMPS**) program at the Animal Health and Diagnostic Center in Ithaca, NY. The study was a randomized design with cows in the herd assigned by DC305 to either the blanket therapy group or the pathogen-based treatment group. If a cow was enrolled a successive time, the same treatment group was assigned.

Microbiological Methods

Standard culture technique was performed on all samples by QMPS employees according to National Mastitis Council guidelines for identification of aerobic organisms and *Mycoplasma* spp. (National Mastitis Council, 1999). Briefly, 0.01 mL of milk was streaked on trypticase soy agar containing 5% sheep blood and 1% esculin (PML Microbiologicals; Mississauga, ON, Canada) and MacConkey's agar (Hardy Diagnostics; Santa Maria, CA) for identification of gram-negative organisms, and plates were incubated aerobically at 37°C for 18-24 h. After

observation of colony morphology and hemolytic patterns, isolates were examined further by means of 3% potassium hydroxide, gram staining, catalase and oxidase testing, PathoDx® Strep Grouping Latex tests (Thermo Fisher Scientific; Lenexa, KS), and additional biochemical and metabolic evaluations as needed. *Mycoplasma* spp. were identified using a dissection microscope after anaerobic incubation at 37°C for seven days on *Mycoplasma* agar (Sigma-Aldrich LLC; St. Louis, MO).

Diagnosis and confirmation of IMM infection by culture of a single quarter milk sample was based on standard published definitions (Dohoo et al., 2011). Confirmation of etiologic cause was defined as the isolation of one or more colonies from ten microliters of milk for all pathogens except for CNS. For CNS, two or more colonies isolated from the sample were needed to establish the presence of an infection. When two or more different bacterial pathogens were identified in the same sample, the result was classified as a mixed infection. If one non-pathogenic or ≥ two dissimilar non-pathogenic colony types were present in low levels (one to five colonies), the culture was defined as having "no significant growth" (*i.e.*, the cow is unlikely to have an intramammary infection). Contamination was defined as the isolation of three or more different colony types (> five colonies each) from the milk sample.

A cow in either treatment group with positive cultures for *Prototheca* spp., *Mycoplasma* spp., *Staphylococcus aureus,* or *Streptococcus agalactiae* was culled when its meat residue was no longer violative. This practice was standard herd policy before the start of the trial. Secondary IMM treatment after the eighth day of enrollment was allowed in cases that did not respond to initial treatment. Any cow not following protocol was excluded from analysis. Each cow was followed until the end of its current lactation, the date it was culled, or 60 d post-enrollment, whichever arrived first.

Treatment Groups

Blanket Therapy Group (BT). Immediately after enrollment, cows received one tube of ceftiofur hydrochloride into the affected quarter(s) (Spectramast LC®; Zoetis, Florham Park, NJ). The treatment was repeated once every 24 h for five days according to label directions.

Pathogen-Based Treatment Group (dPB). Direct upload of culture results to the on-farm computer was performed daily. Dairy Comp 305 automatically assigned the following treatment regimen based on pathogen: cows positive for CNS, *Streptococcus* grp G, *Streptococcus* grp C, *Streptococcus dysgalactiae, Strep. uberis, or Enterococcus* spp. were administered one IMM tube of cephapirin sodium (ToDAY®; Boehringer Ingelheim, St. Joseph, MO) every 12 h for two treatments according to label directions. A cow positive for any other organism or no growth on culture plates was assigned to no treatment. If two organisms were present or mastitis occurred in two quarters, the presence of CNS or *Strep.* spp. determined treatment in all CM quarters with cephapirin.

All CM cows remained in the treatment pen until milk withdrawal times were met and milk returned to normal visual appearance. Entrance and exit dates, as well as the date that clinical signs became absent were recorded by trained on-farm personnel. Any cow that graduated to severe clinical signs exited the trial and was treated systemically according to veterinary recommendations.

Treatment Outcomes

Cows in the study were followed up for: clinical days, post-treatment milk production, post-treatment LS, survival in the herd, and hospital days. The clinical days outcome was defined as the time to clinical cure and was calculated as the number of days from entrance into the

treatment pen until milk, udder, and systemic abnormalities associated with CM were undetectable. Linear score is a transformation of SCC, calculated as [Ln(SCC / 100) / Ln(2)] + 3 (Ali and Shook, 1980). Post-treatment LS and milk production were obtained from the test day 8-43 d post-CM event. The hospital days outcome was defined as the time in the treatment pen and was calculated as the number of days between entrance and exit into and from this pen. All values and dates were retrieved from farm management (clinical days), DHIA test-day records (LS and milk production), and DC305 (culling and hospital days).

Data Analysis

The effects of selected explanatory variables on post-treatment outcomes were analyzed using general linear models for continuous variables and binomial logistic regression models for dichotomous outcomes. Continuous outcomes included clinical days, post-treatment milk production, post-treatment LS, and hospital days. Dichotomous outcomes included retention in the herd 30 d post-enrollment and retention in the herd 60 d post-enrollment. Continuous explanatory variables included milk production and LS at previous DHIA test (8-43 days before the event). Explanatory categorical variables tested were mastitis event (1^{st}, 2^{nd}, or $>2^{nd}$), parity (1^{st}, 2^{nd}, or $\geq 3^{rd}$), and days in milk (0-100 **DIM**, 101-200 DIM, and >200 DIM). All analyses were performed in SAS© version 9.4 (SAS Institute, 2014) using PROC GLM and PROC LOGISTIC. To select the best model, all possible confounding variables were subjected to bivariate analysis by means of Chi-squared, t-tests, or analysis of variance tests using PROC FREQ, PROC TTEST, PROC REG, or PROC ANOVA. Interactions of each variable with the main effect of treatment group were assessed in a similar manner and any terms with a *P*-value < 0.2 were offered into the models. Each variable was then examined by manual backwards

stepwise elimination in the regression until all possible explanatory variables with $P < 0.1$ were included. In many of the models, the treatment variable was forced ($P > 0.1$). We used least squares means (**LSM**) and $\alpha = 0.05$ to describe means and confidence intervals (**CI**), respectively, unless otherwise noted. Additionally, basic graphing techniques were used to assess normality, homoscedasticity, and linearity of variables. Data transformations were performed and reported as necessary.

Six final models were established describing 1) the average number of clinical days, 2) post-event milk production, 3) post-event LS, 4) the odds of survival at 30 d, 5) the odds of survival at 60 d, and 6) the average number of hospital days. Not all included animals had complete LS or milk yield data as some cows experienced the event in early lactation with no prior test day or experienced the event late in lactation with no post-CM test day. Cows were excluded from clinical days or hospital days analyses if pen moves were incomplete in the records. An animal was not included in a model if it was missing a data point for a parameter offered the model; the number of animals with complete data for each analysis is indicated in each table.

RESULTS

Descriptive Data

A total of 725 cases of CM were observed. Of those, 489 cases were enrolled in the study. Distribution between groups was similar with 50% cows (n = 246/489) assigned to the dPB protocol and 50% of cows (n = 243/489) assigned to the BT group. Cows with severe mastitis, amounting to 16% (n = 114/725; dPB, n = 62; BT, n = 52; $P = 0.33$), were not eligible for enrollment. Cows were excluded from analysis if they received: the wrong treatment (n = 24;

dPB, n = 14; BT n = 10, $P = 0.68$), treatment with an additional antimicrobial within 7 d of enrollment (n = 57; dPB, n = 35; BT n = 22, $P = 0.17$), or an event entry error (n = 4; dPB, n = 1; BT, n = 3, $P = 0.35$). Cows excluded due to *Staph. aureus* positive and *Mycoplasma* spp. positive cultures were 4% (n = 28/725; dPB, n = 15; BT, n = 13, $P = 0.85$) and 1% (n = 9/725; dPB, n = 8; BT, n = 1, $P = 0.04$), respectively. Of the 489 cases enrolled in the study, parity distribution was 19% (n = 93/489), 30% (n = 147/489), and 51% (n = 249/489) for first, second, and third lactation and greater. The overall mean and median for DIM was 165 d and 151 d, respectively. Sixty percent of cows (n = 294/489) were experiencing their first mastitis event of the current lactation; this number was greater in the BT group than the dPB group (dPB, n =137; BT, n =157; $P = 0.05$). The relationship is reversed in those cows experiencing their $>2^{nd}$ mastitis event (dPB, n = 57; BT, n = 35; $P = 0.02$). Distribution of these descriptors by treatment group can be seen in Table 3.1. The mean and median number of days between enrollment and retrieval of test day data was 23 d (S.D.=10 d) and 21 d, respectively.

On a herd level, 70% (n = 340/489) of cultures produced diagnostic bacterial growth. The most commonly isolated pathogens at 24 h post-incubation were gram-negative bacilli (34%; n = 164/489), followed by *Streptococcus* spp. (32%; n = 155/489) and CNS (2%; n = 10/489). At 48 h post incubation, gram-negative organisms were further defined: 23% of all CM etiologies were *Escherichia coli* (n = 113/489), 6% *Klebsiella* spp. (n = 30/489), and 1% *Pasteurella* (n = 8/489). Among the gram-positive organisms, *Strep. uberis* was most commonly isolated (14%; n = 66/489) followed by *S. dysgalactiae* (13%; n = 63/489), other *Streptococcus* spp. (4%; n = 21/489), and CNS (2%; n = 11/489). The remaining 48 h diagnoses (gram-positive bacilli, *Enterococcus* spp., *Corynebacterium* spp., *Enterobacter* spp.) each represented less than 1% of all CM cases. Distribution of 24 h culture results by treatment group are listed in Table 3.2.

Treatment assignment and pathogen diagnostics resulted in 79 of 246 cows in the dPB treatment group receiving cephapirin, leaving 68% of CM cases (n = 167/246) in the dPB group not treated with IMM antimicrobials.

Effect of Treatment Protocol

Effect on Clinical Days. Cases were only eligible for clinical days analysis if they had a recorded date for when milk and udder signs returned to normal. Exactly 59 dPB cows were lost to follow up for clinical days analysis due to placement into a non-hospital pen during the first month of the trial. Full data existed for all variables in the model for 150 dPB cows and 219 BT cows. Mixed linear regression involved a log transformation of the clinical days outcome to provide normality of the variance. The resulting model provided a numerically lower number of clinical days for the dPB group, however, this difference was not statistically significant (LSM for dPB group = 4.5 d, BT = 4.8 d; $P = 0.12$). The results of the final regression model are shown in Table 3.3. The noticeable parameter in the model was milk production ($P = 0.02$), which indicated a decrease in clinical days as production increased. There was a trend for clinical days to increase with increasing DIM and increasing parity categories.

Effect on Test-Day Milk. The average post-CM test-day milk for all cows with test-day data (n = 440) was 34.5 kg. Full data existed for 202 dPB cows and 206 BT cows in the model. Although not statistically significant, the mixed linear regression model indicated +0.7 kg milk for the dPB group (LSM dPB = 35.4 kg, BT = 34.7 kg; $P = 0.48$). As expected, pre-CM milk production had an effect on post-event milk production. Cows earlier in lactation (0-100 DIM), on average, produced 5 kg more milk than those later in their lactations (>200 DIM), $P < 0.0001$.

In this herd, parity was important with 1st and 2nd parities producing more milk than those ≥3rd parity (+2.8 kg; $P = 0.04$; +2.2 kg; $P = 0.04$, respectively).

Effect on Test-Day Linear Score. No statistically significant difference existed in LS post-CM between cows assigned to either treatment program; BT cows experienced a lower numerical LS than dPB cows (n = 200 for BT, n = 196 for dPB; LS = 4.2 and 4.3, respectively; $P = 0.58$). As expected, LS before CM event was an important variable; as pre-CM LS increased, post-CM LS increased ($P < 0.0001$). Two other parameters remained important in the model: parity and mastitis event. These effects can be seen in Table 3.4.

Effect on Probability of Survival at 30 d. A total of 451 observations were used in logistic analysis to model the probability of survival at 30 d post-event; 5% of CM cows with full data (n = 23, BT = 14/226; dPB = 9/225) did not remain in the herd beyond this time point. No significant difference was detected in odds of survival 30 d post-CM event between the treatment groups ($OR_{dPB} = 1.6$, 95% CI: 0.7-3.7; $P = 0.31$). The only variable that remained in the model was pre-CM milk production. The odds ratio of this effect was 1.05 (95% CI: 1.02-1.26).

Effect on Probability of Survival at 60 d. Similar to 30 day odds, no significant difference existed in odds of survival 60 d post CM event between the treatment groups ($OR_{dPB} = 1.4$, 95% CI: 0.7-2.6; $P = 0.29$). Out of 451 CM cows, 11% (n = 50, BT = 27/226, dPB = 23/225) were culled by 60 d. Estimates that remained significant in the model were those describing milk production, parity, and mastitis event. Similar to the 30 d model, cows had increased odds of survival as milk production increased ($OR_{dPB} = 1.09$; 95% CI: 1.02-1.17). Parities 1 and 2 had increased odds of survival over parity ≥3, and as the number of mastitis events in the current lactation increased categorically, odds of survival decreased. The logistic regression model describing ORs and CIs is shown in Table 3.5.

Effect on Hospital Days. Hospital days data was recorded for 424 CM cases, 184 in the dPB and 240 in the BT group; 57 dPB cows were lost to follow up for clinical days analysis due to placement into a non-hospital pen during the first month of the trial. The number of cows with full data that were included in the regression model was 388 (dPB = 166, BT = 222). A linear regression model for hospital days was also log-transformed for normality of variance and is shown in Table 3.6. After transformation of the estimates, LSM for hospital days was 5.8 d for the dPB group and 8.8 d for the BT; the BT group spent three additional days in the hospital pen ($P < 0.0001$).

Table 3.1. Cow-level descriptors for each treatment group

	Treatment Group					
	Pathogen Based (n = 246)	Blanket Therapy (n = 243)	Total	Overall	% of group	*P*-value[1]
Parity, no.						0.39
1st	48	45	93			0.82
2nd	67	80	147			0.20
≥3rd	131	118	249			0.32
DIM						
Mean	169	161		165		0.43
Median	154	150		151		
Mastitis Event,[2] no.						0.04
1st	137	157	294			0.05
2nd	52	51	108			> 0.99
>2nd	57	35	92			0.02
Milk yield,[3] kg	n = 232	n = 219		n = 451		
Mean	35.0	34.0		34.5		0.90
Median	35.5	33.2		35.0		
Linear Score[3]	n = 231	n = 216		n = 447		
Mean	3.8	3.5		3.6		0.22
Median	3.2	2.8		3.0		
Intramammary treatment, no.						
Ceftiofur	0	243			100	
No treatment	167	0			68	
Cephapirin	79	0			32	

[1]*P*-values based on bivariate analysis (*t*-test, Fisher exact) of each variable by treatment group
[2]Cow-level mastitis event at time of enrollment for the current lactation
[3]At test d 8 to 43 before enrollment

Table 3.2. Cow-level etiology of infection at detection[1]

	Treatment Group				
	Pathogen- Based (n = 246)		Blanket Therapy (n = 243)		
24 h Culture Result	no.	%	no.	%	*P*-value[2]
No Growth	65	26	51	21	0.28
No Significant Growth	16	7	17	7	0.86
CNS	4	2	6	3	0.54
Streptococcus spp.	75	31	80	33	0.63
Gram-negative bacillus[3]	84	34	80	33	0.77
Yeast spp.	1	0.4	5	2	0.12
Trueperella pyogenes	1	0.4	2	0.8	> 0.99
Corynebacterium spp.	0	0	2	0.8	0.25

[1]Etiological classification is based on aerobic culture results performed by Quality Milk and Production Services.
[2]*P*-values based on bivariate analysis (Fisher exact) of each etiology by treatment group.
[3]Postancillary testing, these organisms were further characterized as *Escherichia coli, Klebsiella* spp., *Pasteurella, Citrobacter* spp., or remained gram-negative bacillus at 48 h reading.

Table 3.3. Parameter estimates and statistics for fixed effects of a linear regression model evaluating log(clinical days)[1]; n = 369

Parameter	Description	Estimate	LSM CD[2]	SE	*P*-value
Intercept		1.90		0.09	<0.0001
Treatment	Pathogen-based	-0.06	4.5	0.04	0.12
	Blanket Therapy	Referent	4.8		
Milk production,[3] kg		-0.005		0.002	0.02
DIM[4]	0-100	-0.11	4.4	0.05	0.04
	101-200	-0.08	4.5	0.05	0.08
	>200	Referent	4.9		
Parity	1	-0.15	4.3	0.06	0.01
	2	-0.07	4.6	0.04	0.10
	≥3	Referent	5.0		

[1]Parameters evaluated for potential confounding included parity, DIM at the clinical mastitis (CM) event, previous occurrence of a clinical mastitis case in the present lactation, and linear score and milk production at test day previous to event.
[2]Least squares means for clinical days.
[3]On test d 8 to 43 d before CM event.
[4]At time of CM event.

Table 3.4. Parameter estimates and statistics for fixed effects of a linear regression model evaluating Linear Score (LS) on test day post clinical mastitis (CM) event (8-43 d)[1]; n = 396

Parameter	Description	Estimate	SE	*P*-value
Intercept		4.21	0.38	<0.0001
Treatment	Pathogen-based	0.12	0.21	0.58
	Blanket Therapy	Referent		
Parity	1	-0.95	0.30	0.002
	2	-0.24	0.24	0.31
	≥3	Referent		
LS[2]		0.26	0.05	<0.0001
Mastitis event[3]	1st case	-1.06	0.30	<0.0001
	2nd case	-0.75	0.33	0.02
	>2nd case	Referent		

[1]Parameters evaluated for potential confounding included parity, DIM at CM event, previous occurrence of a CM case in the present lactation, and LS and milk production at test day previous to event.
[2]On test day 8-43 d before CM event.
[3]At time of CM event.

Table 3.5. Logistic regression for survival to 60 d after clinical mastitis (CM) event[1]; n = 451

Parameter	Description	Estimate	SE	*P*-value	Odds Ratio	95% CI
Intercept		-0.53	0.66	0.42		
Treatment	Pathogen-based	0.33	0.31	0.29	1.4	0.7-2.6
	Blanket Therapy	Referent				
Milk production,[2] kg		0.08	0.03	0.01	1.09	1.02-1.17
Parity	1	0.64	0.47	0.17	1.9	0.8-4.7
	2	0.87	0.40	0.03	2.4	1.1-5.2
	≥3	Referent				
Mastitis event	1st	0.98	0.38	0.01	2.7	1.3-5.6
	2nd	0.40	0.41	0.32	1.5	0.7-3.3
	>2nd	Referent				

[1]Probability modeled was "alive" at 60 d. Covariates evaluated for potential confounding included parity, DIM at CM event, previous occurrence of a CM case in the present lactation, and linear score and milk production at test day previous to event.
[2]On test d 8 to 43 before CM event.

Table 3.6. Parameter estimates and statistics for fixed effects of a linear regression model evaluating log(hospital days)[1]; n = 388

Parameter	Description	Estimate	LSM	SE	*P*-value
Intercept		2.38		0.09	<0.0001
Treatment	Pathogen- based	-0.41	5.8	0.04	<0.0001
	Blanket	Referent	8.8		
Milk production,[2] kg		-0.005		0.002	0.01

[1]Parameters evaluated for potential confounding included parity, DIM at the clinical mastitis event, previous occurrence of a clinical mastitis (CM) case in the present lactation, and linear score and milk production at test day previous to event.
[2]On test d 8 to 43 before the CM event.

DISCUSSION

We found that using a pathogen-based treatment protocol compared with a blanket therapy protocol for mild to moderate clinical mastitis resulted in no significant differences in days to clinical cure, post-event milk production, test-day linear score, and survival to 30 and 60 d. Our study did not provide a direct comparison between a first generation cephalosporin, cephapirin, and a third generation cephalosporin, ceftiofur, as was done in the noninferiority comparison performed by Schukken et al. in 2013. Instead, we described an overall comparison of two distinct protocols; assessments between antimicrobial products were not included nor were important to our objectives. While no differences existed between treatment groups for many of the evaluated outcomes, we found an advantage to the use of a protocol that selectively treated 32% of cows with IMM cephapirin while not treating a majority of others: a significant decrease in the average number of hospital days (three days). No-growth, no significant growth, and gram-negative bacillus together accounted for more than 60% of mild to moderate cases of CM in this trial, meeting the described patterns in recent studies (Schukken et al., 2013; Oliveira and Ruegg, 2014).

Our average clinical days, 4.7, and clinical days by group (4.8 and 4.5) are comparable to the respective nonsevere ceftiofur-treated (4.5 d, n = 262) and cephapirin-treated cows (4.9 d, n = 49) in a recent Wisconsin mastitis survey (Oliveira and Ruegg, 2014). Our result was less than the ceftiofur-treated cows in an earlier study performed by the same group (Pinzón-Sánchez and Ruegg, 2011). The authors expressed that the large value, 5.4 clinical days, was due to the administration of IMM antimicrobials until the complete resolution of clinical signs. Another study detected lower values than those found in the present study, at 2.7 d and 3.2 d for blanket-treated cephapirin and selectively treated gram-positive cases, respectively (Lago et al., 2011a).

Sixty-seven percent of CM cows treated with cephapirin and an equivalent percentage of oxytocin-only treated cows exhibited clinical cure by 4.5 d after treatment in a study by Guterbock et al. (1993). In the current study, 52% (n = 85/163) and 39% (n = 92/235) exhibited clinical cure by 4.5 d for dPB and BT therapies, respectively. The Schukken et al. study (2013) analyzed this outcome using a different method; the authors described noninferiority for overall clinical cure when comparing on-label IMM cephapirin versus 5 d IMM ceftiofur. The 2013 study described no absolute difference (0%) when considering clinical observations at days 10 and 17 post-CM. Our risk difference for a clinical cure by day 4.5 was noted above as 13% between treatments and reflects observations performed at earlier time points. Despite deviations from our findings, all authors referenced concluded no statistical differences between treatment groups in days to clinical cure.

Several reasons influenced our choice of evaluating clinical cure as an index of outcome. While the “gold standard” approach in research studies to evaluate treatment efficacy is serial culture for bacteriological cure, many initial cultures can return negative results, limiting the ability to determine cure. Follow-up samples also may result in a different pathogen or contamination. Bacteriologic cure rates at 14 and 21 days in CM cows treated with pirlimycin were not significantly different for cows with mastitis caused by susceptible or resistant bacteria, indicating that this index of cure may not always be valid (Hoe and Ruegg, 2005). An indirect assessment such as LS or SCC as well as simple assessment of resolution of clinical signs provides a reliable perception of treatment success (Lago et al., 2004; Bradley and Green, 2009; Bradley et al., 2012). In fact, most producers pursue a remission of clinical signs foremost over immediate elimination of the causative organism; when the owner is not actively engaged in milking, the decision-making process is based on evaluation of individual-cow milking

performance and observations by the milking staff (Roberson, 2003; Pinzón-Sánchez and Ruegg, 2011). For these reasons, we measured the number of days to resolution of clinical signs. Resolution of clinical signs is a subjective measure and blinding in regards to treatment was not performed in this trial. However, producers and researchers were unaware of objective primary outcomes, including LS, culling, and milk production. We acknowledge that normal milk may not constitute elimination of the infection, and likewise, the clinical phase may extend beyond the time of bacteriological cure. These are disadvantages to the use of a clinical scoring system (Roberson, 2003). Bacteriological cure using serial cultures can provide an additional indicator of efficacy and could be pursued to quantify differences in response between protocols. Furthermore, bacteriological culture of post-mastitis samples would be advantageous for determining the presence and comparison of new and chronic intramammary infections between groups.

Previous and current research has established that differences in cure rates are associated with etiology, previous milk yield, previous SCC, previous occurrence of CM, treatment duration, parity or lactation of the animal, and stage of lactation (Pinzón-Sánchez and Ruegg, 2011). As the decision to treat BT cows was independent of pathogen result (these cows were treated immediately), a valid comparison of the treatment groups did not include pathogen in the model for clinical days. At diagnosis of CM, milk samples were retrieved and cultures were performed for cows in both groups to ensure that: randomization occurred without creating bias for individual etiologies, cows with contagious organisms were identified, and cows in the dPB group received their treatment assignment. Objectives did not include assessing the impact of culture results on treatment outcomes, but rather the impact of the selected protocols. Subsequent mastitis events at the quarter-level were not followed; this is a limitation of the current study.

Analysis and interpretation of follow-up mastitis events between groups would be difficult to assess as an unequal distribution in the categories of mastitis event number at enrollment existed (Table 3.1). More cows experiencing their first case of mastitis were allocated to the BT group, while more animals experiencing their 3rd or greater event were allocated to the dPB group. Cows with previous cases of CM are less likely to respond to therapy: a previous study by Pinzón-Sánchez et al. (2010) including 143 cases of CM showed that cows treated in the current lactation for the first time were 11 times less likely to have a recurrence than those experiencing a previous case of CM. Also, an increase in mastitis events during a lactation can result in decreased milk production, increased LS, increased risk of culling, and an increase in hospital and clinical days, creating a bias away from the null when considering differences in outcomes between groups. In this trial, any contribution of selection bias creates more opportunity for the dPB cows to experience these negative outcomes. Variables listed in the Pinzón-Sánchez study, including mastitis event number, were initially included as potential confounders when constructing a model and no statistical differences between treatments were observed.

Milk production and LS post-CM event were not significantly different between treatment groups in the current study. Our results were consistent with three previous mastitis trials comparing blanket treated to selectively treated cows ("no growths" and coliform-positive cows did not receive antimicrobials). No significant differences in LS or milk production were detected between groups (Van Eenennaam et al., 1995; Roberson et al., 2004; Lago et al., 2011b). A fourth study depicted no significant differences in post-event LS or in milk production between first and third generation cephalosporin treatments (Schukken et al., 2013). Conversely, a study by Shim et al. (2004) reported a continued loss of production for cows that were not treated with antimicrobials; in comparison, treated animals suffered 1/3 of the stated loss.

Approximately 40% of the cows in the 2004 study were experiencing their second or greater case of mastitis and distribution of cases between treatment groups was not described. Additionally, the author recognized that the continued milk loss in CM cases may have resulted from persistent subclinical mastitis.

The current study found no significant differences between treatment groups when assessing survival in the herd to 30 and 60 d. Similarly, Lago et al. (2011a) found no statistical differences in removal from herd within 21 d when assessing outcomes for blanket-treated cephapirin cows versus a protocol treating only gram-positive CM cows. A subsequent time-to-event analysis from the same data set concluded that days to culling between groups was similar (Lago et al., 2011b). Previous to both studies, a 1995 trial assessing two different IMM treatments versus non-treatment of CM resulted in no differences in removal from the herd, an outcome that was independent of etiology (Van Eenennaam et al., 1995). In our study, milk yield at test was the most important predictor for retention in the herd when assessing survival indices. This agreed with a recent study assessing risk factors associated with short-term post-treatment outcomes (Pinzón-Sánchez and Ruegg, 2011).

Approximately 60 untreated dPB cows were placed into a non-hospital pen at time of diagnosis, creating potential bias in the models for clinical and hospital days. These cows exited the hospital pen at day zero; time to resolution of clinical signs is difficult to determine. Despite omission of these cows from clinical and hospital day analysis, the mean number of hospital days for those cows included was significantly different between groups. The authors were aware of the possibility of a three day difference in outcomes when the trial commenced. Drug labels designate that using an antibiotic with a one-day treatment and 96 h milk withhold versus using an antibiotic with a five-day treatment and 72 h milk withhold would result in a three day

difference. However, dPB cows had a delay in treatment by 24 hours, resulting in a projected difference of two days. Also, we could not assume that ceftiofur-treated cows, cephapirin-treated cows (16% of the 489 enrolled cows; n = 79) nor untreated cows (34%; n = 167) would experience resolution of clinical signs before the withhold date. Cows with etiologies known to have a longer duration of clinical signs at this farm (*E. coli, Klebsiella* spp.) were not treated in the dPB protocol; it was anticipated that these cows would contribute a higher number of clinical days to this group. Additionally, clinical signs proceeded beyond the withhold date for 52% of cephapirin-treated cows with full data (n = 38/73), which included a large percentage of *Streptococcus* spp. positive mastitis cases (n = 36). Of all cows experiencing greater than six clinical days, 56% had *Streptococcus* spp. positive cultures (n = 56/100). Of the cows in the dPB group, 33% (n = 54 of 163 with full data) experienced unresolved clinical signs by day six.

The three day difference in days out of the tank between the BT and the dPB groups is an important attribute when considering economics. Pinzón-Sánchez et al. (2011) performed a decision tree analysis on treatment strategies for mild and moderate first-case CM in early lactation and found that the most cost-effective strategy when implementing OFC was to treat gram-positive pathogens for two days (one treatment per day) while permitting culture-negative and gram-negative results to "self-cure." The mentioned study did not investigate the economics of treating twice daily for one day, which would involve the same costs for treatments and labor, but dependent upon withdrawal time of product used, may decrease costs associated with discarded milk. Regardless, when modeling for pathogen, probability of recurrence, and probability of cure, routine extended therapy ($\geq$ five days) was not economically optimal under any circumstance (Pinzón-Sánchez et al., 2011). Extended antimicrobial treatments, defined as greater than three days of IMM treatments with or without systemic antimicrobials or anti-

inflammatories, did not result in outcome benefits (*i.e.*, fewer follow up treatments, less milk production, less culling) in excess of treatment costs (Steeneveld et al.; 2011).

Until recently, the impact of delaying CM treatment for 24 h to allow for culture was not well defined; the idea that use of a pathogen-based system produced undesirable outcomes as compared with implementation of immediate treatment was a valid consideration. However, several studies have addressed this question, particularly for the delayed treatment of gram-positive CM etiologies. Immediate versus delayed treatment of all CM cases producing growth on agar or delayed treatment of only gram-positives cases indicated no differences in survival indices (Wagner et al., 2007; Lago et al., 2011b), LS and milk production (Lago et al., 2011b), clinical and bacteriological cures (Lago et al., 2011a), or mastitis recurrence (Wagner et al., 2007; Lago et al., 2011b). Conversely, these cases tended to have shorter milk discard time while decreasing antimicrobial use (Lago et al., 2011a). The majority of the referenced studies include data from multiple dairies. The authors recognize that trials using more than one herd may provide additional data that includes or accounts for variation among herds; a weakness in the current study exists if this study population is not representative of an external population. Certainly one should not generalize the results of this study to dairy farms that are not like this one. Despite the use of one dairy, ad-hoc power analysis produced confidence in our results: with a power of 90% and a Type I error rate of .05, we were able to detect differences in clinical and hospital days of 1.2 days or more, a 0.2 or more kg difference in milk yield, odds ratios of 1.2 or greater for 30 and 60 d survival, and differences in linear score of 0.22 or more. While a large sample from one herd may impact external validity and generalizability to heterogeneous dairies, it likely increases the reliability of application of the results to similar dairy farms.

Resistance to antimicrobials may contribute to the similarities found between treatment groups in the current study. Previous research indicates that resistance to cephalosporins may be more prevalent in gram-negative versus gram-positive mastitis-causing pathogens. Ruegg et al. (2015) analyzed phenotypic profiles of gram-positive, mastitis-causing organisms and determined that while ceftiofur and cephapirin were the most commonly used IMM products in the enrolled herds, almost none of the pathogens were phenotypically resistant to those compounds. The latter study agreed with the results of a European trial performed by Thomas et al. (2015). Particularly, among clinical mastitis isolates of *Strep. uberis* and *Staph. aureus*, resistance to beta-lactam antibiotics was absent. For *E. coli*, there was an absence of resistance for ceftiofur and minimal resistance for cephalexin (Thomas et al., 2015). Alternatively, Srinivasan et al. (2007) and Erskine et al. (2002) found cephalosporin resistance in 15.5% and 25.5% of *E. coli* isolates from cows with mastitis, respectively. As over 20% of cows in the current trial were culture-positive for *E.coli*, ceftiofur-treated resistant isolates may have decreased any differences in outcomes that could be realized. Regardless, the objectives of this trial were to compare treatment protocols and the cow-level response for each protocol; despite any resistance in mastitis isolates, benefits to the use of blanket therapy in regards to the explored outcomes were not found. Additionally, herds that use systemic ceftiofur for the treatment of various health conditions are more likely to have cows with less susceptible fecal isolates of *E. coli* than those that did not report ceftiofur use (Tragesser et al., 2006), suggesting that on a herd level, reduction of antimicrobials can decrease the potential for development of resistance.

Protocols that practice judicious and targeted use of antimicrobials by rapid identification and diagnosis not only decrease the potential for development of antimicrobial resistance, but

also address a concern with violative residues. The risk of a residue violation is directly associated with IMM antimicrobial use (McEwen et al., 1991). It has been reported that drugs used for the treatment of CM contribute the highest average daily dose per cow per year (**DDD**) at 2.0 followed by drugs used for dry cow therapy at 1.58. These numbers compare with doses for the treatment of other ailments such as foot rot (0.39 DDD), metritis (0.32 DDD), and respiratory illness (0.14 DDD). The mean overall density of ceftiofur use in the report was significantly greater than the density of use for other compounds labeled for IMM CM treatment (Pol and Ruegg, 2007b). In the current trial, the assignment and administration of an IMM cephalosporin product based on pathogen results apportioned the antimicrobial to less than 40% of nonsevere mastitis cases.

CONCLUSIONS

Many organisms that cause mastitis are successfully cleared by a cow's immune system—a shift in etiologies of current dairies to mostly gram-negatives or "no growths" rather than contagious or gram-positive organisms creates a large opportunity to reduce the use of antimicrobials. Greater than 65% of moderate and mild CM cases would not have been treated if all cows on this trial were enrolled in a pathogen-based protocol. This strategic method of treatment decreased milk withholding time by three days for those cows on a pathogen-based treatment protocol, with no significant differences in days to clinical cure, milk yield, and LS post-mastitis event; nor additional odds of culling in the two months following. If mastitis treatment decisions are based on scientific reasoning and data indices, economic returns and aspects of public health will be protected.

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CHAPTER FOUR

PROMOTING SUSTAINABILITY ON MODERATE-SIZED DAIRIES: USE OF PATHOGEN-BASED PROTOCOLS FOR THE TREATMENT OF NONSEVERE CLINICAL MASTITIS ON 8 NEW YORK HERDS

Final report for:
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ABSTRACT

The purpose was to determine if a pathogen-based treatment protocol for nonsevere clinical mastitis could be effectively implemented on moderate-sized dairies that might not have daily access to a professional diagnostic laboratory. The study was conducted at 8 commercial dairy farms in central New York (500-1,000 milking cows). Two different "diagnostic" groups were investigated. Dairies in the on-farm culture group (n = 2) were trained to use Minnesota tri-plates to diagnose every case of nonsevere clinical mastitis. Dairies in the 5-day pickup group (n = 6) submitted milk samples from all nonsevere mastitis cases to Quality Milk Production Services at the Cornell Animal Health and Diagnostic Laboratory 5 days per week and waited 24 h for diagnostic results prior to the treatment of clinical cases. Upon receipt or interpretation of results, dairies in both groups followed a treatment protocol decided upon by management and the herd veterinarian. Briefly, all dairies chose to treat non-contagious gram-positive pathogens with an intramammary antibiotic according to the manufacturer's label. All dairies chose not to treat any quarter with a negative culture result. Variation existed in regards to treatment of gram-negative pathogens. A total of 1,031 cases of nonsevere mastitis were enrolled. Compliance to the protocol ranged from 59% to 96% and was higher for the dairies in the 5-day-pickup group. When delayed pathogen-based treatment was practiced, no important changes were noted in monthly mastitis incidence on seven of the dairies. Bulk tank somatic cell count was also similar before and after the study for these dairies. No changes in mature equivalent milk production were observed for the 8 dairies. One dairy experienced a drop in the bulk tank somatic cell count of 100,000 cells/mL, which can be explained by their apparent increase in monthly mastitis incidence. This suggests improved proficiency in mastitis detection for this herd. Overall, inconsistencies and irregularity of data recording, in respect to mastitis events and treatments

was drastically improved with implementation of the program. Reductions in antimicrobial usage ranged from 6.8% to 92.5% dependent on farm protocol and pathogen profile of the herd. Decreased antibiotic use resulted in savings between $2,200 and $4,600 per dairy per year when cost of cultures was accounted for. The use of a pathogen-based treatment protocol based on 5-day per week results or on farm-culture results has the potential to decrease antimicrobial use, promote product sustainability, and protect aspects of public health.

INTRODUCTION

Clinical mastitis (CM) is defined as inflammation or infection of the mammary gland and is detected by visible signs of inflammation such as redness, swelling or pain, and alterations in milk such as clots, flakes, discoloration, or abnormal consistency. This disease remains the most common bacterial disease on dairies and its high incidence (20.4-51.0%) accounts for the majority of antimicrobials administered to lactating dairy cattle (Pol and Ruegg, 2007, Olde Riekerink et al., 2008). This disease can incur severe economic losses due to discarded milk, reduced production, decreased conception, premature culling, transmission of infection to other cattle, and treatment costs (Fetrow, 2000, Hertl et al., 2014a, b). Current practice on many dairy farms is treatment of all CM cases or “blanket treatment” with intramammary (IMM; into the mammary gland) antimicrobials. In a Wisconsin study (Pol and Ruegg, 2007), 80% of antimicrobial drugs used on dairies were for treatment or prevention of mastitis. However, many organisms that cause mastitis are successfully cleared by a cow’s immune system—a recent shift in etiologies to a majority of gram-negatives or “no growths” rather than contagious or gram-positive organisms creates an opportunity to reduce the use of antimicrobials. Multiple “treat”

and “no-treat” CM regimens have been tested using on-farm clinical trials; results describe no important differences in clinical outcomes between groups (Lago et al., 2011b, a, McDougall et al., 2018).

A recent study by our group (Vasquez et al., 2017) used a randomized field trial to assign nonsevere CM cases to either the blanket therapy group (BT) or the delayed pathogen-based therapy group (dPB). This work was performed on a 3,500 cow dairy with a 5-6% monthly incidence of CM. Cows in the BT group received immediate IMM treatment with a 3rd generation cephalosporin every day for 5 days. Upon receipt of 24 h culture results, cows in the dPB group followed a protocol automatically assigned via the dairy computer: gram-positive organisms such as *Staphylococcus* spp. and *Streptococcus* spp. were administered IMM treatment with a first generation cephalosporin for 1 day. Others, including cows with negative cultures or gram-negative pathogens received no treatment. Statistical comparisons were performed between treatment groups for clinical outcomes. The strategic method of treatment decreased the time that milk had to be discarded by 3 d for those cows in the dPB group, with no significant differences in days to clinical cure, milk yield, and linear score post-mastitis event; nor additional odds of culling in the 2 months following. Greater than 65% of moderate and mild CM cases would not have been treated if all cows were enrolled in a pathogen-based protocol. An additional economic analysis performed on the same data established that greater than $30,000 per 1,000 cows would be returned if this protocol was used at similar dairies. If mastitis treatment decisions are based on scientific reasoning and data indices, economic returns and aspects of public health will be protected. Non-prudent use of antimicrobials as therapeutics can have a negative impact on sustainable agriculture by reducing drug efficacy, as pathogenic microorganisms can develop resistance, leading to cycles of prolonged use of the same drug or

supplemented use of alternative antimicrobials. Use of antimicrobials on dairies has been shown to have a direct positive correlation to the risk of drug residues in fluid milk sold to processing entities (McEwen et al., 1991).

Our trial was unique in that it involved a 24 h turnaround of culture results 7 d per week. Cows experiencing mastitis that eventually went on to be treated (gram-positive culture results) were only delayed by this short amount of time. Additionally, cultures were performed at a certified diagnostic laboratory allowing for accuracy of results and confirmation of contagious and more obscure organisms. However, many smaller, widespread dairies may not have access to a reference laboratory with a 24 h turnaround. We intended to continue and evaluate our protocol on the more representative, moderate-sized dairies of NY State (500-1,000 cows) using either on-farm culture, which has been assessed as a rapid and reliable way of diagnosis (Mansion-de Vries et al., 2014, Royster et al., 2014) or a 5 d per-week sample pickup, simulating a veterinary facility or diagnostic lab with the capacity to culture less frequently.

We wished to evaluate the practicality of a pathogen-based treatment protocol on dairies that do not have daily access to sample submission. To this end, our objective was to create a network of involved and engaged dairy producers to assess 2 alternatives: less frequent sample retrieval (simulating a 5 d per-week veterinary laboratory) and on-farm 24 h culture diagnosis, or "OFC". If application of our protocol on these dairies can successfully reduce antimicrobial use by greater than 60% without negative outcomes, widespread education of the judicious use of antibiotics by dairy farmers in states such as NY, which have prominent contributions to milk products, can create positive impacts on sustainability by preventing the selection of resistant organisms, thereby increasing product efficacy.

MATERIALS AND METHODS

This study was approved by Cornell IACUC under Quality Milk Production Services #2013-064.

Herd Selection

The study involved 8 Holstein dairy herds in central New York with a herd size target of <1,000 milking cows. Each dairy was targeted to enroll 150 cases of nonsevere clinical mastitis between February 2017 and December 2017. Farm characteristics can be seen in Table 4.1. Herds were selected based on their willingness to take samples from mastitic quarters and to use on-farm culture (OFC), or to refrigerate samples immediately and notify Quality Milk Production Service (QMPS) at the diagnostic lab at Cornell University for prompt sample retrieval (5 d pickup). Farms had to be using IMM antibiotics for the treatment of the majority of mild and moderate mastitis cases to be enrolled. Farms using QMPS diagnostics had to be willing to delay treatment by 24 h and either treat on the days when sample pickup did not occur, or to wait two additional days for culture results. Additionally, all herds sending samples to QMPS had to use Dairy Comp 305 (DC305) computer recording, as 24 h culture results were uploaded to this program.

Enrollment of Cases

All parlor employees were trained to recognize signs of mild-to-moderate clinical mastitis. This included abnormal color or consistency of the milk (flakes, flecks, clots), and/or redness and swelling of the quarter. Any cow that was showing systemic illness including dehydration, pyrexia, or depression was not enrolled in the trial and was treated according to the

herd veterinarian's instructions. Using aseptic technique, milk samples were retrieved from each affected quarter, labeled with the cow number, quarter, and the date, and placed immediately in a 5°C refrigerator.

Diagnostic Groups

On-Farm Culture (OFC) Herds. Herds A and B each nominated one person responsible for the microbiological procedures. Each herd was provided an incubator that was to be maintained at 37°C. Humidity was supplied using a small container of water within the unit. Training was provided by the author and was supplemented with color handouts, including the Minnesota Easy Culture System guide. Training included inoculating the agar with sterile swabs, interpretation of the results, and recording of the data. The media provided was the Minnesota Tri-plate (University of Minnesota, St. Paul, Minnesota), which consisted of three different media on one plate: selective media for gram-negative bacteria (MacConkey media), selective media for *Streptococcus* and *Streptococci*-like species (Modified TKT Media), and selective media for gram-positive bacteria and yeast/fungi (Factor Media). The nominated person initiated cultures on the day that milk samples were collected. A sterile cotton swab was saturated with the milk sample prior to inoculating each portion of the plate. The plate was then incubated at 37°C for 24 h. After 24 h, plates were assessed and the colonies were identified as streptococci, *Staph. aureus*, CNS, *Klebsiella* spp., *E. coli*, gram-negative bacteria, or other organisms. Pure growth of one or more colonies was indicative of an infection for all organisms except CNS. For CNS, more than 3 colonies had to be present to indicate an infection. Growth of two different colony types was a mixed infection and was recorded as the two different etiologies. Growth of three or more colony types was contamination and management was instructed to sample the

quarter again. Results were recorded on paper and in DC305 (Herd B) or on paper only (Herd A). Dependent on result, treatment commenced 24-36 hours after original detection of mastitis. The remaining portion of the milk sample was frozen at -20°C until samples were retrieved on a weekly basis by the author. Obvious disagreements between QMPS and OFC results were addressed with weekly feedback and further training. Technical support was provided throughout the study.

5-Day Sample Pickup Herds. Herds 1-6 were provided instruction for entering culture events into DC305 for each individual case of mastitis. This included recording the date, quarter, and severity of mastitis. A printed submission list was generated each morning for the courier, who retrieved samples for 5 consecutive mornings prior to 10:00am. Results were returned within 24 h of retrieval and marked as specific culture code within an individual cow card. Laminated copies of culture codes and their corresponding organisms were posted at each dairy. Additionally, a printout of each day's culture results was generated and printed at 11:00am from each herd's computer.

Laboratory Procedures

Standard culture technique was performed on all frozen samples from herds A and B and fresh samples from herds 1-6 according to the National Mastitis Council's guidelines for identification of aerobic organisms (National Mastitis Council, 2017). Briefly, 0.01 mL of milk was streaked on trypticase soy agar containing 5% sheep blood and 1% esculin (PML Microbiologicals, Mississauga, ON, Canada) and incubated aerobically at 37°C for 18 to 24 hours. Colonies from pure cultures were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using a Bruker MALDI-TOF Biotyper (version 3.1.66; Bruker

Corp. Billerica, MA). Confidence levels were assigned by the machine software by referencing the Bruker 5989 RUO bacterial library. Levels accepted for species identification were ≥2.0 and for genus identification between 1.8 and 2.0. The same definitions for intramammary infection were used as described earlier.

Treatment Protocols

Managers, owners, and the herd veterinarian determined a treatment protocol specific for each dairy. As not important to our objectives, we allowed each herd's team to decide what IMM antibiotic(s) to use. Following assessment of the culture plates at 24 h, quarters to be treated in the OFC group were treated with the selected IMM product according to the manufacturer's instructions. For example, all gram-positive organisms on herd A and gram-positive organisms and *Klebsiella* spp. on herd B were treated with an IMM antimicrobial. All other organisms were not treated. For the 5 d pickup herds, treatment commenced upon receipt of the computerized results the morning after sample submission. If a herd elected not to wait the weekend to treat a mastitic cow, IMM treatment could begin immediately, prior to receipt of results. Any cow that graduated to systemic clinical signs was removed from the trial and treated according to the veterinarian's recommendations. Farm management was asked to record all treatments in DC305.

Questionnaires

Brief initial and follow-up oral questionnaires were administered to individuals directly involved with the treatment decisions on each dairy. The preliminary questionnaire inquired about the details and costs of the current mastitis treatment protocol, what factors were involved

in selecting cows to treat, the primary reason why management sought to change their current program, and what type of program they see as ideal. The final questionnaire asked whether the trial program was effective at the dairy, the most helpful and beneficial aspects of the program, the cost-savings, whether management thought cows were becoming chronic, and what improvements could be made overall.

Analysis

Initial record analysis prior to the start of the trial revealed that management was not consistent in their recording of mastitis cases nor in recording of individual treatments. Many dairies did not have established protocols, implemented protocols that included extra-label administration of antimicrobials, or had overlapping/redundant protocols within the computer system or within individual cow cards. Therefore, monthly mastitis incidence, number of mastitis cases, and chronicity of mastitis (the number of retreated quarters) prior to the start of the trial are likely inaccurate. As such, any statistical comparison between these benchmarks before and after the trial was not performed. For other analyses, the unit of analysis was the quarter. Quarters were excluded from analysis if no on-farm culture result was recorded (herds A and B). Compliance was calculated as the number of cases for which management followed the decided treatment protocol divided by the total number of cases cultured for that herd. Antimicrobial use was calculated as the number of cases receiving IMM antibiotics divided by the total number of cases cultured for that herd. Chronicity of cases was assessed as the number of repeated mastitis cases over the study period (same cow and same quarter), at least 14 days apart, divided by the total number of mastitis cases for that period.

For comparison between OFC and QMPS culture results for herds A and B, diagnoses were grouped according to the most prevalent mastitis pathogens on the dairy as well as according to their treatment protocol. The following categories of growth were described for herd A: "no growth", *Staph. aureus*, gram-positive, gram-negative, "mixed with gram-positive," and "other". For herd B: "no growth", *Klebsiella* spp., *E.coli*, gram-positive, gram-negative, "mixed with gram-positive," and "other". Where *Staph. aureus* (herd A) or *Klebsiella* (herd B) and another organism were cultured, the result was coded as agreed if the respective organism was reported by the other methodology, irrespective of the second organism. If a result was contaminated, it was classified as "other". If one result was missing, it was coded as null. The percent agreement was calculated as the total number of cultures in agreement over the total number of cultures for each herd. Kappa statistics were used to calculate the overall level of agreement.

RESULTS

Individual Farm Characteristics

Herd size, bedding type, and the number of quarters enrolled for each farm can be seen in Table 4.1. Herds 1, 4, 5, and 6 initially used blanket therapy for all mastitis cases, which was defined as treating each case with an IMM antimicrobial on the date of detection. Herd A treated all cows with IMM antimicrobials on the day of detection, but discontinued treatment for gram-negative and "no growth" cultures when results of the OFC were read. Herd 2 and herd B both used OFC prior to the beginning of the trial, but were not consistent with their treatments and did not feel adequately trained on culture plate diagnostics. Prior to the commencement of the trial, herd 3 submitted samples to the diagnostic laboratory once per week; treatment of gram-positive

cases was delayed by up to 8 days for some cows. Only herds 4, 5, and 6 reliably recorded all mastitis events, and only herds 4 and 6 reliably recorded mastitis treatments. Dairies on the project were taught to record single mastitis events rather than treatments (as some cows would not be treated) and to record all events, regardless of severity. Consistency was encouraged by creating standard protocols within the computer program. Overall, this allowed for better epidemiological analysis of mastitis cases, and easier communication between the herd veterinarian and the client.

Initial Questionnaire Responses

Two of the 8 dairies were unsatisfied with the current mastitis program. Half of the dairies believed that the current protocol worked well, but that the overall mastitis program needed improvement. The remaining dairies (n = 2) noted that the current program worked well and needed no improvement; however, they were willing to try something new. Of all dairies on the project, the largest dairy had the highest expense for the treatment of CM at $25,000/yr. When expenditures per year were standardized for the number of cows per dairy, between $0.83 and $23.81 was spent on treatments per head, per year. The most common reason given for enrolling in the program was projected savings on treatment costs. The remaining reasons were knowledge of etiology (or knowledge of etiology in a timelier manner), and establishing a more effective treatment protocol. The main factor driving the written mastitis protocols was: the veterinarian (4), the withhold time of the treatment chosen (1), the ease of implementation (1), the effectiveness of the treatment chosen (1), or the individual cow characteristics (1). The main factors driving whether a cow was treated or not (assuming culling as an a “non-treatment” for those herds using blanket therapy) were: milk production, clinical signs, chronicity, and

pregnancy status. According to management, characteristics of an ideal selective treatment protocol would be: one that offers a prevention strategy or minimizes the need for antibiotics and one that offers diagnostic driven decisions with minimal turnaround time for results.

Culture Results

A total of 1,031 quarters were enrolled in the study. Using aerobic culture, a pathogen was identified in 65.6% of the mastitis cases. About 1/3 (34.4%) of cases had no-growth on culture, about 1/3 had gram-positive organisms (27.3%), and about 1/3 of cultures revealed a gram-negative pathogen (30.9%). Culture results by herd are listed in Table 4.2.

Compliance, Milk Production, and Milk Quality

Descriptors, including treatment scheme, compliance, and milk quality and production indices for each dairy can be seen in Table 4.3. All herds elected to treat non-contagious gram-positive organisms and not treat culture-negative cases, but there were several variations on treatment of gram-negative pathogens and contagious gram-positive pathogens. Three dairies in the 5 d pickup group extended their "wait-to-treat" period over the days when samples were not picked up. On average, 82.5% of cows were treated according to the established protocol (n = 851 cases). Compliance was generally higher for the 5 d pickup herds as compared with herds using OFC. Protocols and compliance by herd is shown in Table 4.3. The most common form of non-compliance was IMM treatment of cows with gram-negative pathogens and cows with negative culture results (41.7% of non-compliant cases, n = 75). These were treated due to worsening clinical signs. The next most common form of non-compliance was non-treatment of gram-positive cases (due to resolution of clinical signs during the 24 h waiting period; 30.6% of

non-compliant cases, n = 55). Other reasons for non-treatment of cases that should have been treated were: culling of the animal (n = 12, 6.7%), drying the cow early (n = 2, 1.1%), and cases refractory to treatment that management elected not to treat (for example, *S. aureus;* n = 10, 5.6%). Finally, 26 culture-negative cows were treated that should not have been in one herd due to general miscommunication between management and hospital staff (14.4%).

Bulk tank somatic cell counts before and after the trial were similar for seven of the dairies. Herd B experienced a dramatic drop in BTSCC over the course of the trial (Table 4.3). Similarly, only small changes were noted in mature equivalent milk production (ME305), a standardized estimation of how much an average cow in the herd would have produced for 305 days, in pounds.

Retreatment for Clinical Mastitis/Chronicity

Dependent on farm, between 10% and 55% of quarters were retreated for CM over the months of the trial and up to 2 months post-mastitis event (Table 4.4). For half of the farms, the percentage of retreats appeared to increase as compared with the percentage of retreats before the start of the trial. Two dairies showed a decrease in the percentage of retreats and one remained similar. However, mastitis event data was not consistently nor reliably recorded prior to the trial and these comparisons are likely inaccurate. Monthly mastitis incidence remained similar or decreased for most herds, except for herd B which appeared to increase 1.4 fold. This is due to better detection and separation/treatment of mastitis cases during the study as compared with the year before. This is supported by the large decrease observed in herd B's BTSCC (Table 4.3).

Agreement Between On-Farm and Laboratory Cultures for Herds A and B

The distribution of results between the culture categories that were used to compare OFC diagnoses and QMPS diagnoses for herds A and B can be seen in Tables 4.5 and 4.6. The overall agreement for herd A was 67.3% (kappa = 0.58; 95% C.I. 0.49-0.67), with the highest agreement occurring for the gram-positive cultures (80.5% agreement) and lowest for "mixed gram-positive" and "other" categories (<10% agreement). For herd B the overall agreement was 55.9% (kappa = 0.42; 95% C.I. 0.32-0.51), with the highest agreement occurring for negative cultures (84.3% agreement) and the lowest for "other gram-negative", "mixed gram-positive", and "other" categories (<10% agreement). Combined results for herds A and B resulted in 188/304 correct diagnoses, or 61.8% agreement.

Antimicrobial Usage

The average reduction in antimicrobial usage was 46.4% (range = 6.8% to 92.5%). The percentage of mastitis cases treated with IMM antimicrobials before and after the study can be seen in Figure 4.1. As previously stated, prior to the trial, herds 2 and B were practicing pathogen-based treatment using OFC without guidance. Guidance reduced antimicrobial use by 6.8% and 20% for these herds, respectively. Herd 3 was also using a pathogen-based treatment protocol, but only submitting cultures once per week. Quicker diagnostic turn-around led to a decrease in use of 17.2%. All other dairies, represented by the dark "before" bars amounting to 100% (Figure 4.1), were practicing blanket treatment at time of mastitis detection.

Table 4.1. Herd characteristics for enrolled dairies. Herds 1-6 used 24 h sample pickup 5 days per week, herds A and B were trained to use on-farm culture to drive treatment decisions

Herd	Bedding	MMI[1]	County	Herd Size[2]	Start Date	End Date	Duration, d	Quarters enrolled, no.
1	Sand	2.2	Onondaga	820	1/30/2017	9/21/2017	234	150
2	Sand	1.8	Cortland	600	1/29/2017	12/20/2017	325	113
3	Peat Moss	4.6	Cortland	510	1/29/2017	10/28/2017	272	153
4	Sand	3.0	Onondaga	910	2/1/2017	7/30/2017	179	154
5	Sand and Sawdust	3.0	Cortland	1,040	2/1/2017	12/21/2017	323	96
6	Sawdust	5.3	Onondaga	630	10/17/2017	12/6/2017	50	53
A	Sawdust	5.7	Broome	1,050	1/23/2017	3/30/2017	66	159
B	Sand	3.6	Cortland	560	2/21/2017	7/4/2017	133	153
Total							198	1,031

[1]Apparent monthly mastitis incidence. Calculated from computer data at time of enrollment as number of treated mastitis cases per month. For an individual cow, treatments had to occur at least 14 d apart to be considered a new case.
[2]Number of lactating cows at time of enrollment.

Table 4.2. Etiology of infection at time of mastitis detection for each enrolled herd[1]

	Herd								
	1	2	3	4	5	6	A	B	Total
Culture Result	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)
Negative	43 (28.7)	42 (37.2)	33 (21.6)	75 (48.7)	26 (27.1)	30 (56.6)	53 (33.3)	53 (34.6)	355 (34.4)
Gram-negative	51 (34)	34 (30)	64 (41.8)	47 (30.5)	47 (49)	15 (28.3)	24 (15.1)	37 (24.1)	319 (30.9)
E.coli	15 (10)	13 (11.5)	62 (40.5)	24 (15.6)	21 (21.9)	1 (1.9)	9 (5.7)	5 (3.3)	149 (14.5)
Klebsiella spp.	23 (15.3)	17 (15)	0 (0)	13 (8.4)	20 (20.8)	6 (11.3)	6 (3.8)	21 (13.7)	105 (10..2)
Other[2]	13 (8.7)	4 (3.5)	2 (1.3)	10 (6.5)	6 (6.3)	8 (15.1)	9 (5.7)	11 (7.2)	65 (6.3)
Gram-positive	42 (28)	33 (29.2)	42 (27.5)	28 (18.2)	13 (13.5)	4 (7.5)	71 (44.7)	48 (31.4)	281 (27.3)
Staph aureus	2 (1.3)	0 (0)	10 (6.5)	0 (0)	1 (1)	0 (0)	32 (20.1)	0 (0)	45 (4.4)
Staph spp.	2 (1.3)	7 (6.2)	4 (2.6)	4 (2.6)	1 (1)	0 (0)	7 (4.4)	8 (5.2)	33 (3.2)
Strep dysgalactiae	5 (3.3)	6 (5.3)	23 (15)	7 (4.5)	3 (3.1)	0 (0)	21 (13.2)	8 (5.2)	73 (7.1)
Strep uberis	8 (5.3)	3 (2.7)	4 (2.6)	4 (2.6)	1 (1)	1 (1.9)	4 (2.5)	0 (0)	25 (2.4)
Strep spp.	5 (3.3)	3 (2.7)	0 (0)	1 (0.6)	1 (1)	0 (0)	3 (1.9)	1 (0.7)	14 (1.4)
Lactococcus spp.	11 (7.3)	6 (5.3)	0 (0)	5 (3.2)	1 (1)	0 (0)	1 (0.6)	20 (13.1)	44 (4.3)
Mixed[3]	6 (4)	4 (3.5)	0 (0)	3 (1.9)	4 (4.2)	1 (1.9)	3 (1.9)	10 (6.5)	31 (3.0)
Other[4]	3 (2)	4 (3.5)	1 (0.7)	4 (2.6)	1 (1)	2 (3.8)	0 (0)	1 (0.7)	16 (1.6)
Other[5]	13 (8.7)	3 (2.7)	9 (5.9)	0 (0)	3 (3.1)	0 (0)	5 (3.1)	9 (5.9)	42 (4.1)
Contamination	1 (0.7)	1 (0.9)	5 (3.3)	4 (2.6)	7 (7.3)	4 (7.5)	6 (3.8)	6 (3.9)	34 (3.3)
Total	150	113	153	154	96	53	159	153	1,031

[1]Etiological classification is based on aerobic culture results and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) performed by Quality Milk and Production Services for all dairies.

[2]Includes *Enterobacter* spp., *Citrobacter* spp., gram-negative bacilli, *Pasteurella*, *Pseudomonas*, and *Serratia*.

[3]Had at least 1 gram-positive organism in addition to another organism, dictating treatment.

[4]Includes gram-positive bacilli and *Enterococcus* spp.

[5]Includes *Trueperella pyogenes* and *Corynebacterium* spp.

Table 4.3. Treatment protocols, compliance, and bulk tank and milk production characteristics for moderate-sized NY dairy herds enrolled in a pathogen-based mastitis treatment protocol. herds 1-6 used 24 h sample pickup 5 d per week, herds A and B were trained to use on- farm culture to drive treatment decisions

Herd	Treatment Protocol	Compliance[1]	Wait Weekend?[2]	BTSCC Before[3]	BTSCC After[4]	ME305 Before[5]	ME305 After[6]
1	Gram +	UNK[7]	no	169,000	125,000	32,650	26,083
2	Gram +	85%	yes	188,000	146,000	32,275	31,135
3	Gram +, cull *S. aureus*	96%	yes	139,000	178,000	30,777	30,793
4	Gram +	94%	yes	90,000	89,000	31,360	31,800
5	All but "No-Growth"	73%	no	109,000	116,000	28,694	28,915
6	Gram +	85%	no	208,000	195,000	27,744	27,744
A	Gram +	59%	N/A	184,000	198,000	*	*
B	Gram +, *Klebsiella spp.*	76%	N/A	240,000	133,000	33,169	31,218
Average				165,875	147,500	30,953	29,670

[1]The percentage of cases treated according to the treatment protocol agreed upon by veterinarians and management; also considers whether farm decided to wait to treat over the weekend or not.

[2]For 5 d per week herds, whether management waited until results were returned prior to treatment.

[3]Bulk tank somatic cell count prior to the start of the trial (see Table 4.1 for individual herd dates).

[4]Bulk tank somatic cell count after the trial was complete (see Table 4.1 for individual herd dates).

[5]Mature equivalent milk production (lbs.) at the start of the trial. Herd A's computer program did not allow for this calculation. The average daily production for a cow in herd A was 87.3lbs.

[6]Mature equivalent milk production (lbs.) at the end of the trial. Herd A's computer program did not allow for this calculation. The average daily production for a cow in herd A was 86.4lbs.

[7]Due to non-compliance of entrance of treatment records into the computer system, we were unable to retrieve this information from this dairy.

Table 4.4. Monthly mastitis incidence (MMI) and percentage of cases retreated before and after implementation of a pathogen-based protocol for treatment of nonsevere mastitis. Herds 1-6 used 24 h sample pickup 5 d per week, herds A and B were trained to use on-farm culture to drive their treatment decisions

	Prior to Trial[1]				During/After Trial[2]			
Herd	Cows, no.	Cases, no.	MMI,%	Retreats, %	Cows, no.	Cases, no.	MMI,%	Retreats,%
1	125	151	2.2	20.8	122	150	2.3	23.0
2	20	21	1.8	5.0	89	113	1.7	27.0
3	50	55	4.6	10.0	122	153	3.3	25.4
4	73	92	3.0	26.0	109	154	2.8	41.3
5	207	237	3.0	14.5	320	354[a]	3.2	10.6
6	54	69	5.3	27.8	48	53	5.0	10.4
A	UNK	UNK	5.7	UNK	125	159	6.9	27.2
B	72	90	3.6	25.0	99	153	6.2	54.5

[1]Values are reflective of the number of cows over the identical period as the trial period, but from the previous year's data. These numbers are likely inaccurate as recording of mastitis events was inconsistent.

[2]Values are reflective of the cases enrolled in the trial (all nonsevere clinical cases).These values are appropriate as management was trained to consistently record events.

[a]Herd 5 did not submit cultures from all mastitis cases during the trial period. This number reflects the number of cases occurring during the trial period, according to computer records.

Table 4.5. Agreement between on-farm culture and Quality Milk Production Services ("Laboratory Culture") results for herd A. Shaded boxes represent agreement between the two methods

	Laboratory Culture						
On-farm culture	Negative	*Staph. aureus*	Gram-positive	Gram-negative	Mixed with gram-positive[1]	Other[2]	Total
Negative	34	1	5	3	0	1	44
Staph. aureus	0	25	1	0	2	0	28
Gram-positive	12	5	29	1	0	6	53
Gram-negative	6	0	0	18	1	0	25
Mixed with gram-positive[1]	1	1	1	2	0	3	8
Other[2]	0	0	0	0	0	1	1
Total	53	32	36	24	3	11	159

[1]Culture had at least one gram-positive organism in addition to one other organism.
[2]Included contamination and *Corynebacterium* spp.

Table 4.6. Agreement between on-farm culture and Quality Milk Production Services ("Laboratory Culture") results for herd A. Shaded boxes represent agreement between the two methods

	Laboratory Culture							
On-farm culture	Negative	*Klebsiella* spp.	*E. coli*	Other gram-negative	Gram-positive	Mixed with gram-positive[1]	Other[2]	Total
Negative	43	6	1	2	8	1	4	65
Klebsiella spp.	0	13	0	2	0	1	0	16
E. coli	1	0	4	2	0	0	1	8
Other gram-negative	0	0	0	0	0	0	0	0
Gram-positive	6	0	0	1	21	5	7	40
Mixed with gram-positive[1]	0	0	0	0	0	0	1	1
Other[2]	1	1	0	3	0	10	0	15
Total	51	20	5	10	29	17	13	145[a]

[1]Culture had at least one gram-positive organism in addition to one other organism.
[2]Included contamination, *Trueperella pyogenes*, yeast, and *Corynebacterium* spp.
[a]Eight results were not classified due to incubator error (machine was accidentally turned off).

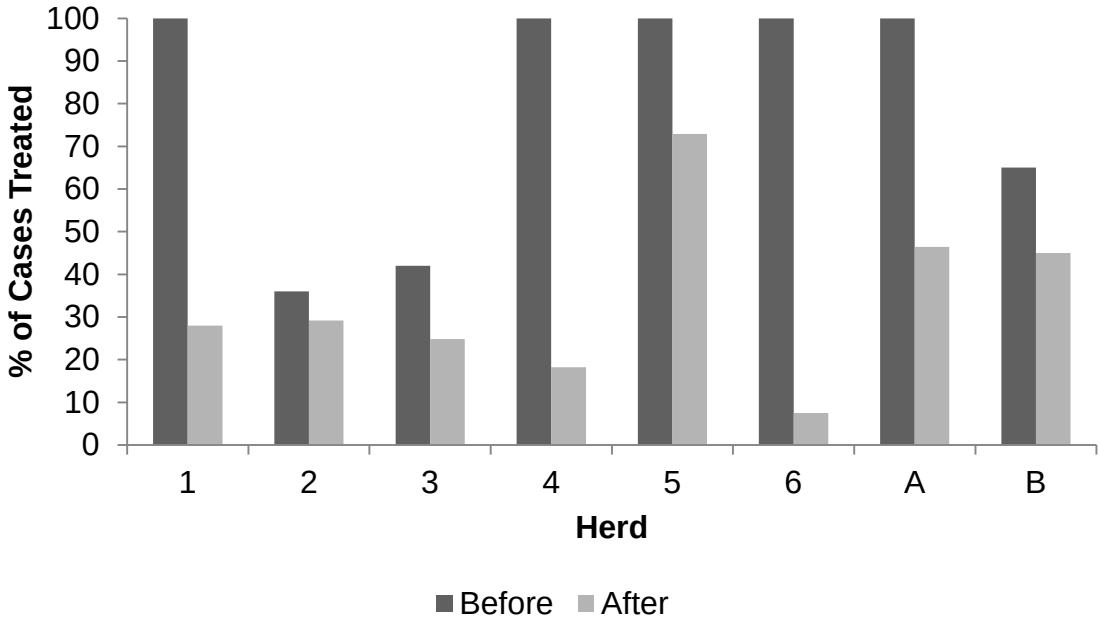

Figure 4.1. Percent of nonsevere clinical mastitis cases treated with antimicrobials before and after implementation of a pathogen-based treatment protocol by herd. Herds 1-6 used 24 h sample pickup 5 d per week, herds A and B were trained to use on-farm culture to drive their treatment decisions

DISCUSSION

This study demonstrated that on on-site farm culture systems or provision of off-site culture results 5 d per week can effectively guide pathogen-based treatment decisions for nonsevere clinical mastitis cases on moderate-sized dairies. On average, a reduction in antibiotic use of 46.4% was experienced. The variation between farms in terms of protocol design, compliance, and diagnostic accuracy was apparent. Overall, compliance and satisfaction with the program was high.

As the follow-up questionnaire generates much of the discussion for this project, detailed responses are mentioned here rather than in the results section above. This study was not performed as a clinical trial, like the one described in Vasquez et al. (2017); comparisons could not be made between outcomes for cows treated according to the pathogen-based treatment protocol and cows treated using a blanket therapy protocol. Originally proposed benchmarks for assessment of this program were evaluations of chronicity and monthly mastitis incidences before and after the trial period. What became apparent was the inconsistency regarding the recording of mastitis events and treatments. As such, the best indications for success of the program were 1) maintained or decreased BTSCC, which occurred for all dairies during the trial, 2) maintained or decreasing numbers of mastitis cases in the hospital pen on a monthly basis, which was described by similar monthly mastitis incidences over the trial period, and 3) anecdotal reports from farm management. Seven out of eight dairies believed that pathogen-based treatment was effective at their dairies. One dairy was not convinced the program was helpful, but moving forward was willing to explore not treating culture-negative cows that were experiencing mild clinical signs. This dairy owner admitted that he was informed and surprised

about the pathogen distribution on his dairy; a positive aspect of the program would be that 26% of his cases would not be treated with antibiotics.

Compliance ranged between 59% and 96% and was likely influenced on OFC herds by confidence in diagnostics. On-farm culture herds listed the following benefits of the trial: continued guidance, knowing etiology quickly, and the ability to compare QMPS results to their results. However, a drawback of the program for OFC herds was the variation in interpretation between QMPS and on-farm results. For herds A and B, the kappa agreement statistics were 0.58 and 0.42. These values fall within the "moderate" category of strength of agreement, the third category on a 5-point scale (Altmant, 1991). Regardless, the level of agreement is better than by chance alone. Levels of agreement would have been lower if categorization of culture data was on the species level. However, categorization was based on groupings of organisms used to guide treatment for each farm. Errors in diagnosis can lead to different therapeutic choices and over- or under-treatment of mastitis cases. For herd A, 15.7% and 8.0% of cases were over- and under-treated, respectively, due to misdiagnosis, assuming that laboratory culture is the gold standard. For herd B, 12% and 18% of cases were over- and under-treated, respectively. Evaluation of accurate data would allow one to determine whether under-treatment was contributing to chronicity of cases. However, given that this was not a randomized clinical trial, it would be difficult to determine if those cases would become chronic, regardless of treatment. None of the dairies believed that the cows became chronic as a result of implementation of the protocol.

Greater compliance on all farms and agreement between on-farm culture and laboratory cultures for OFC herds could have been accomplished with greater levels of veterinary input. Also, herd management was instructed to exclude any animal with systemic signs. A large

proportion of gram-negative cases were eventually treated with antimicrobials, indicating that perhaps these cases should not have been enrolled. One must also consider that aside from protocol designation, a combination of factors including parity, stage of lactation, CM history, and SCC history contribute to the decisions that herd owners make. Thus the value of culture data, particularly when highlighting a chronic or unresponsive case, can provide information to assist with culling decisions. Accordingly, benefits mentioned by dairies in either group during the follow-up questionnaire were the ability to make decisions quickly, particularly for cows that they would cull (not treat), or for those organisms they may elect to treat with additional supportive care. Decreased treatment costs, and seamless data entry due to loop access between QMPS and the dairies were also mentioned as benefits. Reductions in antimicrobial usage ranged from 6.8% to 92.5%. Differences between farms occurred due to dissimilarities in pathogen profiles and in choice of treatment protocols. For example, the combined effect of only treating gram-positive pathogens in addition to having only 7.5% of cultures positive for these organisms led to a decrease of 92.5% in antibiotic use on herd 6. This compares with herd 5, which elected not to treat only negative cultures, of which they only had 27.1%, contributing to a 27.1% reduction.

Three dairies were intrigued by the recovery rates of untreated cases and believed they were more educated on the behavior of pathogens on their dairies. Negative aspects of the program were the need to coordinate sampling with parlor workers, difficulty segregating cows once for sampling and again for treatment, and the frustration of experiencing negative culture results. Suggested improvements were adding weekend pickup, more frequent training of sampling personnel, and establishing more interpretable codes for results within DC305.

Accounting for the cost of cultures, savings on antibiotics alone was between $2,200 and $4,600 ($3.00-$6.00 per cow) over the course of one year. Several dairies indicated the difficulty of placing value on cows that they were able to cull sooner due to knowledge of etiology. One of these dairies provided additional supportive care to *E.coli*-positive, nonsevere cases when results were returned; management declared that survivability of these cases increased. One dairy owner in the 5 d pickup group noted that decreased labor costs should be factored into analysis as he had used OFC prior to the study.

Despite the few negative comments on the follow-up survey, all 6 dairies currently pay for continued courier and culture services on a 5-day-per-week basis. Though no changes in BTSCC were seen for the specific dairy, one herd relayed that mastitis detection has improved due to positive reception of the program by employees. The 2 OFC farms continue to culture their mastitis cases, though both now prefer the use of AccuMast plates (FERA Animal Health, Ithaca, NY) over Minnesota Tri-plates.

CONCLUSIONS

Implementation of culture systems or prompt reporting of culture results by nearby laboratories can effectively drive pathogen-based clinical mastitis treatment decisions on moderate sized dairies, resulting in decreased use of antimicrobials on all dairies involved. While the two on farm systems experienced moderate agreement with laboratory results, cultures from all eight farms provided data that was useful for decision making, whether for IMM treatments or culling decisions. For most dairies, no negative outcomes were noted when evaluating chronicity, monthly mastitis incidence, BTSCC, or milk production.

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CHAPTER FIVE

THE MICROBIOME OF *ESCHERICHIA COLI* AND CULTURE-NEGATIVE NONSEVERE CLINICAL MASTITIS: CHARACTERIZATION AND ASSOCIATIONS WITH LINEAR SCORE AND MILK PRODUCTION

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ABSTRACT

Culture-negative and *Escherichia coli* cases are uncommonly treated in pathogen-based protocols for nonsevere mastitis. High-throughput 16S rRNA sequencing might reveal the presence of other pathogens and can provide information on microbial diversity. The objective was to explore the milk microbiome and its association with survival in the herd, milk production, and postevent linear score (LS) for cows with clinical mastitis characterized as negative or *E. coli* by culture. Fifty *E. coli*-positive and 35 culture-negative samples from mastitis cases were enrolled. No cases were treated with antimicrobials. Follow-up samples were taken 14 d post-mastitis for the *E.coli*-positive quarters to determine chronicity. On culture of the follow-up samples, none were positive for *E. coli*. A difference in α-diversity (Shannon index) was present between enrollment and follow-up samples (3.8 versus 5.1). When α-diversity was explored for enrollment *E. coli* samples, there was no relationship between the Shannon indices of these samples and post-mastitis LS. Alpha-diversity of the enrollment samples was lower for *E. coli*-positive cows that subsequently had greater losses in milk production. This difference was explained by a greater relative abundance of the family *Enterobacteriaceae* (67.8% versus 38.4%) for cows that dropped in production. Analysis of composition of the microbiome identified one phylum, Proteobacteria, that differed between *E. coli*-positive cows that dropped in production and those that did not. Evaluation of β-diversity found no statistical relationship between post-mastitis LS and microbiome. Nine culture-negative quarters had profiles dominated by taxonomic families containing common mastitis pathogens. When evaluating α- and β-diversities and composition of the microbiomes for culture-negative quarters, no associations were found for milk production changes and post-mastitis LS. Three cows did not remain in the herd, limiting the ability to analyze survival. The findings suggest that a

contributing factor to negative outcomes in *E. coli*-positive cows is relative abundance of this pathogen, and that no single or collective group of bacterial families is associated with milk production changes or post-mastitis LS in culture-negative quarters. While additional studies should be performed, the absence of associations between outcomes explored and microbial profiles in this study suggests that we are not missing opportunities by not treating nonsevere *E.coli* or culture-negative mastitis.

Key words: mastitis, *Escherichia coli*, milk microbiome, metagenomics

INTRODUCTION

As outlined in *Journal of Dairy Science's* 100-year review, mastitis, the inflammation or infection of the mammary gland, remains the most common bacterial disease on the majority of dairies and its treatment or prevention accounts for the majority of antimicrobials administered to adult dairy cattle (Pol and Ruegg, 2007; Stevens et al., 2016; Ruegg, 2017). Pathogen-based treatment strategies for clinical mastitis (**CM**) have been useful in decreasing antibiotic use without consequential negative outcomes (Lago et al., 2011b; a; Vasquez et al., 2017). These strategies use aerobic culture results to guide therapy, often resulting in treatment of only gram-positive organisms. Pathogen-based protocols were developed in response to research that suggested a lack of efficacy of intramammary (**IMM**) antimicrobials for gram-negative mastitis pathogens (Pyörälä et al., 1994; Suojala et al., 2013). Additionally, the use of antimicrobials on culture-negative quarters can be difficult to justify; provided that 25-35% of cases produce negative results, accurate diagnostic tools should replace the routine use of treating all cases prior to etiologic diagnosis (Hogeveen et al., 2011; Oliveira and Ruegg, 2014). Though aerobic culture remains the gold standard for diagnosis, it is inherently biased toward bacteria that can

grow in specific conditions, and for organisms that are present in high numbers in the commonly aliquoted inoculum of 10µL.

Alternatively, sequencing and analysis of hypervariable regions in the 16S rRNA gene can assess bacterial diversity in milk from affected cows and might elucidate what organisms are contributing to culture-negative and other etiologic disease (Kuehn et al., 2013; Oikonomou et al., 2014; Oultram et al., 2017). Profiles generated from mastitic human and bovine milk suggest that CM is associated with dysbacteriosis and a robust microbial community in the mammary gland might aid in the prevention of an intramammary infection (**IMI**) (Fernández et al., 2013; Oikonomou et al., 2014). A dysbacteriosis detected using sequencing methods might provide information for treatment or culling decisions, however, analyses of the relationships of bacterial composition and cow-level outcomes should also be assessed. Although relationships to production and health outcomes were not investigated, Kuehn et al. (2013) identified bacterial genera that might be responsible for the microbial differences between mastitic and healthy quarters. The presence of some of genera suggested that well-known mastitis pathogens were present below culture detection thresholds. Alternatively, if a dominant pathogen such as *E.coli* is present, its disruption to the diversity of the microbiome might contribute to the development of clinical disease. As such, one could speculate that the non-treatment of these profiles in a selective treatment protocol represents a missed opportunity. Independent of treatment, the impact of microbial profiles at diagnosis on health and production outcomes must be assessed. In this study, we chose to focus on *E.coli* and culture-negative mastitic quarters, as in a selective treatment protocol, these animals do not receive IMM treatment.

Our first objective was to determine if non-treatment of nonsevere *E. coli* cases would result in chronicity. Our second objective was to explore the microbiome of cows with nonsevere

E. coli CM to determine if microbial profiles differ between cows that had positive (survival in the herd, no drop in milk production, or a next-test linear score [**LS]** of <4.0) or negative (culling from the herd, a drop in milk production, or a next test LS of ≥4.0) outcomes when not treated. Our last objective was to explore the microbiome of cows with non-treated culture-negative CM to determine if microbial profiles differ between cows that had positive or negative outcomes as defined above. Characterizing these associations will improve our understanding of CM, allowing for more effective treatment strategies.

MATERIALS AND METHODS

Study Animals

Mild to moderate CM cases that were positive for *E. coli* or negative for growth on aerobic culture were assessed for inclusion at a 3500 Holstein-cow commercial dairy in central New York between December 2014 and April 2015 under Institutional Animal Care and Use Committee approval. The resulting cases were a subset from a larger group (n = 725) of clinical cases that were used to investigate a pathogen-based treatment protocol. Herd characteristics including distribution of mastitis pathogens are described elsewhere (Vasquez et al., 2017). The J-vac coliform vaccine (Merial Limited, Duluth, GA) was given at dry off, 3-4 weeks pre-calving, and 3-4 weeks post-freshening according to the labeled directions. The farm used Dairy Herd Information Association (**DHIA**) services, which included monthly somatic cell counts (**SCC**) and milk weights. Health records were recorded using Dairy Comp 305 (**DC305**; Valley Ag. Software, Tulare, CA, USA).

Case Definition

Each mastitis case was detected by trained on-farm employees by observing abnormalities in milk such as changes in consistency and color or "udder" signs including hard, swollen, or red quarters. Cows exhibiting severe symptoms such as depression, anorexia, dehydration, or fever received systemic antimicrobials and anti-inflammatories and were excluded from the study. Other exclusion criteria were treatment with antimicrobials or anti-inflammatories in the previous 15 d or impending sale of the animal.

Sample Collection and Treatment Assignment

Using aseptic technique, a milk sample was collected from each affected quarter into a milk culture tube. These samples were defined as "enrollment" samples. The tube was labeled and promptly placed in a 5°C refrigerator. Culture results within 24 h of sample collection were achieved through the use of daily sample pick-up via the Quality Milk Production Services (**QMPS**) program at the Animal Health and Diagnostic Center in Ithaca, NY. This study focused on non-treated clinical quarters; only culture-negative and quarters positive for *E. coli* were included in the analysis. To assess for chronicity of *E.coli*-positive quarters, post-enrollment samples were targeted to be retrieved from the same quarter 14 ± 3 d after each mastitis event. These samples were defined as "follow-up" samples.

Microbiological Methods

Standard culture technique was performed on all samples by *QMPS* employees according to National Mastitis Council guidelines for identification of aerobic organisms and *Mycoplasma* spp. (National Mastitis Council, 2017). Briefly, 0.01 mL of milk was streaked on trypticase soy

agar containing 5% sheep blood and 1% esculin (PML Microbiologicals; Mississauga, ON, Canada) and MacConkey's agar (Hardy Diagnostics; Santa Maria, CA), and plates were incubated aerobically at 37°C for 18 to 24 h. Colonies from pure cultures were subjected to matrix assisted laser desorption/ionization time-of-flight (**MALDI-TOF**) using a Bruker MALDI-TOF Biotyper version 3.1.66 (Bruker Corp., Billerica, MA). Confidence levels were assigned by the machine software by referencing the Bruker 5989 RUO bacterial library. Levels accepted for species identification were ≥2.0 and for genus identification between 1.8 and 2.0. Diagnosis and confirmation of an IMI by culture of a single quarter milk sample was based on standard published definitions (Dohoo et al., 2011). Confirmation of *E. coli* as the etiologic cause was defined as the isolation of one or more colonies from ten microliters of milk.

Any cow not following treatment protocol was excluded from all analyses; the distribution of cows in each group not following protocol was described in Vasquez et al. (2017). Cows in the study were followed up for post-mastitis milk production, post-mastitis LS, and survival in the herd. All values and dates were retrieved from farm management, DHIA test-day records, or DC305. Each cow was followed until the end of its current lactation, the date it was culled, or 60 d post-enrollment, whichever arrived first. All cows remained in the treatment pen until milk returned to normal visual appearance.

Metagenomic Analysis

DNA Extraction, Isolation, and Purification. Milk samples were thawed, vortexed, and a 2 mL aliquot was removed for isolation and purification. The samples were centrifuged at 4°C and 16,000 x *g* for 3 min. The supernatant and fat were discarded and DNA was extracted from the pellet using the Powerfood DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA)

according the manufacturer's protocol. Concentration of DNA was first evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) with the following wavelengths: 230, 260, 280 nm.

Amplification of the Hypervariable Region of the 16S rRNA gene. Amplification of the V4 hypervariable region of the bacterial 16S rRNA gene was performed via PCR using the primers 515F and 806R optimized for the Illumina MiSeq platform and 140 different 12 base-pair error-correcting Golay barcodes (Illumina Inc., San Diego, CA) as previously described (Gilbert et al., 2010; Caporaso et al., 2012). Positive and negative controls were included in each plate and consisted of the following, respectively: DNA extracted from a pure colony of *Streptococcus uberis* (ATCC 700407) and DNA-free water. Amplicons were generated in triplicate using 3µL of DNA template, 1 X EconoTaq® Plus Green Master Mix (Lucigen®, Middleton, WI, USA), and 5 µM of each primer. Amplification of the 16S rRNA gene consisted of the following: an initial denaturing step of 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, 72°C for 90 s, and an elongation step of 72°C for 10 min. Amplicons from the triplicate wells were pooled and subsequently purified with a Gel/PCR DNA Fragment Extraction kit (IBI Scientific, Peosta, IA) according to the manufacturer's guidelines. Amplicons and controls were visualized by electrophoresis using 1.2% (wt/vol) agarose gels stained with 0.5 mg/mL ethidium bromide. Quantification of amplicon DNA was performed using the Qubit Fluorimeter (Life Technologies Corporation, Carlsbad, CA).

Library preparation and sequence analysis. Amplicons from individual samples were standardized to the same concentration and pooled. Equimolar libraries were sequenced on the Illumina MiSeq platform in 2 runs using the MiSeq reagent kit V2 for 300 cycles. Each run included 279 samples from multiple bovine projects and one sequencing control (purified

barcoded PCR product of DNA extracted from *Streptococcus uberis*). The raw single-end sequences generated were processed through the Quantitative Insights Into Microbial Ecology 2 (**QIIME2**; version 2017.9) open source software pipeline (Caporaso et al., 2010a). Quality control was performed on demultiplexed sequences using the DADA2 pipeline, which removed phiX artifacts and chimeric sequences, and a truncation at 180 bases was chosen (Callahan et al., 2016). Open-reference taxonomic assignment into operational taxonomic units (**OTU**s) with 97% identity was performed using UCLUST, RDP classifier, PyNAST, and Greengenes databases (DeSantis et al., 2006; Caporaso et al., 2010b; Edgar, 2010; McDonald et al., 2012). Within-sample diversity, alpha-diversity (α-diversity) was assessed using the Shannon index calculated in a randomly selected subset of the OTU database obtained through the script "diversity α-group-significance" after creation of rooted and unrooted phylogenetic trees using the mafft and FastTree programs in QIIME2 at a rarefication level of 1500 reads per sample. Shannon index evaluates richness and abundance of OTU. Between-sample diversity, beta-diversity (β-diversity) was assessed through phylogenetic-based weighted and unweighted Unifrac distances, calculated in QIIME2 through the script "diversity beta-group-significance" and the distance matrix produced was used for comparisons between outcome groups (Lozupone and Knight, 2005).

Statistical comparisons

The UNIVARIATE procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC) was used for descriptive analysis. Tabulation of relative abundances for the 24 most abundant bacterial families was performed using the tabulate function of JMP Pro 12 (SAS Institute Inc., Cary, NC). The remaining families were compiled into a single category defined as "Other." As

relative abundances and measures of diversity were not normally distributed, outcomes of interest were evaluated using nonparametric statistics. The outcomes investigated were culling or sale prior to 60 d post-mastitis, drop in milk production between the test days before and after the CM event, and LS at the test day after the mastitis event. Linear score and loss in milk production outcomes were also explored as dichotomous outcomes: loss in production was dichotomized into a reduction of >2.2 kg or not, and LS was dichotomized into ≥4.0 or <4.0. A milk production drop of 2.2 kg was chosen (approximately 5lbs) for several reasons. First, it accounts for more than the milk production declines of a normal-mid lactation curve between test days, and it is an economically meaningful loss for dairy herds. Additionally, the distribution of cattle for each outcome was similar. A linear score cutoff of 4.0 (SCC of 200,000 cells/mL) was chosen as it is commonly used as an indicator of IMI (Dohoo and Leslie, 1991; Schepers et al., 1997). PROC CORR procedures of SAS were used to determine Spearman's rank correlation to test whether α-diversity of the samples was associated with the outcomes. This test was also used to determine whether α-diversity of the samples was associated with the relative abundance of *Enterobacteriaceae* or *Ruminococcaceae* in the enrollment samples or associated with the change in relative abundance of *Enterobacteriaceae* between the enrollment and follow-up samples in the *E. coli* group. When α-diversity comparisons were made between enrollment and follow-up samples, the Wilcoxon test was used to account for repeated measures. The NPAR1WAY (Kruskal-Wallis or Wilcoxon test) procedure in SAS was used to determine the association between the relative abundance of *Enterobacteriaceae* or *Ruminococcaceae* and dichotomous outcomes. Changes in relative abundance of *Enterobacteriaceae* and the relationships to dichotomous outcomes were also explored. PROC REG in SAS was used to

explore the relationship between three continuous variables: change in milk production, Shannon index, and relative abundance of *Enterobacteriaceae* in the enrollment samples.

Multivariate analysis of the milk microbial profile was performed in QIIME2 and R (R Core Team, Vienna, Australia). Unifrac distances are quantitative measures of community dissimilarity that incorporate phylogenetic relationships between the samples. Unweighted UniFrac distances (most efficient for detecting abundance changes in rare lineages) and weighted UniFrac distances (most sensitive to detect changes in abundant lineages) were both investigated. Initially, the non-parametric test, Permutational Analysis of Variance (PERMANOVA), was used on Unifrac distance matrices in QIIME2 to determine relationships to the outcomes (Anderson, 2001). Analysis of similarities (ANOSIM) was performed on non-rarefied data using the vegan package in R. Groups that had statistical differences using ANOSIM underwent Analysis of Composition of Microbiomes (ANCOM) in QIIME2 to identify which OTUs were driving these differences. Visualization of the differences were permitted by performing principal coordinate analysis of weighted Unifrac distances in EMPeror (Vázquez-Baeza, et al., 2013). To test whether β-diversity of the samples in each dataset (culture-negative samples and *E. coli* enrollment samples) was associated with the dichotomized production outcomes, the "diversity beta-group-significance" command was used in QIIME2 to compare the Unifrac distance matrices between the categorical descriptors. Descriptive analyses of results are presented as medians, means, and standard errors.

RESULTS

Chronicity of Escherichia coli: Aerobic Culture Results

Of the 489 qualifying mild and moderate quarter-level mastitis events outlined in Vasquez et al. (2017), 113 were culture-positive for *E.coli* (23%). A total of 56 cases were assigned to the non-treated group (49.6% of *E. coli* cases). Of these, 6 were omitted from the analysis of chronicity for the following reasons: cow was dried early (n = 4), cow died (n = 1), and quarter became agalactic (n = 1). The remaining 50 cases were each contributed by an individual cow. The mean and median number of DIM at the culture event was 127 d (S.E. 12 d) and 115 d. The majority of cows (n = 37; 74%) were experiencing their first case of mastitis in the current lactation. Mastitis events were distributed across each lactation category as follows: 28% first parity (n = 14), 34% second parity (n = 17), and 38% third and greater parities (n = 19). The mean number of days between mastitis event and follow-up culture was 16 ± 3. At the time of the follow-up sample, no cows were experiencing clinical signs. The most commonly reported follow-up culture result was "negative" (n = 44, 88%). The remaining 6 cases were characterized as *Streptococcus* spp., CNS, yeast, gram-positive bacillus, *Streptococcus dysgalactiae,* or *Trueperella pyogenes*. No follow-up cultures were characterized as *E. coli*.

Descriptive Data: All Samples

Mean DIM and distribution of parity at mastitis event for each outcome can be seen in Table 5.1. These variables were not different between outcome groups for *E. coli* positive quarters. Days in milk at mastitis event were also similar for all outcome groups for culture-negative quarters. Statistically, a greater number of higher parity cows experienced negative outcomes (LS ≥4, milk drop) as compared with the number of higher parity cows that

experienced positive outcomes ($P = 0.03$, $P = 0.007$, respectively). As milk drop was calculated as the difference in milk production between the test days before and after the mastitis event, the distribution of days surrounding the event and each test day was evaluated. There was no statistical association between the number of days between the prior test date and the mastitis date and the milk drop outcome. However, when the same was evaluated for the postevent test date, a fewer number of days between the mastitis date and postevent test day was associated with the higher LS category ($P = 0.03$). The number of days between the mastitis event and the postevent test day was not associated with the other outcomes evaluated.

Sequencing Results and Taxonomic Classification

On average across all samples, 15.4% of all reads were not classified on the family level (SE = 0.9%); the number of unclassified reads at the genus level was 37.7% (SE = 1.4%). A total of 3,193,730 sequences were used for downstream analysis of the *E.coli* positive samples (n = 100 samples; 50 enrollment samples and 50 follow-up samples). The mean number of reads was 31,937 reads/sample (SE = 1,654). A total of 981,958 sequences were used for downstream analysis of the negative samples (n = 35). The mean number of reads was 28,056 reads/sample (SE = 2,886).

For *E.coli* samples, the prior test day occurred at 22 d before the mastitis event (SE = 1.5 d; range 8-41 d), while the post-mastitis test day occurred 24.5 d after the event (SE = 1.4 d; range 8-45 d). The mean number of days between retrieval of the enrollment and follow-up samples was 15.2 d (SE = 0.3 d; range, 12-21 d). For culture negative samples, the prior test day occurred at 26.0 d before the mastitis event (SE = 1.4 d; range, 9-41 d), while the post-mastitis test day occurred 22.6 d after the event (SE = 2.0 d; range, 8-41 d).

The most abundant families in the enrollment samples that were culture positive for *E. coli* were *Enterobacteriaceae* (mean = 55.7%, SE = 5.6%), *Ruminococcaceae* (mean = 11.9%, SE = 2.3%,), *Lachnospiraceae* (mean = 4.7%, SE = 0.6%), *Corynebacteriaceae* (mean = 2.5%, SE 0.7%), *Bacteroidaceae* (mean = 2.2%, SE = 0.4%), *Aerococcaceae* (mean = 1.8%, SE = 0.4%), *Paraprevotellaceae* (mean = 1.6%, SE = 0.3%), *Peptostreptococcaceae* (mean = 1.5% SE = 0.3%), *Succinivibrionaceae* (mean = 1.3%, SE = 0.3%), and *Dermabacteraceae* (mean = 1.1%, SE = 0.4%). The average reduction in relative abundance of *Enterobacteriaceae* between the enrollment and follow-up sample was 40.4% (SE = 7.8%). Detailed information on bacterial profile per study animal is shown in Figures 5.1 and 5.2.

The most abundant families in the enrollment samples that were culture-negative were *Ruminococcaceae* (mean = 31.4%, SE = 4.5%), *Streptococcaceae* (mean = 11.7%; SE = 4.5%), *Lachnospiraceae* (6.9%, SE = 0.8%), *Corynebacteriaceae* (mean = 6.6%; SE = 2.6%), *Bacteroidaceae* (mean = 3.6%, SE = 0.4%), *Moraxellaceae* (mean = 1.4%, SE = 0.6%), *Enterobacteriaceae* (mean = 2.9%, SE = 2.0%), *Bacillaceae* (mean = 2.2%; SE = 1.0%), *Planococcaceae* (mean = 2.4%, SE = 2.1%), and *Paraprevotellaceae* (mean = 2.7%, SE = 0.3%). Nine quarters had >25% abundance of a taxonomic family that includes species associated with major or minor mastitis-causing pathogens. These animals had profiles dominated by *Streptococcaceae* (n = 4: 84.0%; 71.6%, 86.9%, 99.9%), *Enterobacteriaceae* (n = 1: 72.5%), *Corynebacteriaceae* (n = 2: 42.8%, 86.7%), *Staphylococcaceae* (n = 1: 38.3%), and *Clostridiaceae* (n = 1: 86.0%). Two quarters had >25% abundance of a taxonomic family not currently documented to include mastitis-causing pathogens: *Planococcaceae* (75.6%) and *Methylobacteriaceae* (72.4%). Detailed information on bacterial profile per study animal is shown in Figures 5.3 and 5.4.

Alpha-Diversity: Associations with Outcomes for E. coli Positive Quarters

Survival data was dichotomized into cows surviving in the herd 60 d post-mastitis event, and those that did not. Only one cow (2.0%) did not remain in the herd. As this sample number was not ideal for statistical comparisons of microbiome data, analysis was not performed for survival in the herd, however, the profile of this quarter is indicated with a symbol in Figures 5.1 and 5.2.

Milk production change was normally distributed with a mean drop in milk production for culture-positive *E.coli* samples (n = 43 with full data) of 3.6 kg (SE = 1.2 kg). There was a relationship between the measure of α-diversity and changes in milk production. As Shannon index increased (Spearman r = 0.36, $P = 0.02$), production loss decreased (Figure 5.1A). When the outcome was dichotomized into cows that dropped in milk production by more than 2.2 kg and those that did not, the difference in Shannon index was 1.7. Cows that dropped in milk production had a mean loss of 3.1 kg (SE = 0.5); those that did not had a mean loss of 4.8 kg (SE = 0.5; $P = 0.02$) (Figure 5.5A). Likely associated with these differences is the relative abundance of *Enterobacteriaceae* in the enrollment samples, which was 38.4% (SE = 9.0%) for cows that did not experience a drop in milk production and 67.8% (SE = 7.0%) for cows that did ($P = 0.01$; Figures 5.1B and 5.6A). When both the initial relative abundance of *Enterobacteriaceae* and change in milk production are used as parameters in linear regression to predict Shannon index, only *Enterobacteriaceae* ($P < 0.001$) remained important in the model. Despite these relationships, there was no statistical difference between the cows that experienced a milk drop and those that did not when assessing the change in relative abundance of *Enterobacteriaceae* between the enrollment and follow-up samples ($P = 0.17$; Figures 5.1B and 5.6C).

Linear score at next test was normally distributed with a mean of 3.4 and a SE of 0.3 (n = 47). The relationship between the measure of α-diversity and post-mastitis linear score was not statistically important (Spearman r = -0.18, $P = 0.23$) (Figure 5.2A). When the outcome was dichotomized into cows that had a LS ≥4.0 (SCC ≥200,000 cells/mL) or LS <4.0 (SCC <200,000 cells/mL), the difference in Shannon index was 0.6; cows in the lower LS group had a mean Shannon index of 4.0 (SE = 0.5), those in the higher group had a mean of 3.4 (SE = 0.6; $P = 0.31$) (Figure 5.5B). Unlike the milk production outcome, the relative abundance of *Enterobacteriaceae* in the enrollment samples was not associated with the LS outcome ($P = 0.32$; Figures 5.2B and 5.6B). There was no statistical difference between the cows that experienced a LS <4.0 and those that did not when assessing the change in relative abundance of *Enterobacteriaceae* between the enrollment and follow-up samples ($P = 0.32$; Figures 5.2B and 5.6D).

Also associated with α-diversity and independent of outcome was the relative abundance of *Enterobacteriaceae* in the enrollment sample (n = 50). As relative abundance increased, the Shannon index decreased (Spearman r = -0.91, $P < 0.0001$). This relationship was also present when comparing the linear correlation between change in relative abundance of *Enterobacteriaceae* between enrollment and follow-up samples and the Shannon index of the enrollment samples (Spearman r = 0.79, $P < 0.0001$).

The difference in α-diversity between enrollment and follow-up samples for *E. coli*-positive cows (n = 50) was also investigated. Shannon diversity was statistically different between the sample types (enrollment = 3.8, SE = 0.3; follow up = 5.1, SE = 0.3, $P = 0.01$) (Figure 5.5C).

Beta-Diversity: Associations with Outcomes for E. coli Positive Quarters

Test statistics and *P*-values for β-diversity comparisons can be found in Table 5.2. The relative abundance of the family *Enterobacteriaceae*, as also noted in the α-diversity comparisons, was the main driver of the variation in UniFrac distances (Figure 5.7A) in samples from *E. coli*-positive cows. A difference in the milk microbiome was detected between cows that did and did not experience a drop in milk production of >2.2 kg (PERMANOVA unweighted $P = 0.03$, weighted $P = 0.01$). This is corroborated by the ANOSIM test ($P = 0.05$; Figure 5.7B), and by visualization of PCoA biplots in which the outcomes are somewhat clustered. Several highly influential samples from cows that experienced a drop in milk production (n = 5) might be driving this difference. These samples each had >99% abundance of *Enterobacteriaceae* and accounted for 5 out of 6 of the samples with >99% abundance; 1 sample with high abundance of *Enterobacteriaceae* was contributed by a cow that did not experience a drop in milk production. Analysis of composition of the microbiome (ANCOM) identified one OTU that differed statistically between groups, assigned to the phyla Proteobacteria. No differences were found at the family level.

Enrollment Samples: Culture-Negative Quarters

The subset of cows chosen from the larger study for metagenomic analysis of negative culture results (n = 35) had no previous cases of mastitis in the current lactation, nor were they treated with IMM antimicrobials. The mean and median number of DIM at the time of the mastitis event were both 108 d (SE = 11.7). Mastitis events were distributed across each lactation category as follows: 25.7% first parity (n = 9), 20.0% second parity (n = 7), and 54.3% third and greater parities (n = 19).

Alpha-Diversity: Associations with Outcomes for Culture-Negative Quarters

Survival data was dichotomized into cows surviving in the herd 60 d post-mastitis event, and those that did not. Only 2 cows (5.7%) did not remain in the herd. As sample number was not ideal for statistical comparisons of microbiome data, analysis was not performed for survival in the herd.

Milk production change was normally distributed with a mean drop in milk production for culture-negative samples of 2.4 kg (SE = 2.3 kg). There was no statistical relationship between the measures of α-diversity and changes in milk production (Spearman r = -0.18, P = 0.32) (Figure 5.3B). Results were similar when the outcome was dichotomized into cows that dropped in milk production by more than 2.2 kg and those that did not; the difference in Shannon index was 0.1 (cows that dropped in production = 4.3 SE = 0.5; those that did not = 4.4, SE = 0.4; P = 0.95) (Figure 5.8A).

Linear score at next test was normally distributed with a mean of 3.3 and SE of 0.4. The relationship between the measures of α-diversity and post-mastitis linear scores was not statistically significant (Spearman r = 0.04, P = 0.84) (Figure 5.4). When the outcome was dichotomized into cows that had a LS ≥4.0 or LS <4.0, the difference in Shannon index was 0.1; cows in the lower LS group had a mean Shannon index of 4.4 (SE = 0.6) and those in the higher group had a mean of 4.5 (SE = 0.4; P =0.90) (Figure 5.8B).

Also explored was the relative abundance of *Ruminococcaceae* and Shannon index, as milk from healthy cows has been documented to have comparatively larger relative abundances of this family. However, a relationship between the two parameters was not statistically significant (Spearman r = 0.24, P = 0.17). The relative abundance of *Ruminococcaceae* was also not associated with a linear milk production change or the dichotomous milk drop outcome

(linear: r = 0.27, *P* = 0.26; dichotomous: cows that dropped in production = 24%, SE = 0.06, those that did not = 39%, SE = 0.07, *P* = 0.13). Linear score analysis produced similar findings, though abundances of *Ruminococcaceae* were numerically higher for those cows that did not experience negative outcomes (linear: r = -0.19, *P* = 0.26; dichotomous: LS ≥4.0 mean = 25.2%, LS <4.0 mean = 34.2%, *P* = 0.37).

Beta-Diversity: Associations with Outcomes for Culture-Negative Quarters

Test statistics and *P*-values for β-diversity comparisons can be found in Table 5.2. No statistical differences were found for comparisons made between cows experiencing positive and negative outcomes within the dataset of culture-negative cows. Analysis of composition of microbiomes was explored but no differential OTUs were present at any taxonomic level when comparing outcomes.

Table 5.1. Cow and data retrieval descriptors for each clinical mastitis outcome analyzed. Mastitis cases were characterized and analyzed separately for *E. coli*-positive or culture-negative quarters

	***E. coli* Positive**					
	LS[1] <4	**LS[1] ≥4**	***P-value*[2]**	**No MD[3]**	**MD[3]**	***P-value*[2]**
DIM[4]	130	122	0.73	128	146	0.46
Parity, no. (%)			0.16			0.76
1st	11 (37.9)	3 (16.7)		7 (35.0)	6 (26.1)	
2nd	11 (37.9)	5 (27.8)		6 (30.0)	10 (43.5)	
3rd	4 (13.8)	5 (27.8)		5 (25.0)	4 (17.4)	
>3rd	3 (10.3)	5 (27.8)		2 (10.0)	3 (13.0)	
Test before[5]	N/A	N/A		20	23	0.45
Test after[6]	26	25	0.76	28	25	0.45
	Culture-Negative					
	LS[1] <4	**LS[1] ≥4**	***P-value*[2]**	**No MD[3]**	**MD[3]**	***P-value*[2]**
DIM[4]	110	104	0.82	110	114	0.57
Parity, no. (%)			0.03			0.007
1st	7 (29.2)	2 (18.2)		8 (44.4)		
2nd	7 (29.2)			3 (16.7)	3 (21.4)	
3rd	6 (25.0)	2 (18.2)		2 (11.1)	5 (35.7)	
>3rd	4 (16.7)	7 (63.6)		5 (35.7)	6 (42.9)	
Test before[5]	N/A	N/A		27	24	0.26
Test after[6]	26	16	0.03	25	20	0.29

[1]Linear Score at next test (8 to 43 d) after the mastitis event, dichotomized.

[2]Student's t-test for continuous data, Pearson's chi-squared for parity comparison.

[3]Milk production drop between test day previous to mastitis event (8 to 43 d) and test day after the mastitis event (8 to 43 d), dichotomized into ≤2.2 kg or >2.2 kg.

[4]Days in milk at the mastitis event.

[5]Mean number of days prior to the mastitis event that data was retrieved to analyze milk production drop, not applicable to LS outcome.

[6]Mean number of days post-mastitis event that data was retrieved for analysis of LS and MD.

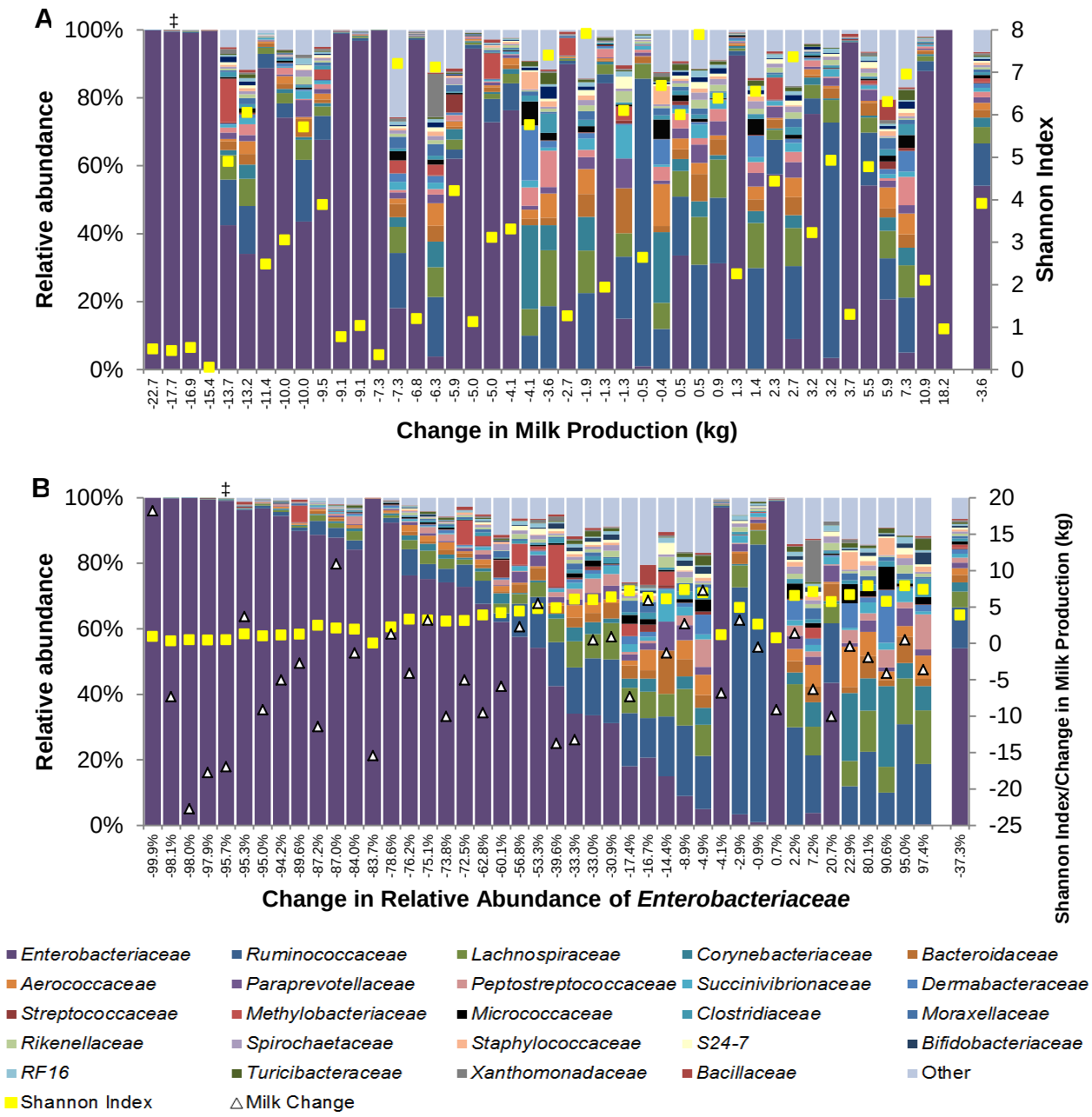

Figure 5.1. Investigating the relationship between metagenomic profile and milk production outcomes. The top 25 most abundant families in individual cows (n = 43) experiencing mild to moderate clinical mastitis characterized on aerobic culture as *E. coli* (final column is mean of all cows). **A**. The relationship of the microbial profile to the Shannon index (yellow squares), plotted by the change in milk production (kg) between the test day before and after the mastitis event (x-axis) **B.** The relationship of the microbial profile to the Shannon index (yellow squares), change in milk production (white triangles) plotted by the change in relative abundance of *Enterobacteriaceae* between enrollment and follow-up samples (x-axis; 14 days after mastitis event), ‡ = cow did not survive in the herd > 60 d

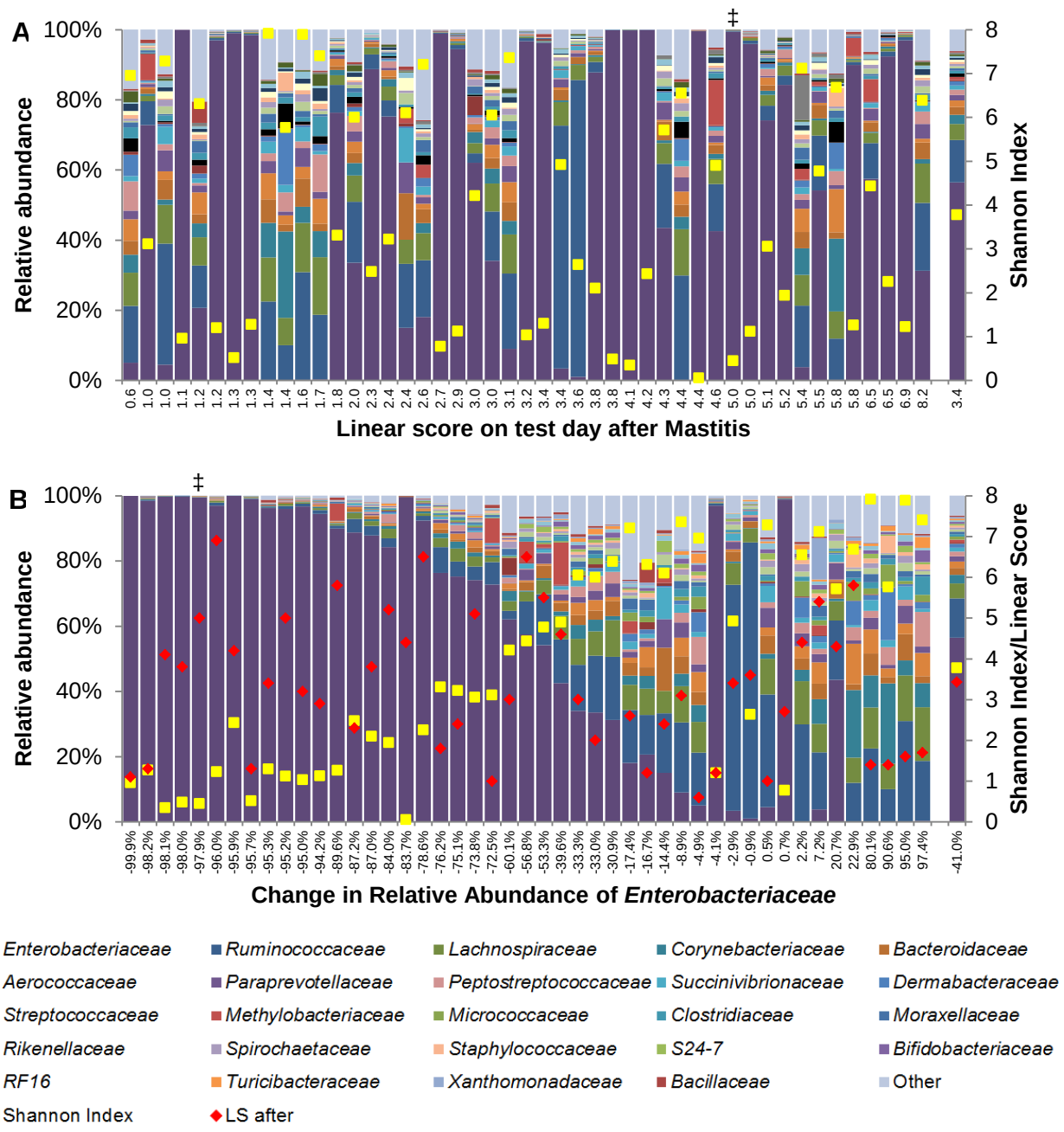

Figure 5.2. Investigating the relationship between metagenomic profile and linear score outcomes. The top 25 most abundant families in individual cows (n = 47) experiencing mild to moderate clinical mastitis characterized on aerobic culture as *E. coli* (final column is mean of all cows). **A**. The relationship of the microbial profile to the Shannon index (yellow squares), plotted by the linear score on the DHIA test day 8-43 d after the mastitis event (x-axis) **B.** The relationship of the microbial profile to the Shannon index (yellow squares), linear score (red diamonds) plotted by the change in relative abundance of *Enterobacteriaceae* between enrollment and follow-up samples (x-axis; 14 days after mastitis event), ‡ = cow did not survive in the herd > 60 d

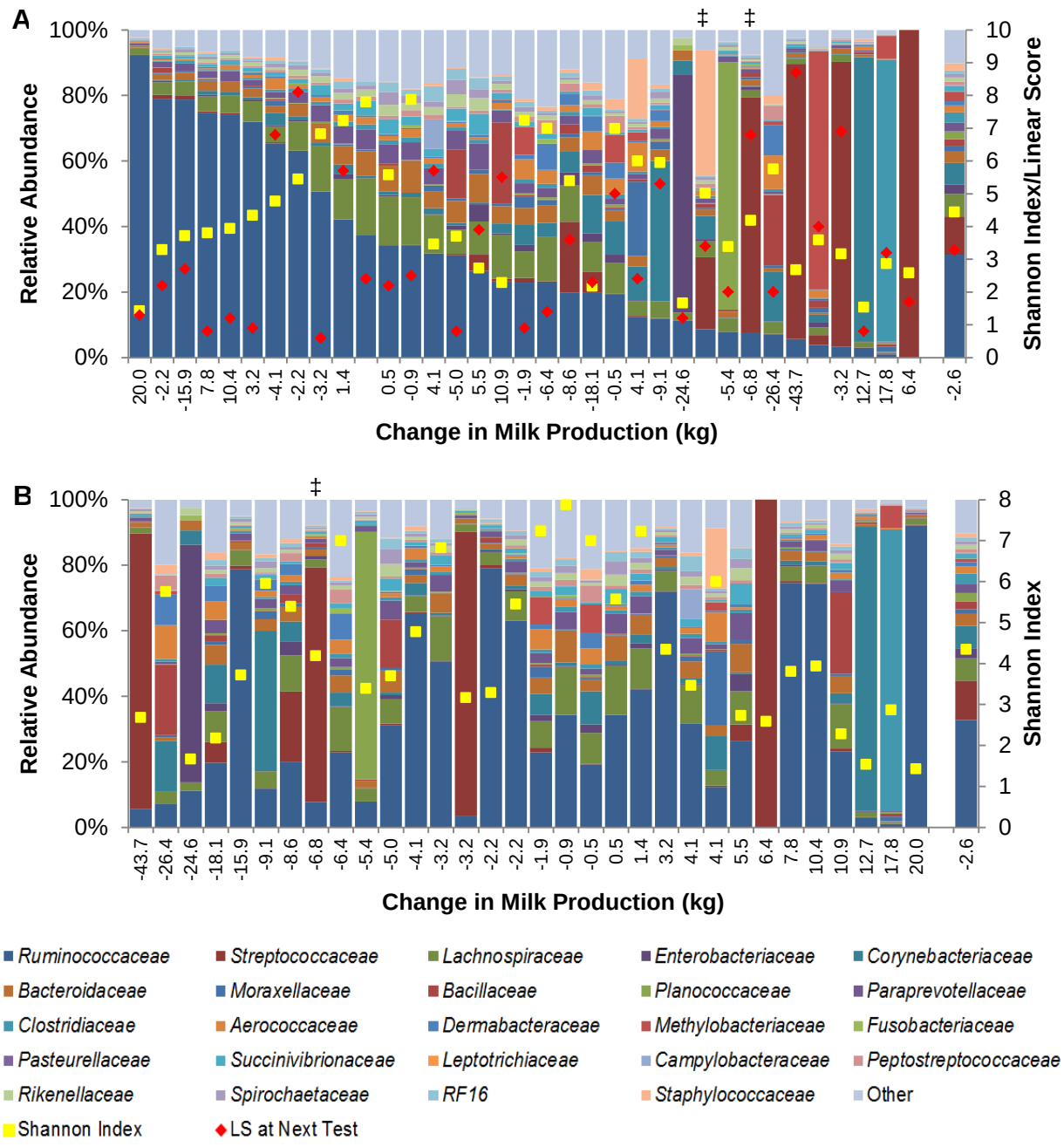

Figure 5.3. Investigating the relationship between metagenomic profile and milk production outcomes for individual cows experiencing mild to moderate clinical mastitis and negative aerobic culture results. Final column is mean of all cows. **A.** Relationships of the microbial profile to linear score at next test (red triangles), the Shannon index (yellow squares), and change in milk production (x-axis) between the test dates before and after the mastitis event, plotted by the relative abundance of *Ruminococcaceae* for all cows (n = 35) in the dataset. **B.** Relationships of the microbial profile to the Shannon index and increasing positive milk production changes (n = 32), ‡ = cows that did not survive in the herd > 60 d

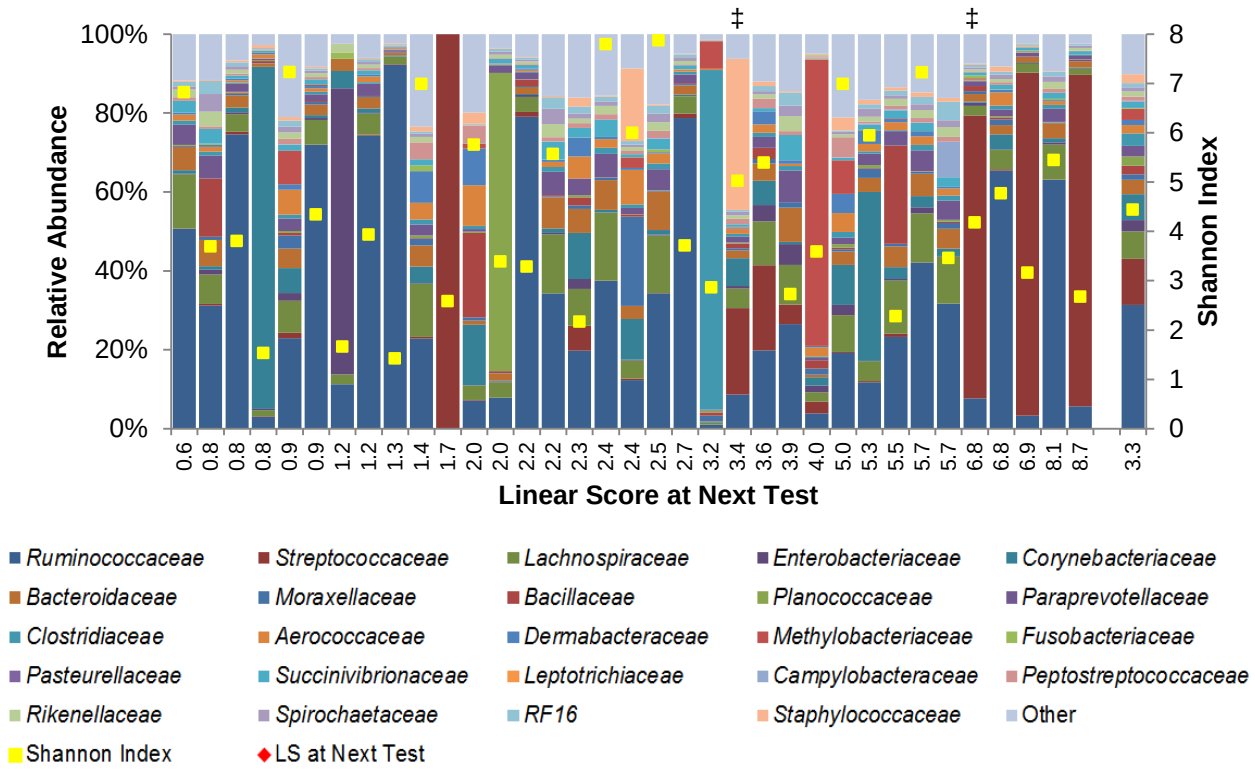

Figure 5.4. Investigating the relationship between metagenomic profile, Shannon index, and linear score outcomes for individual cows experiencing mild to moderate clinical mastitis and negative aerobic culture results. Final column is mean of all cows (n = 35), ‡ = cows that did not survive in the herd > 60 d

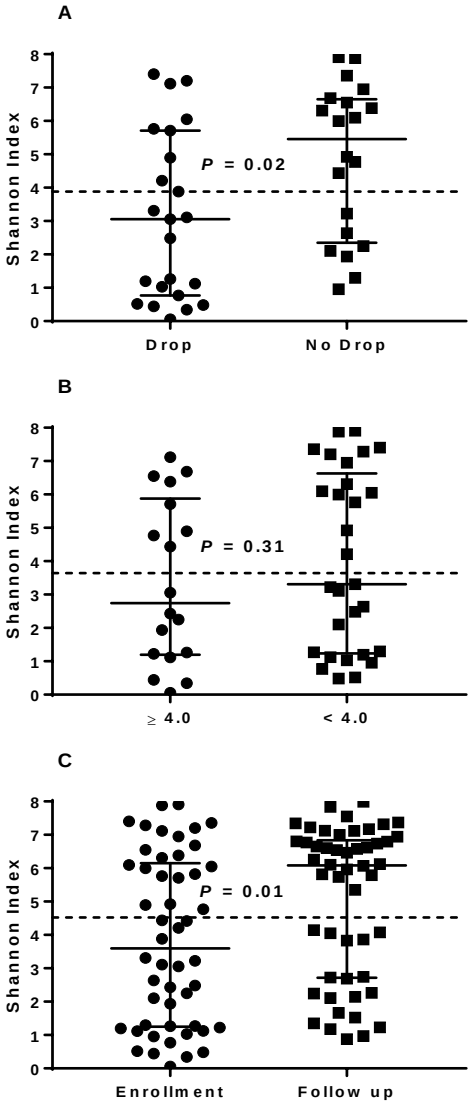

Figure 5.5. Scatterplots investigating the relationship between alpha-diversity (Shannon index) of milk samples and outcomes for cows experiencing mild to moderate clinical mastitis characterized on aerobic culture as *E. coli*. **A.** Shannon index comparison of enrollment samples when milk production has been dichotomized into cows that have decreased in production by 2.2 kg (Drop) or did not (No Drop) between the test day prior to mastitis and the test day after (n = 43). **B**. Shannon index comparison of enrollment samples when linear score is dichotomized into cows that have a post-mastitis test-day linear score of ≥4.0 and those <4.0 (n = 47). **C.** Shannon index comparison of enrollment samples versus follow-up samples (n = 50). Medians and quartiles represented, dotted line represents grand mean. Statistical testing with Kruskal-Wallis (A and B) and Wilcoxon (C).

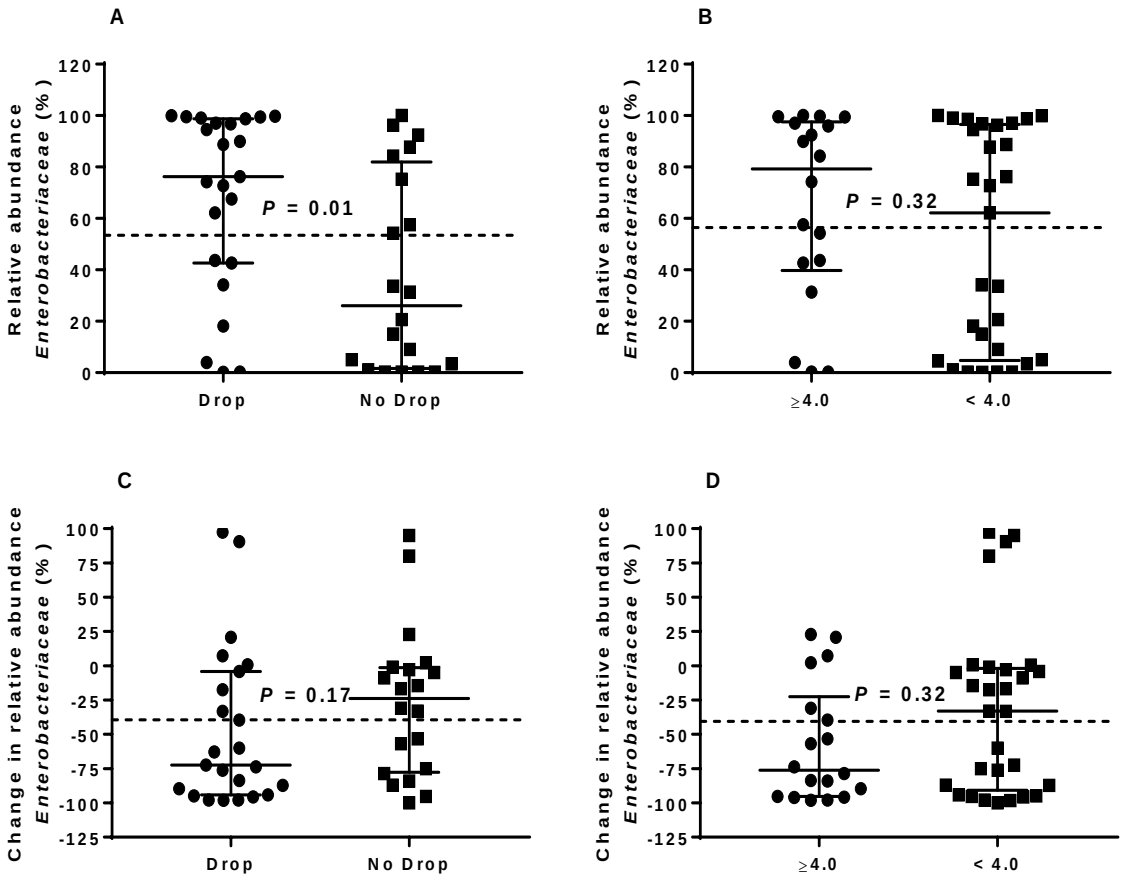

Figure 5.6. Scatterplots investigating the relationship between relative abundance of *Enterobacteriaceae* (A, B) and change in relative abundance of *Enterobacteriaceae* between enrollment and follow-up samples (C, D) for the following outcomes: drop in milk production (n = 43) (A, C) or post-mastitis linear score <4.0 or ≥4.0 (n = 47) (B, D). Medians and quartiles represented, dotted line represents grand mean. Statistical testing with Kruskal-Wallis (A and B) and Wilcoxon (C and D)

Table 5.2. Beta-diversity statistical comparisons between metagenomic profiles of cows with negative versus positive outcomes for cows experiencing mild to moderate clinical mastitis characterized as *E. coli* or negative on aerobic culture

		Unweighted Unifrac		Weighted Unifrac	
Outcome	no.	Test Statistic	*P-value*	Test Statistic	*P-value*
Milk production drop vs. no drop[1]					
E. coli on culture	43	1.97	0.03	4.11	0.01
Negative on culture	32	0.85	0.70	0.97	0.38
Linear Score <4.0 vs. ≥4.0					
E. coli on culture	47	0.92	0.50	0.44	0.76
Negative on culture	35	0.84	0.73	0.45	0.75

[1]Drop in milk production dichotomized into ≤2.2 kg or >2.2 kg.

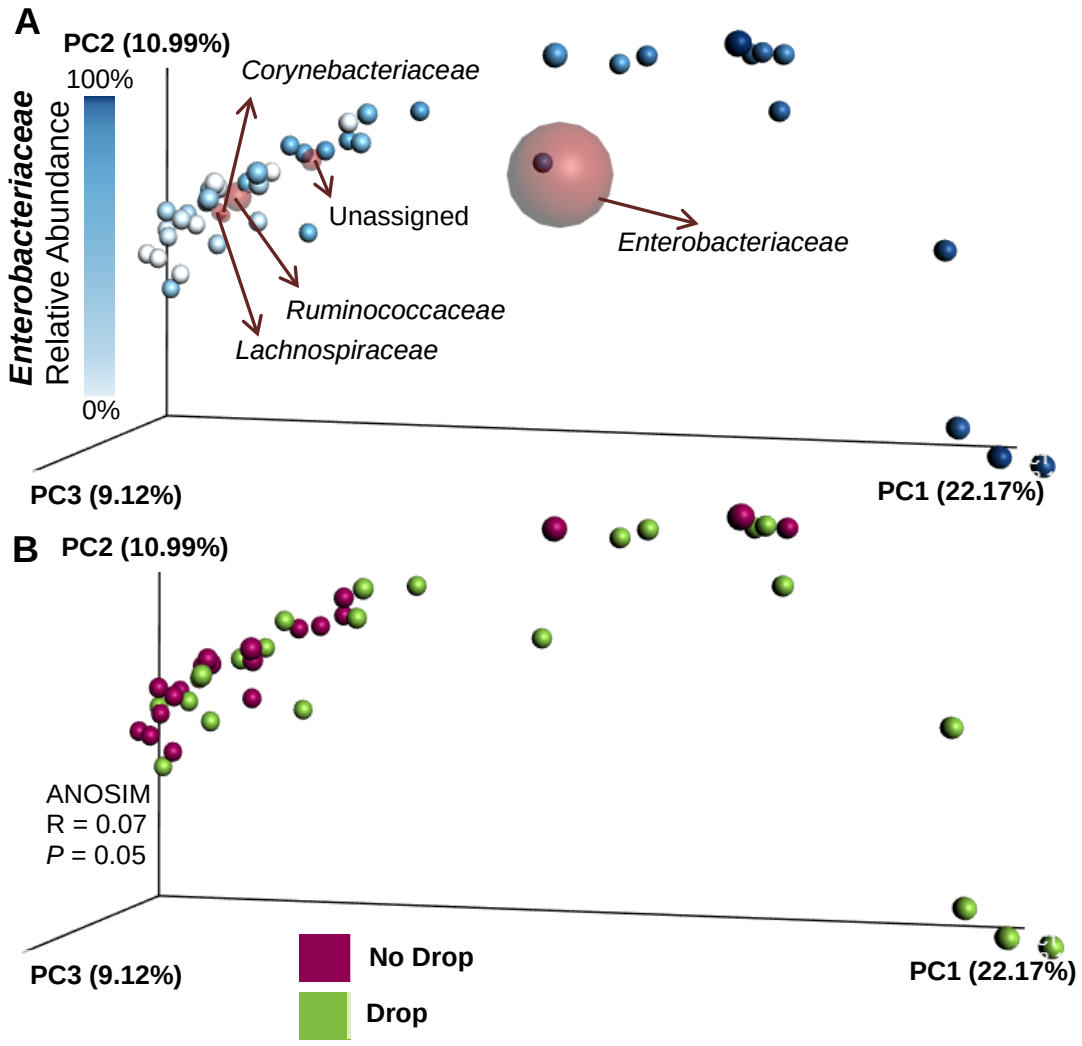

Figure 5.7. Biplots depicting use of weighted UniFrac distances for principal coordinate analysis (PCoA) of *E. coli* culture-positive samples (n = 43) and **(A)** the coordinates of the five most abundant family-level taxa (red spheres) in the context of relative abundance of *Enterobacteriaceae.* (**B)** Relationship of the same samples comparing samples of cows that dropped in milk production , "Drop" (>2.2 kg) and those that did not, "No Drop" in analysis of similarity (ANOSIM)

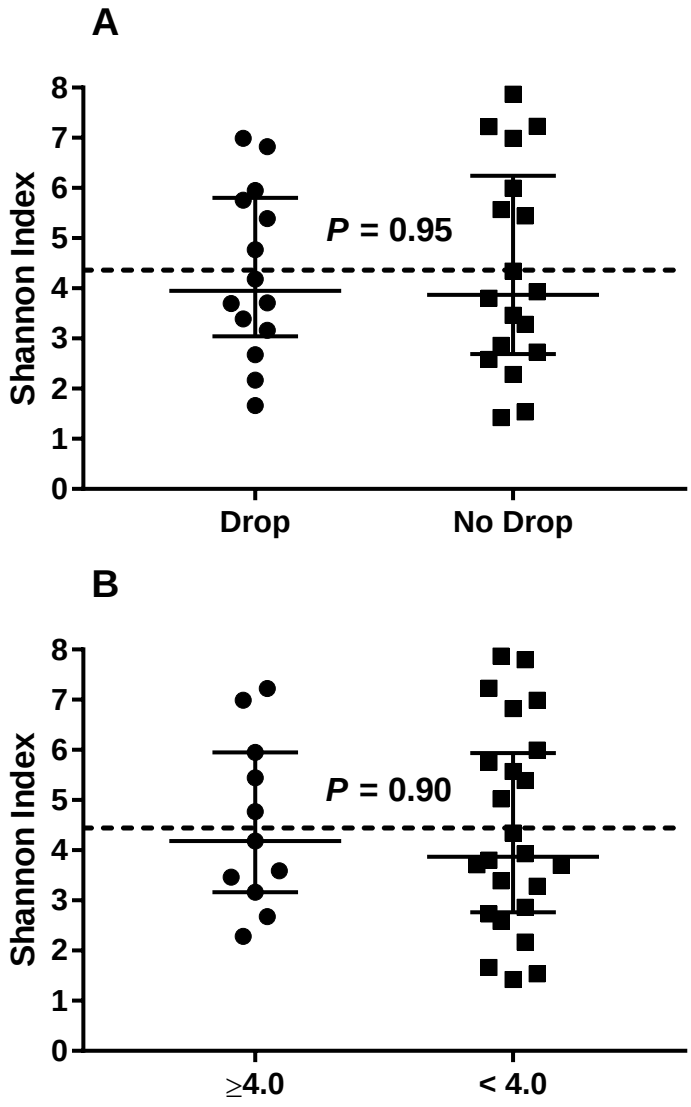

Figure 5.8. Scatterplots investigating the relationship between alpha diversity (Shannon index) and outcomes for cows experiencing mild to moderate clinical mastitis characterized on aerobic culture as negative. **A.** Shannon index comparison when milk production has been dichotomized into cows that had a decrease in production by 2.2 kg (Drop) or did not (No Drop) between the test day prior to mastitis and the test day after (n = 32). **B.** Shannon index comparison when linear score is dichotomized into cows that have a post-mastitis test-day linear score of ≥4.0 and those <4.0 (n = 35). Median and Quartiles represented, dotted line represents grand mean. Statistical testing with Kruskal-Wallis

DISCUSSION

Reduced microbial diversity has been associated with several human diseases such as inflammatory bowel disease and obesity (Ley et al., 2006; Turnbaugh et al., 2009). Additionally, primiparous cows that would go on to experience mastitis within the first 30 d postpartum had colostrum with a lower diversity index than those that did not (Lima et al., 2017). Using these concepts we wished to determine if there is a characteristic "mastitis microbiome" associated with LS or changes in milk production, in the absence of treatment. This study applied 16S metagenomic characterization of culture-negative and *E. coli*-positive nonsevere mastitis cases for this purpose. When milk production changes and post-mastitis LS were investigated for *E. coli*-positive cows, associations between α- and β-diversity measurements, milk production changes, and initial abundance of *Enterobacteriaceae* were present. No associations were found for α- and β-diversity measurements and outcomes for culture-negative cows. There was no set of microorganisms in *E. coli*-positive or culture-negative samples that was associated with the outcomes explored.

Alpha- and β-diversities of the *E.coli*-positive samples were associated with relative abundance of *Enterobacteriaceae* and milk production changes. The larger relative abundance of *Enterobacteriaceae* in samples from cows that experienced larger drops in milk production is perhaps reflective of a higher relative abundance of *E. coli*, though the current taxonomic analysis did not classify organisms to the species level. If relative abundance is used as a proxy for bacterial numbers, which is often true when the pathogen identified is the etiologic cause, the association between diversity measurements and milk production changes found is likely due a greater concentration of lipopolysaccharide (**LPS**) in the mammary gland. This ascribes to the knowledge that the outcome and severity of *E.coli* mastitis is mainly attributed to environmental

and immune factors in response to LPS, rather than virulence of the *E. coli* strain (Burvenich et al., 2003; Mehrzad et al., 2008). Likewise, increased cytokine synthesis is associated with an increased concentration of *E. coli* particles (Günther et al., 2011). The authors speculate that associations were found for changes in milk production, but not for post-mastitis LS, due to the transient nature of the organism. Initial growth of *E.coli* is decelerated by a quick spike in somatic cells in the milk (Hill, 1981), and while LS recovers, perhaps secretory epithelial damage in the gland undergoes a more delayed resolution, if any. Further investigation using quantitative PCR, LPS bioassays, or biopsy would provide answers to these hypotheses.

Upon analysis of composition of microbiomes (ANCOM), the phylum Proteobacteria was differentially abundant between the *E.coli* samples from cows that experienced a drop in production and from those that did not. Proteobacteria was also higher in abundance in *E.coli*-positive quarters compared with healthy quarters in Ganda et al. (2016), and was driven by greater abundances of *Enterobacteriaceae*, as observed in the current study. Other investigators have observed that drops in milk production were highest and most persistent for cows with *E.coli* infections (Gröhn et al., 2004; Schukken et al., 2009), which agree with our findings for these quarters. Also, clinical cure was statistically different between cows having higher versus lower abundances of *Enterobacteriaceae* in Ganda et al. (2016), indicating that clinical outcomes are affected by this measure.

Despite the presence of only *E. coli* on aerobic culture or the absence of pathogens for the culture-negative samples, metagenomic analysis of milk from these quarters revealed the presence of a large diversity of taxonomic families. This is consistent with previous characterization of mastitic and healthy human and bovine milk microbiomes and also supports the idea of an endogenous entero-mammary pathway, as many families present in the

gastrointestinal tract such as *Ruminococcaceae, Clostridiaceae,* and *Lachnospiraceae* were present in the milk samples (Kuehn et al., 2013; Oikonomou et al., 2014; Addis et al., 2016; Ganda et al., 2016). Using quantitative methods, groups have shown that despite the vast representation of different microbial species in both healthy and mastitic milk samples, mastitic samples contain statistically larger bacterial loads or higher overall DNA concentrations than their healthy counterparts (Kuehn et al., 2013; Ganda et al., 2016). Additionally, negative correlations between the Shannon index and bacterial load have been described, indicating that when overall microbial concentration increases, there is a reduction in microbial diversity (Bonsaglia et al., 2017). This can be observed for the Shannon indices of the mastitic quarters in the current study, which were 3.5 for *E.coli*-positive quarters and 4.5 for culture-negative quarters. Values are similar to those documented for mastitic quarters and much lower than values observed in healthy quarters, which range from 7.0 to 9.0 (Oikonomou et al., 2014; Ganda et al., 2016; Rodrigues et al., 2017). In the 14 d follow-up samples, quarters in the current study reached a mean value of 5.1; in the Ganda et al. study, mean values for *E.coli* and culture-negative mastitic quarters at 14 d were approximately 6.5. This could be due to a greater decrease in relative abundance of *Enterobacteriaceae* from days 1 to 14 (reduction from 62.6% to 9.7%) in Ganda et al. as compared to our findings (reduction from 55.7% to 15.2%).

Although a relative abundance of 9.7% *Enterobacteriaceae* on 16S analysis of post-mastitis samples was observed, all cows with cultures positive for *E. coli* experienced bacteriological cure by day 14. Unfortunately, as the risk for cure was 100%, the differences between the microbiomes of quarters that did and did not cure could not be evaluated. Our high spontaneous cure rate agrees with Ganda et al. (2016) who found that 80% of non-treated and 85% of treated *E. coli*-positive quarters experienced bacteriological cure. Roberson et al. (2004)

also found that 85% of *E. coli* cases (17/20) were cured bacteriologically by 7 d, regardless of treatment. Collectively, these findings agree with conclusions made by Döpfer et al. (1999): less than 5% of "chronic" or recurrent *E.coli* mastitis cases are genotypically identical. Regardless, if we had identified chronic strains of *E.coli* in the current study, that would not necessarily indicate the need for IMM antimicrobial treatment as research shows that persistency of *E. coli* is associated with higher odds of resistance to ampicillin and ceftiofur, common drugs labeled for the treatment of CM (Fairbrother et al., 2015).

Bacteriological cure was not assessed for culture-negative quarters as no pathogen was isolated on aerobic culture. Indeed, a major finding in this study was the inability to identify a potentially causative taxonomic family in the microbial profiles of 24 of the 35 samples. This is similar to results outlined in Ganda et al. (2016) and Oikonomou (2014): none of the culture-negative clinical quarters contained a taxonomic family representing over 25% relative abundance. Our findings are also similar to those described in Kuehn et al. (2013), where no genus represented greater than 25% of the microbiome in 9 out of 10 culture-negative clinical mastitis samples. The largest mean relative abundance, 20.4%, in Kuehn et al. (2013) was for the genus *Sphingomonas.* Culture-negative quarters in Oikonomou et al. (2014) had a mean relative abundance of 20.6% *Caulobacter leidyi*, a poorly-characterized species also associated with the *Sphingomonas* genus. Conflicting with this idea is the finding by Hunt et al. (2011), who listed *Sphingomonas* among the 15 most abundant genera found in healthy human milk. However, given that its relative abundance in healthy human milk is 2.4% (Hunt et al., 2011), establishment of a dysbacteriosis during the clinical mastitis event should not be ruled out. The family *Sphingomonadaceae*, while present in the current study, was found in very low abundances (<1%) across all culture-negative samples. A longitudinal or challenge study needs

to be performed to provide proof of pathogenicity for these organisms as a primary cause of bovine CM. This argument can also be made for the families *Planococcaceae* and *Methylobacteriaceae* which are present in high abundance in two culture-negative samples from the current study; neither of which is reported in the samples from Hunt et al. (2011), Kuehn et al. (2013), or Oikonomou et al. (2014).

Nine culture-negative quarters were dominated by one taxonomic family that includes species associated with major or minor mastitis-causing pathogens. If these pathogens were the primary cause of mastitis, they would likely have been apparent by culture if the bacteria were at higher concentrations, or more importantly, if the bacteria were still viable—a characteristic that cannot be assessed through sequencing methods. Therefore, the presence of a dominant family on 16S analysis of culture-negative samples may be indicative of a primary infection that has spontaneously resolved. The dominant families identified in 7 of the samples (*Streptococcaceae*, *Corynebacteriaceae*, and *Staphylococcaceae*) have also been identified in healthy human and bovine milk, suggesting that they are a normal part of the milk microbiota (Hunt et al., 2011; Ward et al., 2013; Ganda et al., 2016; Bonsaglia et al., 2017). Therefore, again, we cannot rule out a dysbacteriosis or disruption of the normal microbiota as an alternative to, or an addition to, a primary infection. Alternatively, members of the *Staphylococcaceae* and *Corynebacteriaceae* families can be commensals habituating teat skin, which, taking into account our procedure for collecting milk, might be contaminants or might support the overlap of the microbiota of the environments (Verdier-Metz et al., 2012; Kuehn et al., 2013). The finding of several anaerobic families such as *Lachnospiraceae, Paraprevotellaceae, Clostridiaceae,* and *Ruminococcaceae* indicate an accurate description of the microbiomes and not simply contamination. This agrees

with findings from bovine colostrum (Lima et al., 2017), bovine milk (Bonsaglia et al., 2017; Rodrigues et al., 2017), and human milk (Fernández et al., 2013; Jiménez et al., 2015).

Characterization of the microbiomes of culture-negative samples infrequently identified a dominant pathogen. Likewise, no differential taxa or group of pathogens was associated with LS or milk production outcomes. Additionally, α- and β-diversity measures within and between outcome groups were similar. The inability to detect a "pathogenic profile" sheds light on previous findings from culture-based selective treatment protocols where authors showed no differences in several health and production outcomes between cows assigned to blanket IMM therapy and cows assigned to selective treatment (Lago et al., 2011b; a; Vasquez et al., 2017; McDougall et al., 2018). For example, in Lago et al. (2011), linear SCC, days to clinical cure, bacteriologic cure risk, new infection risk, treatment failure risk, and milk production were similar between untreated culture-negative cases and treated culture-negative cases as well as between untreated gram-negative cases and treated gram-negative cases (Lago et al., 2011b). Not all outcomes investigated in Lago et al. were explored for their relationship to the microbiome in the current study. Few cows with no-growth or *E. coli*-positive cultures experienced negative outcomes related to culling, clinical cure, or treatment failure; low sample numbers prevented the ability to assess these outcomes in the current study. While inherently bacteriological cure risk cannot be evaluated for culture-negative cases, follow-up cultures would have allowed for exploration of new infection risk and the relationship to aspects of the mastitis microbiome, something that was not performed in the current study.

Perhaps no associations were present at the family level between culture-negative cows that experienced negative outcomes and those that experienced positive outcomes because the samples were similar to profiles of healthy quarters. The resilient nature of the milk microbiome

was described by Ganda et al. (2016). In that study, differences were found between profiles of culture-negative mastitis samples versus healthy samples on day 0 when evaluating analysis of similarities (ANOSIM), but a clear separation in principal coordinate analysis (β-diversity) was not observed. When comparing healthy to culture-negative mastitic quarters, shifts in the milk microbiome for several different phyla (Firmicutes, Bacteroidetes, Tenericutes, and Spirochaetes) were found in Ganda et al. (2016). At day 14, however, the profiles were indistinguishable. Evaluation on the phyla level was evaluated for culture-negative samples in the current study, but no differential phyla were found. The addition of more cows to the current study might have allowed us to detect differences in microbial profiles. In the advent of big data, there are no guidelines dictating or suggesting an appropriate sample size to ensure the ability to detect differences in microbiomes between multiple groups, given a specified level of Type I error.

A limitation of our study design is the absence of healthy quarter samples or follow-up cultures from each affected quarter. Benefits exist for having a healthy quarter comparison as individuals can have diverse microbial profiles dependent on parity, stage of lactation, environment, or bodyweight (Hunt et al., 2011; Cabrera-Rubio et al., 2016; Lima et al., 2017). In the current study, we were therefore unable to determine if the samples could be discriminated, in profile, from healthy milk from the same animal. Characterization of a healthy contralateral quarter or a post-resolution sample would allow us to discriminate between a primary infection or a dysbacteriosis. From a healthy quarter comparison, Oultram et al. (2017) used a 2-fold increase in bacterial relative abundance to diagnose 80% of the potentially causative pathogens. As noted above, Kuehn et al. (2013) also used a healthy quarter comparison to identify 7 differentially present genera in culture-negative mastitis samples versus healthy samples. If

follow up samples were available, similar techniques could have been used in the current study to determine whether certain bacteria in the culture-negative quarters were causative; healthy versus mastitic analysis would allow us to rule out the contribution of specific taxonomic families to negative outcomes.

There were a statistically higher number of later lactation cows in the LS ≥4.0 and milk drop groups versus the respective positive outcome groups for culture-negative quarters. This finding is well supported by research that shows that higher-parity cows experience greater impacts on milk production during mastitis events, and are overall more susceptible to repeat mastitis events (Gröhn et al., 2004; Pinzón-Sánchez et al., 2011; Hiitiö et al., 2017). As microbiota can be more diverse for higher parity animals, a selection of cows from the same lactation may have provided a better comparison (Lima et al., 2017). Similarly, a shorter time period between the mastitis event and the next-test day was associated with the higher LS category. Therefore, cows in the dataset that were allowed more time to recover from the mastitis event prior to data retrieval appeared to experience more positive outcomes. Collection of a milk sample from the affected quarter at a standard number of days after the event would eliminate this bias. Ideally, to minimize bias associated with time between the mastitis event and test day data, and to best characterize the effects of microbiota on milk production and linear score, daily milk weights and LS could be recorded, though statistical comparisons in a multivariate model could prove difficult. Despite discrepancies between demographics of the outcome groups, the results for comparisons of diversity measurements of pathogen profiles do not support the need to treat *E. coli* and culture-negative cases with currently available antibiotics. However, cow characteristics and historical LS should also be considered when making treatment decisions.

CONCLUSIONS

The current study presented an analysis of the milk microbiome of culture-negative and *E.coli*-positive CM quarters. Irrespective of metagenomic characteristics, many of the cases experience positive outcomes without treatment. There was no pathogen profile in culture-negative cases that was associated with linear score or milk production outcomes. Relative abundance of *Enterobacteriaceae* was associated with greater losses in milk production between test days for cows that were experiencing nonsevere *E.coli* mastitis. However, no *E.coli*-positive cow cultured positive for this organism 14 d after the mastitis event. As dairies are more commonly using diagnostic tools to aid in treatment decisions, more research is needed for those cases that will not be treated, specifically for characterizing or confirming a dysbacteriosis and for exploring associations between the microbiome and additional outcomes.

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CHAPTER SIX

USE OF A CULTURE-INDEPENDENT ON-FARM ALGORITHM TO GUIDE THE USE OF SELECTIVE DRY COW ANTIBIOTIC THERAPY

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ABSTRACT

An algorithm using only computer-based records to guide selective dry cow therapy was evaluated at a New York dairy farm via a randomized field trial. Dairy Comp 305 and Dairy Herd Information Association test-day data were used to identify cows as low risk (cows that might not benefit from dry cow antibiotics) or high risk (cows that will likely benefit). Low-risk cows were those that had all of: somatic cell count (SCC) ≤200,000 cells/mL at last test, an average SCC ≤200,000 over the last 3 tests, no signs of clinical mastitis at dry-off, and have not had greater than one clinical mastitis event in the current lactation. Low-risk cows were randomly assigned to receive intramammary antibiotics and external teat sealant (ABXTS) or external teat sealant only (TS) at dry-off. Using pre-dry-off and post-calving quarter-level culture results, low-risk quarters were assessed for microbiological cure risk and new infection risk. Groups were also assessed for differences in first-test milk yield and linear scores (LS), individual milk weights for the first 30 days, and culling and mastitis events before 30 days in milk. A total of 304 cows and 1,040 quarters in the ABXTS group and 307 cows and 1,058 quarters in the TS group were enrolled. Among cows to be dried, the proportion of cows that met low-risk criteria was 64% (n = 611/953). Of cultures eligible for bacteriological cure analysis (n = 171), 93% of ABXTS cured, while 88% of TS experienced cure. Of the non-cures, 95% were contributed by the minor pathogens coagulase-negative staphylococci (CNS; n = 19/20). These organisms also accounted for 57.5% of new infections (n = 77/134). There were no statistical differences between treatment groups for new infection risk (TS = 7.3% quarters experiencing new infections; ABXTS = 5.5%), milk production (ABXTS = 40.5 kg; TS = 41.2 kg), LS (ABXTS = 2.5; TS = 2.7), culling events (ABXTS n = 18; TS n = 15), or clinical mastitis events (ABXTS n = 9; TS n = 5). Results suggest that the employed algorithm decreased dry-cow

antibiotic use by approximately 60% without adversely impacting production or health outcomes.

Key words: selective dry cow therapy, mastitis

INTRODUCTION

The rate of clinical mastitis for cows is highest within the 2 wks post-calving as compared with any other time during lactation and is associated with intramammary infections (IMI) with major pathogens during the dry period (Green et al., 2002). Using serial culture throughout this period, groups have confirmed that a majority of clinical mastitis cases are contributed by chronic subclinical or newly acquired cases from dry-off to calving (Todhunter et al., 1991; Bradley and Green, 2000; Green et al., 2002).

Dry cow antimicrobial administration was developed and implemented in the 1960s as part of a series of management strategies to mitigate the high incidence of IMI in early lactation (Neave et al., 1966; Smith et al., 1966). A survey by the National Animal Health Monitoring System in 2014 indicated that over 90% of cows are treated and 90% of operations use antimicrobial products at dry-off (USDA, 2016a). This suggests that blanket dry cow therapy (BDCT) is widely employed; all quarters are treated with a long-acting antimicrobial immediately after the last milking. Success of mastitis control programs is indicated by the increase in negative quarter-level culture results at dry-off from 44% in 1985 to between 73 and 95% of quarters within the last decade (du Preez and Greeff, 1985; Pantoja et al., 2009; Rajala-Schultz et al., 2011). The decreased prevalence of contagious mastitis pathogens and reduction of bulk tank somatic cell counts (BTSCC) also suggests that BDCT is not currently a necessity in all herds (Ekman and Osteras, 2003; Robert et al., 2006b). Finally, concerns exist for non-

prudent use of antimicrobials in regards to public health consequences. For these reasons, Nordic countries have adopted restrictions that permit only selective use of antimicrobials, leading to reductions of approximately 80% and 40% for dry cow and clinical mastitis treatments, respectively (Ekman and Osteras, 2003). External or internal teat sealants are additional technologies that can offer further protection against new intramammary infections and are readily available in the US (Crispie et al., 2004; Timms et al., 2004; Krömer et al., 2014). Selective treatment of cows at dry-off might produce economic returns via decreased labor and dry-tube costs, even when increases in incidence of mastitis are accounted for (Huijps and Hogeveen, 2007; Scherpenzeel et al., 2016).

Selective dry cow therapy (SDCT) identifies and treats cows currently infected or at higher risk for infection during the dry period and is an alternative to BDCT. There are several approaches to SDCT that have been investigated including culture-based diagnostics (Cameron et al., 2014; Patel et al., 2017). While culture remains the gold standard for diagnosing an IMI, there are disadvantages to its use including costs and time associated with materials, sampling, and labor. Culture-independent cow-side tools such as the California Mastitis Test and milk leukocyte differential tests are available and have been used in SDCT protocols (Poutrel and Rainard, 1981; Hockett et al., 2014). Fair to good sensitivities and specificities have been reported for diagnosing IMIs from late lactation milk samples using these tests, dependent upon cut-point and interpretation (Godden et al., 2017). Use of a single composite somatic cell count (SCC) before dry-off to serve as a proxy for inflammation and infection has also been used to identify cows to be treated (Schukken et al., 1993; Scherpenzeel et al., 2014). However, identification and treatment of high SCC cows or quarters at dry-off based upon one time-point was shown to have negative consequences on udder health in several studies (Schukken et al.,

1993; Scherpenzeel et al., 2014). Lastly, combination screening using SCC, mastitis history, plus or minus bacteriologic culture has produced beneficial effects for several outcomes, but successful implementation can be farm-dependent (Rajala-Schultz et al., 2011; Cameron et al., 2014; Cameron et al., 2015).

Our group tested a culture-independent method using only on-farm data in the form of a computer-driven algorithm to guide SDCT. Ninety-eight percent of large dairies (>500 cows) use an on-farm computer record-keeping system and 82.7% of cows in the United States are housed on operations that use these systems (USDA, 2016b). Our approach was to use DHIA and Dairy Comp 305 records employed by 72.4% and 68% of large US dairies, respectively (USDA, 2016b), to determine whether a cow was at low or high risk for having or acquiring an infection during the dry period. Our objective was to use an algorithm to identify low-risk cows and then compare outcomes (bacteriological cure, new infection risk, milk production, linear scores, mastitis cases, and culling) between those receiving IMM antibiotics and external teat sealant at dry-off and those receiving external teat sealant only.

MATERIALS AND METHODS

This randomized field trial was performed at a New York commercial dairy, milking 1,800 cows, between June 2016 and March 2017. The rolling herd average milk production, BTSCC, culling rate, monthly clinical mastitis incidence, and 21-day pregnancy rate were 13,388 kg, 201,000 cells/mL, 36.2%, 2%, and 20%, respectively. Cows were housed in freestalls with reclaimed sand and calved in a pen bedded with straw. The trial herd milked most cows three times per day while late lactation animals were milked twice per day. All cows eligible for dry-off according to guidelines defined by the dairy (pregnant >220 d or pregnant >180 d and

producing <11.4 kg of milk) were considered for inclusion. This farm used DHIA services, which included monthly SCC and milk weights and Dairy Comp 305 (DC305; Valley Ag. Software, Tulare, CA) for recording mastitis and culling events.

Sample Size Calculation

The primary outcome was new IMI at freshening, which has a documented prevalence of 6.4% to 25% at the quarter level (Godden et al., 2003; Pantoja et al., 2009; Arruda et al., 2013). Using a prevalence of 15%, an alpha of 0.05, a power of 80%, and the ability to detect a change in risk of 5%, a sample size of 1,884 (942 quarters per group; 236 cows) would be required. After accounting for a 20% loss to follow-up, the sample size amounts to 2,261 quarters, or a total of 565 cows (approximately 300 cows per group).

Algorithm Details, Treatment Allocation, and Sampling Procedures

This study was approved by Cornell University Institutional Animal Care and Use Committee, approval #2013-064. Each cow eligible for dry-off was classified into low- or high-risk groups based on the following algorithm: a low-risk cow was defined as having an average SCC over the last 3 tests before dry-off of ≤200,000 cells/mL, a SCC ≤200,000 cells/mL on the last test, and having no more than one case of clinical mastitis in the current lactation. The expected dry period had to be <100 d and each low-risk cow could not have been treated with antibiotics in the last 30 d. All other cows were categorized as high-risk by the algorithm. The computer-based algorithm was automated within DC305. All previous mastitis events were detected by farm personnel and all events were recorded in DC305 regardless of treatment protocol. Aside from computer-based assignment, if a cow had clinical mastitis on dry-off day or

had <3 functional quarters, she was considered a high-risk cow. All high-risk cows received dry cow intramammary antibiotics and external teat sealant. Eligible low-risk cows were randomized to either the external teat sealant only (TS) group or the antibiotic and external teat sealant (ABXTS) group using a random number generator in Microsoft Excel (Microsoft Corp.; Redmond, WA). Low-risk cows were blocked within dry-off day, aiming to enroll an equivalent number of cows into each treatment group that day.

The trial farm had a designated weekly dry-off day. Procedures, including dry tube antibiotic selection and use of external teat sealant, were not altered from the farm's normal routine. Subsequent to the afternoon milking, dry cows were held in a pen to await dry-off procedures. They were individually placed into a tilt-table chute, teats were cleaned thoroughly with a dilute (1:128) chlorhexidine solution (2%; AgriLabs, St. Louis, MO) and teat ends were scrubbed with individual alcohol towelettes. Following initial fore stripping of milk, single quarter-level samples were collected into a culture tube by study personnel using sterile technique and promptly placed on ice. Immediately after sample collection, cows in the ABXTS group received one tube of cephapirin benzathine into each quarter according to label directions (Tomorrow; Boehringer Ingleheim, St. Joseph, MO). Subsequent to administration of the dry cow antibiotic, an external teat sealant (T-hexx Dry; Hydromer, Inc., Branchburg, NJ) was applied once to each barrel and teat end. For cows in the TS group, only external teat sealant was applied once to each barrel and teat end. Treatments were applied on a cow level; all quarters of cows in each group received antibiotics (ABXTS) or did not (TS). All cows were dried off abruptly and dry cow treatments and external teat sealants were administered by farm personnel.

A red leg band was placed on every dry cow regardless of administration of antibiotics and management did not maintain records as to which cows received treatments though the study

investigators did. Each cow was also marked as treated with antibiotics in an effort to minimize bias when milkers or management detected cases of mastitis or made culling decisions as well as avoid residue issues. All trial cows remained in the dry cow pens or maternity pens until milk withdrawal times were met and milk production commenced. Any mastitis case defined during the trial was detected by on-farm employees, trained by Quality Milk Production Services (QMPS), by observing abnormalities in milk, inflammation of the quarter, or systemic signs in the cow. Milk samples from all mastitis cases were submitted to QMPS at the Animal Health and Diagnostic Center in Ithaca, NY for culture. Events (mastitis, sold, died) were recorded in DC305 by on-farm employees.

Quarter-level samples were obtained from each low-risk cow after milking in the parlor at 1-7 DIM. Teat ends were scrubbed with individual alcohol towelettes and samples were taken following an initial forestrip procedure. If a cow received antimicrobial treatment before collection of the post-calving sample, its samples were removed from quarter-level analyses.

Quarter-level samples were also retrieved on dry-off day and at 1-7 d fresh from a subset of high-risk cows to describe organism distribution and to calculate the positive and negative predictive values of the algorithm. Positive predictive value (PPV) is the probability that a cow assigned to the high-risk group has one or more positive quarters. Negative predictive value (NPV) is the probability that a cow assigned to the low-risk group had 4 negative quarters on culture.

Microbiological Diagnosis

Standard culture technique was performed on all milk samples by QMPS following procedures recommended in the Laboratory Handbook on Bovine Mastitis (National Mastitis

Council, 2017). Briefly, 0.01 mL of milk was streaked on trypticase soy agar containing 5% sheep blood and 1% esculin (PML Microbiologicals, Mississauga, ON, Canada), and plates were incubated aerobically at 37°C for 18-24 h. Colonies from pure cultures were subjected to matrix assisted laser desorption/ionization (MALDI-TOF) using a Bruker MALDI-TOF Biotyper version 3.1.66 (Bruker Corp., Billerica, MA). Confidence levels were assigned by the machine software by referencing the Bruker 5989 RUO bacterial library. Levels accepted for species identification were ≥2.0 and for genus identification between 1.8 and 2.0. A composite sample was created by combining 300µL of milk from each quarter for each cow for mycoplasma testing. Pools of milk from 5 cows were subjected for Mycoplasma screening using a dissection microscope after anaerobic incubation at 37°C for 7 d on Mycoplasma agar (Sigma-Aldrich LLC, St. Louis, MO).

Treatment Outcomes

Quarters were assessed for bacteriological cure and new infection risk. Only quarters with pure, non-contaminated cultures were considered. Exclusions for these reasons were not different between groups (Tables 6.2 and 6.4). An infection was defined as the isolation of one or more colonies from 10µL of milk for all pathogens except for CNS. For CNS 2 or more colonies isolated from the sample were needed to establish the presence of an infection (Dohoo et al., 2011). If one nonpathogenic or ≥2 dissimilar nonpathogenic colony types were present in low levels, the culture was defined as negative. Contamination was defined as the isolation of ≥3 different colony types from the milk sample. A quarter was defined as a bacteriological cure when the initial pathogen present at enrollment was not present in the fresh quarter sample. Negative quarters at dry-off were not included in analysis for bacteriological cure. A quarter was

defined as a new infection when the organisms cultured at dry-off and at fresh were different. If a cow left the trial due to culling during the dry period, or was missed for any reason at fresh, it was lost to follow-up for both bacteriological cure and new infection risk. Cows in the study were also followed up for first-test (1-41 d) milk production and linear scores, longitudinal milk production up to 30 DIM, mastitis events before 30 DIM, and survival/removal from the herd before 30 DIM. These values and dates were retrieved from DHIA monthly reports or DC305. Linear score (LS) is a transformation of SCC, calculated as [ln(SCC/100)/ln(2)] + 3 (Ali and Shook, 1980).

Statistical Analysis

The effects of treatment on outcomes were analyzed using generalized linear mixed models for continuous variables and binomial logistic regression for dichotomous outcomes. Analysis of outcomes accounted for important covariates such as parity at fresh (2^{nd}, 3^{rd}, 4^{th}, or $>4^{th}$), DIM at sample collection, length of the dry period, previous milk yield and LS, mastitis events before and after the dry period (up to 30 DIM), and pathogen present at dry-off and at fresh. The primary explanatory variable of interest was the treatment group and all interactions with this variable were tested. All analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary NC) using the PROC LOGISTIC command for binary outcomes: bacteriological cure, new infection risk, mastitis before 30 DIM, and culling before 30 DIM. The PROC MIXED command was used for milk production (longitudinal and at first test) and LS at first test. Each quarter was treated as an independent observation in the analysis of bacteriological cure and new infection risk. To select the best model, all possible confounding variables were subjected to bivariate analysis by means of Chi-squared, t-tests, or ANOVA tests using PROC FREQ, PROC

TTEST, PROC REG, or PROC ANOVA. Any terms with a $P < 0.2$ were offered into the models. Each variable was then examined by backwards stepwise removal from the regression until all covariates with $P < 0.1$ were identified and included. In many of the models, the treatment variable was forced. Quarter nested in cow was tested as a random effect to determine the contribution of interdependence of quarters. Basic graphing techniques were used to assess normality, homoscedasticity, and linearity of variables. Data transformations were performed and reported as necessary. Seven final models were established describing (1) the odds of bacteriological cure (2) the odds of new infection (3) first-test milk production, (4) longitudinal milk production, (5) first-test linear score, (6) the odds of culling before 30 DIM, and (7) the odds of mastitis before 30 DIM. An animal was not included in a model if it was missing a data point for a parameter included in the model; the number of animals with complete data for each analysis is indicated in each table.

RESULTS

Descriptive Statistics for Low- and High-Risk Cows and Quarters

A total of 953 cows were dried between June 2016 and January 2017. Cows were allocated into the high-risk group (n = 321) by the algorithm for combinations of the following criteria: high average SCC, high last-test SCC, an anticipated dry period >100 d and >1 mastitis event in the current lactation. More than half of the excluded cows experienced more than one reason for assignment into the high-risk group (n =169). High last-test SCC is commonly used in SDCT protocols as criteria for administration of an IMM antimicrobial. The addition of criteria other than last-test SCC, for which 244 cows would have been excluded, resulted in an additional 77 cows assigned to the high-risk group. The 2 most common reasons for placement into the

high-risk group were high SCC at last test and high average SCC over the last 3 tests (n = 268 cows equaling 28% of cows eligible for dry-off and 83% of exclusions). Cows added last minute to the dry-off list (not due to dry-off by the farm's criteria, but elected to dry-off by the management) were not sampled nor included in the either risk group (n = 21). Details of cow and quarter enrollment can be found in Figure 6.1. A total of 611 low-risk cows and 2,386 quarters were enrolled in the study. Fifty-eight cows were 3-quartered. Allocation to the low-risk group was 64%. Distribution between groups was similar with 50% of cows/quarters assigned to the TS group (n = 307/611; 1,202/2,386) and 50% assigned to the ABXTS group (n = 304/611; 1,184/2,386). Post-enrollment exclusions of low-risk cows resulted in 37 cows lost for analysis of milk production and LS due to death or culling during the trial period (n = 33) or no recorded LS (n = 4). The number of quarters lost to follow up for both bacteriological cure and new infection risk analyses was 288. Quarters were lost for the following reasons: no post-calving sample was collected (n = 34), sample was retrieved >7 DIM (n = 2), death or culling during early fresh or during the dry period (n = 75 quarters, 19 cows), and antimicrobial treatment before post-calving milk samples were retrieved (n = 52 quarters). An additional 125 quarters were excluded from bacteriological cure and new infection risk analyses due to contamination or mixed culture results. No statistical differences were observed between study groups with respect to post-enrollment exclusions. Cows experiencing culling or missing milk samples over the dry or early fresh period were still considered for culling and mastitis outcomes and for longitudinal milk production for as long as they remained in the herd. Overall, 1,058 TS quarters and 1,040 ABXTS quarters were considered for analysis of bacteriological cure and new infection risk. Cow inclusion numbers for analyses of culling and mastitis risk was 307 TS cows and 304

ABXTS cows. Analyses of milk production and LS included 291 TS and 283 ABXTS cows (Figure 6.1.).

Descriptors for each treatment group are found in Table 6.1. The distribution of cows experiencing one case of clinical mastitis in the lactation of enrollment was different between groups, with cows in the TS group experiencing more cases (17 versus 6). Mean last-test-day milk production and LS were 23.6 kg and 2.3, respectively. The mean and median number of DIM when the fresh quarter milk sample was retrieved was 2 and 3 d. The mean and median number of DIM that milk production and LS data were retrieved were each 16 d. Distribution of these variables were not statistically different between treatment groups.

Infection Status at Dry-Off and Freshening

A total of 142 high-risk cows (n = 553 quarters) were sampled to determine the ability of the algorithm to correctly classify cows as high or low risk. The cow-level IMI prevalence at dry-off for high-risk and low-risk cows was 70.7% (n = 99/140) and 29.4% (n = 178/605), respectively. The PPV and NPV for the algorithm were 70.7% (95% CI: 65.4-76.0) and 70.6% (95% CI: 66.4-74.7) at the observed apparent prevalence of infection (37.2%). Including mixed culture results and excluding contaminated quarters, the quarter-level prevalence for IMI was 9.6% (n = 229/2,379) and 29.8% (n = 165/553), for low and high-risk quarters, respectively (13.4% overall). Distribution of dry-off culture results for low and high-risk quarters are described in Tables 6.2 and 6.3. Randomization created a similar distribution of organisms between treatment groups ($P > 0.05$). The most commonly isolated organisms for both low and high-risk cows were coagulase-negative staphylococci (CNS), contributing to 4.9% and 13.9% of overall quarters and 59.8% and 46.6% of infections, respectively. Infection proportion with

Corynebacterium spp. was similar between high- and low-risk quarters, but *Lactococcus* spp. were more prevalent in high-risk quarters.

Following calving, 7.3% of quarters in the high-risk group were culture positive (n = 36/495), while 8.2% of low-risk quarters were (n = 182/2,225). The most common positive culture result was CNS, with 4.6% of the low-risk quarters and 3.0% of the high-risk quarters infected. The infection status of quarters in low-risk groups is seen in Table 6.4.

Treatment Effects

Bacteriologic Cure Risk. A quarter was not eligible for bacteriological cure if the dry-off sample had no growth (n = 1,927), the dry-off or fresh sample was contaminated (n = 69), or if the dry-off or fresh sample had mixed growth (n = 56). The number of quarters meeting inclusion criteria for analysis was 171. Bacteriological cure was seen in 151 of the quarters (88.3%): 72 (83.7%) of the TS quarters and 79 (92.9%) of the ABXTS quarters (Fisher's exact *P* = 0.09). This compares with high-risk cows that experienced a cure in 94.9% (n = 112/118) of quarters. Of the non-cures, 95.0% were contributed by the minor pathogens CNS in low-risk cows (n = 19/20) and 100% in high-risk cows (n = 6). Mixed model logistic regression of bacteriological cure produced a model that included etiology of infection and DIM at retrieval of fresh quarter sample (Table 6.5). While controlling for these variables, the odds ratio (OR) for bacteriological cure between TS and ABXTS was 0.32; bacteriological cure was approximately 3.1 times higher for ABXTS quarters than for TS quarters (*P* = 0.04). Extrapolation of least squares means (LSM) for bacteriological cure for this model indicated a cure of 93.2% of TS quarters and 97.7% of ABXTS quarters. To permit for the inclusion of etiology of IMIs in a regression model, the variable was condensed so that an acceptable number of quarters within

each category was established. Etiology was therefore dichotomized into “CNS” and “other.” When controlling for treatment group, odds of bacteriological cure for CNS-infected quarters at dry-off were lower than the grouping of other organisms (P = 0.03).

New Infection Risk. A new infection was defined as the presence of new or different growth on the dry-off quarter sample from the fresh quarter sample. Contaminated and mixed growth quarters were not included in the analysis. Chronic quarters, or growth of the same organism for both samples, were included but not defined as new infections. New infections totalled 6.4% with 57.5% (n = 77/134) of new infections described as CNS. Quarters in the TS group experienced a numerically higher number of new infections (n = 77/1,058; 7.3%) than the ABXTS group (n = 57/1,040; 5.5%). Chi-squared analysis indicates a P-value for this difference of 0.09. A logistic regression model includes parity and dry-off quarter growth (categorized into “CNS”, “Other”, and “No Growth”) (Table 6.6). When controlling for these variables, the odds of new infection for TS versus ABXTS is 1.29 (95% CI: 0.9-1.85; P = 0.17). New infections in the high-risk group amounted to 6.8% (n = 31/456).

Milk Production and LS. All cows with milk yield and LS values for the first test day after calving were included in the analysis. The mean test-day milk for all low-risk cows was 40.8 kg. A mixed linear regression model used 574 observations (TS = 290, ABXTS = 284) and included LS at first test day, milk production at last test day before dry-off, and DIM on the first test day. Treatment group was not important in the model, though numerically the TS cows produced 0.69 kg more milk (LSM TS = 41.2 kg, ABXTS = 40.5 kg; P = 0.35). As expected, as LS at first test day increased, milk production decreased (P < 0.001). As milk production at last test day or DIM at first test day increased, milk production increased (P < 0.001). A repeated measures analysis performed on daily milk yields (n = 15,987) over the first 30 DIM by

treatment group is shown in Figure 6.2. Means for milk production were numerically greater in the TS group for each of the first 26 DIM, but the effect of treatment nor the treatment by time interaction was important in the model (P = 0.20, P = 0.40, respectively). The same cows as in the milk production outcome were included in a linear regression model for first-test-day LS (n = 574; Table 6.7). The mean test-day LS for all cows was 2.6. When controlling for other independent variables included in a linear regression model, the LSM for LS was numerically higher for the TS group (P = 0.10). Important in the model were the type of growth on dry-off and fresh samples, parity, mastitis before 30 DIM (dichotomized into "yes" or "no"), DIM at first test, and milk production at first test. A negative culture result for all 4 quarters at dry-off or fresh sampling was associated with a lower LS than a result of "CNS" or "other" for at least one quarter. Cows entering their second lactation had a lower LS than any other category of parity (P < 0.05). A cow that experienced mastitis within the first 30 DIM had a higher linear score than one that had not (P < 0.05).

Culling and Mastitis Events. All cows enrolled were included in logistic regression models for culling and mastitis events up to 30 DIM. Overall, 94.6% of enrolled cows remained in the herd until they reached 30 DIM. A total of 33 cows were culled or died, 18 in the ABXTS group and 15 in the TS group (Chi-squared P-value = 0.57). In a logistic regression model, cows in the TS group had lower odds of leaving the herd than cows in the ABXTS group, but we did not observe an important difference between treatment groups (P = 0.60). The model for this outcome included parity only, with cows in parity 2 or 3 experiencing lower odds than parity > 4 (P < 0.05; Table 6.8). The total number of cases of clinical mastitis in the first 30 DIM was 14 (2.3%) with TS cows contributing 5 cases and ABXTS contributing 9 cases (Chi-squared P-value = 0.27). Each cow (n = 14) had a single case of mastitis. No cases of clinical mastitis were

recorded during the dry period for cows in either group. Treatment group was not important in a logistic regression model, although the odds of clinical mastitis in the ABXTS group was 2.3 times higher than the TS group (P = 0.21, Table 6.9). Regression analysis included LS at first test and whether the cow had one mastitis event during the lactation of enrollment ("yes" or "no"). As expected, when controlling for important variables, the odds for mastitis were numerically higher for a cow that had experienced one case of mastitis during the lactation of enrollment than a cow that had not (P = 0.08). Finally, as first-test-day LS increased, the odds of clinical mastitis increased (P = 0.003).

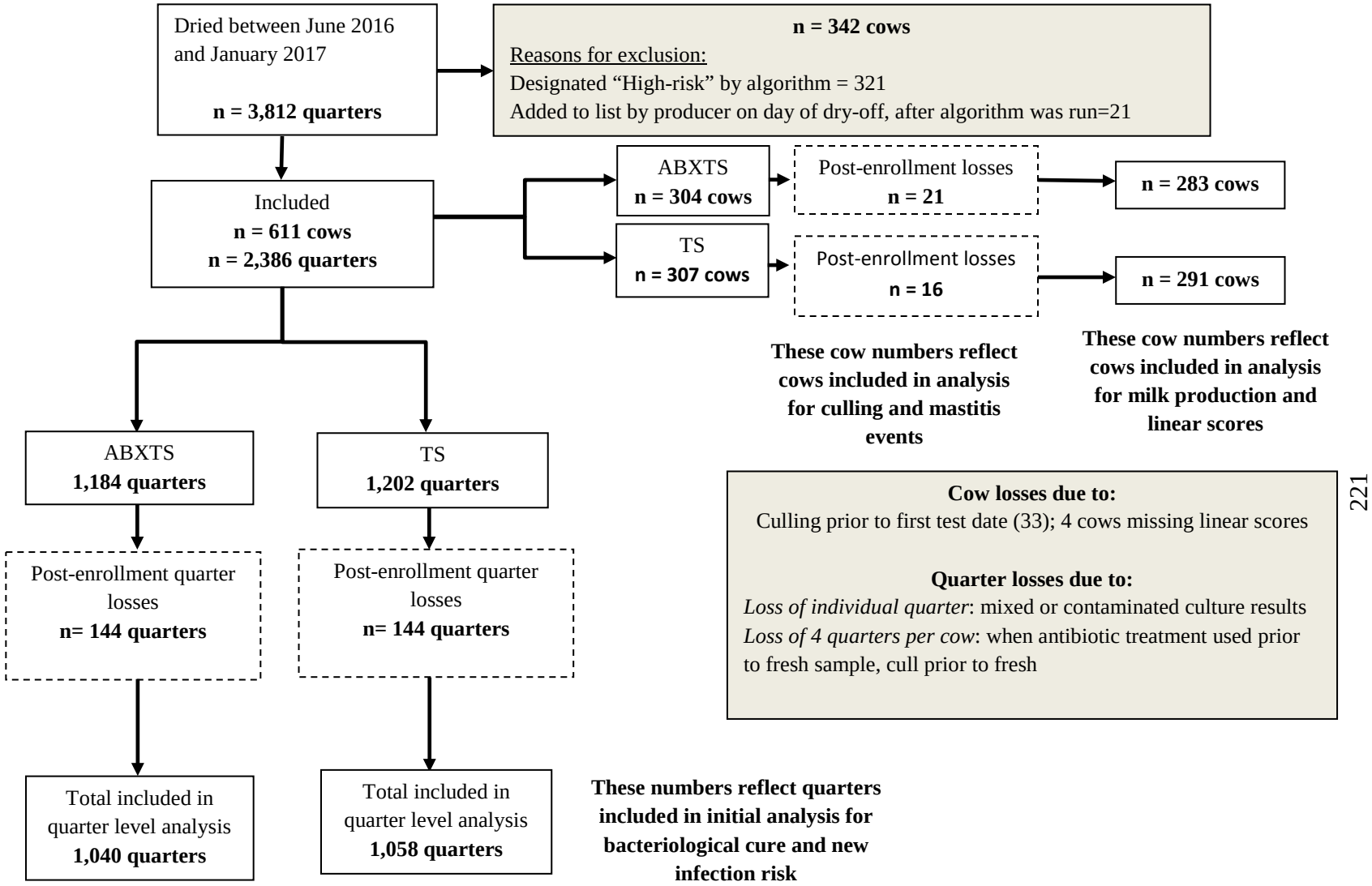

Figure 6.1. Enrollment of cows and quarters in a randomized field trial to evaluate the use of an algorithm that identifies high- and low-risk cows to guide selective dry-cow therapy. ABXTS = low-risk cows that receive antibiotic therapy and external teat sealant at dry-off; TS = low-risk cows treated with external teat sealant only.

Table 6.1. Descriptors for cows in a randomized field trial evaluating treatment of low-risk cows with external teat sealant (TS; n = 307) or antibiotics and external teat sealant (ABXTS; n = 304) at dry-off

	Treatment Group			
Item	TS	ABXTS	All cows	*P*-value[1]
Parity[2] (no.)				0.65
First	136	140	276	0.68
Second	84	81	165	0.86
Third	52	42	94	0.31
>Third	35	41	76	0.46
DIM[2] (d)				
Mean	338	343	340	0.27
Median	313	330	340	
Actual dry period (d)				0.06
Mean	56	54	55	
Median	54	53	54	
Mastitis Event[3] (no.)				0.02
Yes	17	6	23	
No	290	298	588	
Milk yield[4] (kg)				
Mean	23.6	23.5	23.6	0.87
Median	24.5	23.6	24.0	
Linear Score[4]				
Mean	2.2	2.3	2.3	0.18
Median	2.2	2.4	2.3	

[1]*P*-values based on bivariate analysis (t-test, Fisher's exact) of each variable by treatment group.
[2]At enrollment.
[3]If the cow had 1 mastitis event in the lactation before enrollment.
[4]At test day before enrollment.

Table 6.2. Quarter-level culture results at dry-off for low-risk cows randomly assigned to receive either external teat sealant (TS; n = 1,204) or antibiotics and external teat sealant (ABXTS; n = 1,183) at dry-off [1]

	TS		ABXTS		
Culture result	No.	%	No.	%	*P*-value[2]
Negative	1,086	90.2	1,064	90.0	0.84
CNS	59	4.9	78	6.6	0.08
Mixed Growth	22	1.8	20	1.7	0.88
Corynebacterium spp.	24	2.0	12	1.0	0.06
Lactococcus spp.	5	0.4	4	0.3	> 0.99
Streptococcus spp.[3]	2	0.2	1	0.1	> 0.99
Other[4]	6	0.5	4	0.3	0.75
Total IMI[5]	114	9.5	115	9.7	0.84

[1]Etiological classification is based on aerobic culture results and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) performed by Quality Milk and Production Services (Ithaca, NY).
[2]*P*-values based on bivariate analysis (Fisher's exact) of each etiology by treatment group.
[3]Includes *Streptococcus* spp. unclassified to the species level and *Streptococcus dysgalactiae*.
[4]Includes contamination, Gram-negative and Gram-positive bacilli.
[5]Excludes contamination.

Table 6.3. Quarter-level culture results at dry-off for high-risk cows as designated by a computer algorithm in a selective dry-cow therapy program[1]

Culture result	No. (553)	%	IMI (%)
Negative	384	69.4	
CNS	77	13.9	45.6
Mixed Growth	27	4.9	16.0
Corynebacterium spp.	14	2.5	8.3
Lactococcus spp.	35	6.3	20.7
Streptococcus spp.[2]	8	1.4	4.7
Other[3]	8	1.4	4.7
Total IMI[4]	165	29.8	

[1] Etiological classification is based on aerobic culture results and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) performed by Quality Milk and Production Services (Ithaca, NY).
[2] Includes *Streptococcus* spp. unclassified to the species level and *Streptococcus dysgalactiae*.
[3]Includes contamination, *Pseudomonas* spp., *Serratia* spp., and Gram-positive bacilli.
[4]Excludes contamination.

Table 6.4. Quarter-level culture results at fresh for low-risk cows randomly assigned to receive either external teat sealant (TS; n = 1,128) or antibiotics and external teat sealant (ABXTS; n = 1,097) at dry-off [1]

	TS		ABXTS		
Culture result	No.	%	No.	%	*P*-value[2]
Negative	983	87.1	998	91.0	0.004
CNS	68	6.0	34	3.1	0.001
Contamination	35	3.1	27	2.5	0.37
Mixed Growth	12	1.1	7	0.6	0.36
Lactococcus spp.	13	1.2	7	0.6	0.26
Streptococcus spp.[3]	8	0.7	10	0.9	0.64
Corynebacterium spp.	2	0.2	6	0.5	0.17
Other[4]	7	0.6	8	0.7	0.8
Total IMI[5]	110	9.8	72	6.7	0.007

[1]Etiological classification is based on aerobic culture results and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) performed by Quality Milk and Production Services (Ithaca, NY) on samples 1 to 7 DIM.
[2]*P*-values based on bivariate analysis (Fisher's exact) of each etiology by treatment group.
[3]Includes *Streptococcus* spp. unclassified to the species level, *Streptococcus dysgalactiae,* and *Streptococcus uberis*
[4]Includes Gram-negative and Gram-positive bacilli, *Escherichia coli, Klebsiella, Trueperella pyogenes,* and *Enterococcus* spp.
[5] Excludes contamination.

Table 6.5. Logistic regression model for the risk of bacteriological cure at freshening in quarters receiving external teat sealant only (TS; n = 86) or antibiotic dry-cow therapy and teat sealant (ABXTS; n = 85).

Parameter	Estimate	SE	*P*-value	Odds Ratio	95% CI
Intercept	6.25	1.30	<0.0001		
Treatment group			0.04		
TS	-1.12	0.55		0.32	0.11-0.96
ABXTS	Referent				
Organism cultured at dry-off			0.03		
CNS	-2.33	1.05		0.10	0.01-0.80
Other	Referent				
Days in milk at fresh sample	-0.52	0.16	0.002		

Table 6.6. Logistic regression model for the risk of new infection at fresh in quarters receiving external teat sealant only (TS; n = 1,058) or antibiotic dry-cow therapy and teat sealant (ABXTS; n = 1,040).

Parameter	Estimate	SE	*P*-value	Odds Ratio	95% CI
Intercept	-2.75	0.27	<.0001		
Treatment group	0.25	0.18	0.17		
TS				1.29	0.90-1.85
ABXTS					
Parity at fresh			0.02		
2	-0.45	0.29		0.64[a]	0.36-1.14
3	0.23	0.29		1.25[b]	0.71-2.23
4	-0.20	0.34		0.82[ab]	0.42-1.60
>4	Referent			Referent[ab]	
Growth on dry-off sample			<.0001		
CNS	-0.10	0.40		0.91[a]	0.41-2.0
Other	1.70	0.36		5.49[b]	2.7-11.0
No Growth	Referent			Referent[b]	

[a,b] Values within the same column and parameter with different superscripts are different ($P < 0.05$)

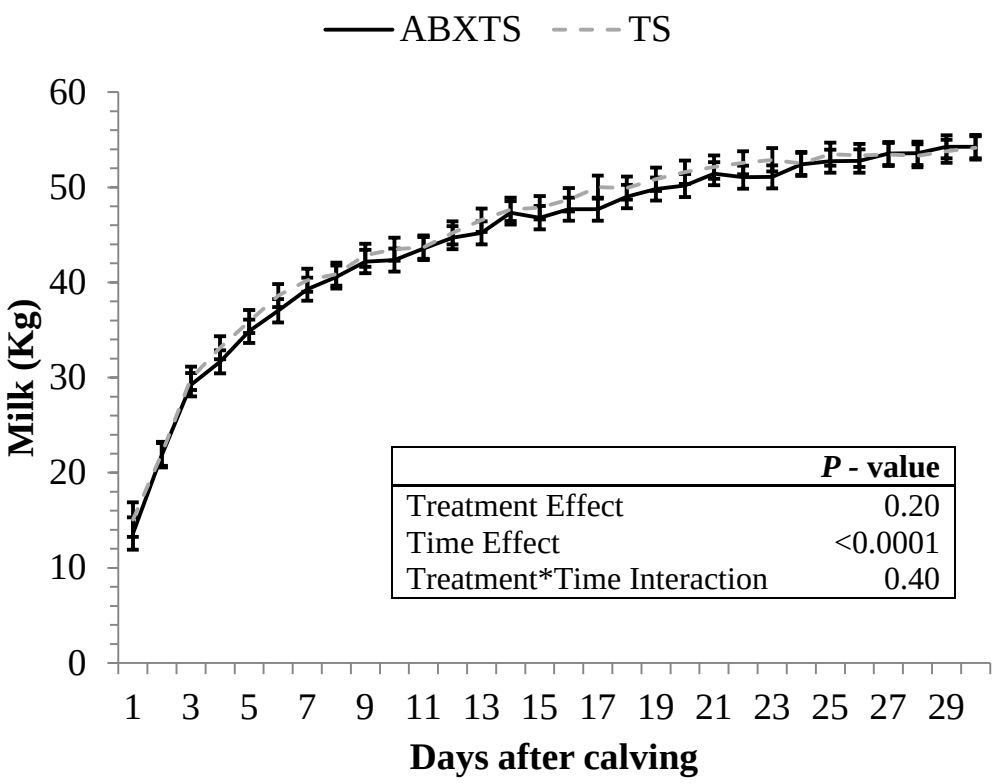

Figure 6.2. Longitudinal milk production (kg) over the first 30 DIM for cows receiving external teat sealant only (TS) or antibiotic therapy and teat sealant (ABXTS) at dry-off. Error bars signify 95% CI

Table 6.7. Linear regression model for linear score at first test (average DIM = 16 d) for cows receiving external teat sealant only (TS; n = 291) or antibiotic therapy and teat sealant (ABXTS; n = 283) at dry-off

Parameter	Estimate	SE	*P*-value	LSM	95% CI
Intercept	5.8	0.53	<0.0001		
Treatment group			0.10		
TS	0.22	0.13		2.7	2.5-2.9
ABXTS	Referent			2.5	2.3-2.6
Growth on dry-off sample[1]			0.002		
CNS	0.36	0.18		2.8[b]	2.5-3.1
Other	0.65	0.19		3.1[b]	2.7-3.4
No Growth	Referent			2.4[a]	2.3-2.6
Growth on Fresh Sample[1]			<0.0001		
CNS	1.30	0.21		3.6[b]	3.2-4.0
Other	0.72	0.18		3.0[b]	2.7-3.3
No Growth	Referent			2.3[a]	2.2-2.5
Parity at fresh			<0.0001		
2	-0.67	0.22		2.2[a]	2.1-2.4
3	-0.18	0.23		2.7[b]	2.5-3.0
4	0.09	0.26		3.0[b]	2.7-3.3
>4	Referent			2.9[b]	2.5-3.3
Mastitis before 30 DIM			0.0006		
No	-0.18	0.15		2.5[a]	2.4-2.7
Yes	Referent			4.2[b]	3.2-5.1
DIM at 1st test	-0.03	0.01	0.008		
Milk (kg) at first test	-0.03	0.007	<0.0001		

[a,b] Values within the same column and parameter with different superscripts are different ($P < 0.05$).

[1]A cow had to have no growth on culture of all 4 quarters to be characterized as "No Growth"; if one or more quarters contained CNS, but no additional organism, she was characterized as "CNS"; Any other growth was "Other".

Table 6.8. Logistic regression model for the risk of culling before 30 DIM for cows receiving external teat sealant only (TS; n = 307) or antibiotic dry-cow therapy and teat sealant (ABXTS; n = 304).

Parameter	Estimate	SE	*P*-value	Odds Ratio	95% CI
Intercept	-2.73	0.18	<0.0001		
Treatment group			0.60		
TS	-0.09	0.18		0.83	0.40-1.69
ABXTS	Referent				
Parity at fresh			0.002		
2	-0.78	0.31		0.18[a]	0.07-0.46
3	-0.38	0.33		0.26[a]	0.10-0.71
4	0.22	0.33		0.48[ab]	0.18-1.32
>4	Referent			Referent[b]	

[a,b] Values within the same column and parameter parameter with different superscripts are different ($P < 0.05$).

Table 6.9. Logistic regression model for the risk of clinical mastitis before 30 DIM for cows receiving external teat sealant only (TS; n = 291) or antibiotic dry-cow therapy and teat sealant (ABXTS; n = 283)

Parameter	Estimate	SE	*P*-value	Odds Ratio	95% CI
Intercept	-4.63	0.74	<.0001		
Treatment group			0.21		
TS	-0.42	0.33		0.44	0.12-1.60
ABXTS	Referent				
Mastitis event in lactation before enrollment			0.08		
Yes	0.80	0.45		4.95	0.83-29.4
No	Referent				
First Test Linear Score	0.39	0.13	0.003		

DISCUSSION

In this study, removal of an antibiotic dry treatment in cows determined to be low-risk by a computer-based algorithm did not have adverse effects on new infection risk, milk yield, first-test linear score, clinical mastitis, or culling compared with low-risk cows that were treated. Bacteriological cure was statistically different between the groups, with antibiotic-treated quarters at higher odds for cure. Coagulase-negative staphylococci comprised 95% of the non-cures on the trial dairy, yet these minor pathogens only contributed one case of clinical mastitis in low-risk cows over the trial period. Using the already incurred costs of computer and testing programs implemented on-farm, a culture-independent algorithm that selectively distinguishes between high- and low-risk cows could result in a reduction in dry cow antibiotic use of approximately 60% when low-risk cows are not treated.

It is difficult to describe the representativeness of one or more dairies by referencing an expected prevalence of dry period infection; much variation exists for new and current IMI, both at cow and quarter levels. In the current study, the prevalence for quarter level dry-off IMI was 29.8% and 9.6% for high- and low-risk quarters, respectively, which is lower than an average quarter-level prevalence (34.7%) in a Wisconsin study surveying 4 dairies (Johnson et al., 2016). However, 2 studies described a range of subclinical infection between 13 and 35% when collectively assessing 7 dairies in 4 states (Pantoja et al., 2009; Arruda et al., 2013). One common finding is a majority of infections are characterized as CNS: in the current trial, 60% of IMI in low-risk quarters at dry-off were CNS. This is similar to the 69% documented in the study by Johnson et al. (2016).

Despite a greater number of CNS-positive quarters allocated to the ABXTS group, creating potential bias against these quarters, the odds for bacteriological cure was greater for

this group. Our higher odds of cure for antibiotic-treated quarters versus untreated quarters agreed with the conclusions of a meta-analysis of 4 SDCT trials and 13 BDCT trials, although our odds ratio of 3.1 was greater than the meta-analytic pooled relative risks of 1.76 and 1.78, respectively (Halasa et al., 2009b). A recent trial by Cameron et al. (2014) found no statistical difference in bacteriological cure between BDCT quarters and SDCT quarters from 16 Canadian herds when treatment was performed at the cow level (n = 729 cows). A similar study on a dairy in Minnesota (n= 56 cows) implemented assignment on the cow level and treatment on the quarter level. Statistical differences were not found between BDCT and SDCT cows, though a cure risk difference of 5.7% was stated (Patel et al., 2017). This is comparable to our statistical cure risk difference of 4.5% with a larger sample size. Culture screening was performed on all SDCT cows in the Cameron et al. (2014, 2015) and Patel et al. trials (2017) before treatment of positive cows/quarters; the study design dictated that comparisons be made between blanket-treated cows and a group comprised of treated and untreated cows/quarters. This is one reason why differences in this outcome were observed in the current trial in which we made comparisons between treated and untreated low-risk quarters. We did not analyze differences between treated high-risk quarters and treated low-risk quarters, nor did we include a group of untreated high-risk quarters. We have structured the algorithm to determine, independently of culture and based on thresholds, which animals would likely benefit from antibiotic treatment. As high-risk quarters were projected to have a higher prevalence of pathogens, and given that commercial dry cow preparations must meet label and efficacy requirements for Food and Drug Administration approval, IMM antimicrobials were assumed likely to be beneficial and were not withheld from these animals. However, we are unable to conclude whether antibiotic therapy was appropriate in these cows.

In the current trial, the majority of quarters included in analysis for bacteriological cure (171 quarters) were contributed by the minor pathogens CNS (n = 126) and *Corynebacterium* spp. (n = 31) with 77% and 91% of non-treated and treated CNS cases curing, respectively. Similarly, the meta-analysis by Halasa et al. (2009b) found that while antimicrobial dry cow therapy led to a 77% cure risk for CNS, spontaneous cure was on average 44%, suggesting that antimicrobial therapy is effective against these organisms. In a survey performed in Belgian herds of CNS present at parturition, 20% of all quarters of multiparous cows had IMIs characterized by 8 different CNS species and over 20 different species have been found in bovine milk (Vanderhaeghen et al., 2015; De Visscher et al., 2016). When analyzed as 3 different groupings of 8 different species, those quarters treated with antimicrobials at dry-off experienced statistically lower odds of having an IMI characterized by CNS at calving relative to those that were not treated (De Visscher et al., 2016). However, there might be an advantage to the presence of CNS in quarters: some groups have observed higher milk production in CNS-infected animals relative to uninfected animals (Schukken et al., 2009; Piepers et al., 2013) and other groups, as reflected in a meta-analysis, see a protective effect of CNS against major pathogens in challenge studies (Reyher et al., 2012). Conversely, Berry and Hillerton (2002) found that untreated quarters with CNS infections had a 4.2-4.7 times greater risk of new infections with *Streptococcus uberis* or coliforms at calving compared with uninfected, untreated quarters when assessing 499 quarters. Characterization of the effects of individual CNS species requires further investigation. As no important differences in LS, milk production, risk of removal, or clinical mastitis cases caused by CNS were found between groups in the current study, we do not believe that influences of these pathogens on bacteriological cure nor new infection risk is concerning on this particular dairy.

New IMI during the dry period is also variable and has been documented to range from 6.4% to 25% at the quarter level (Godden et al., 2003; Pantoja et al., 2009; Arruda et al., 2013). Intramammary infections present at calving have important economic consequences that result from declines in milk quantity and quality and increases in cases of clinical mastitis (Bradley and Green, 2000, Archer et al., 2014). Accordingly, dairies attempt to minimize these consequences with the use of dry cow products. While antimicrobial products might result in reductions in new IMI, the ranges provided were described for herds on which BDCT was performed; total elimination of IMIs is difficult to achieve. Variation in the prevalence of IMI at calving between herds demonstrates that infection status is influenced by many factors including cow (e.g. parity, SCC), management (e.g. frequency of cleaning bedding), and farm characteristics [e.g. facilities, pathogens present on the farm (Green et al., 2007; Green et al., 2008)]. Alterations of these factors, when appropriate, could reduce IMIs. With use of SDCT, we found the proportions (LSM) of untreated low-risk quarters acquiring new IMI over the dry period to be 11.2%. This compares to the treated low-risk quarters at 8.9%. These values were similar to the weighted mean incidences calculated by Robert et al. (2006b) of 9.9% and 6.5% for untreated and treated quarters, respectively, when considering new IMIs in 13 studies that compared SDCT with BDCT. When a model was generated for risk of new IMI in the current study, we were unable to find a statistical difference between the treatment groups. No statistical differences were found for new IMIs when culture diagnostics were used for targeting treatment at the cow or quarter level in a recent trial and a pilot study (Cameron et al., 2014; Patel et al., 2017). Our odds ratio of 0.77 was similar to the pooled relative risks of 0.58 and 0.55 described in 2 meta-analyses that compared BDCT to SDCT (Robert et al., 2006b; Halasa et al., 2009a). The meta-analysis performed by Halasa et al. (2009a), noted that while significant differences in relative risk were

seen for protection against new quarter IMI, no statistical differences were calculated when the selection unit was the cow. When the Robert et a. (2006b) meta-analysis considered comparisons between a non-treated group and a BDCT group, differences in new IMI between groups were significant in 15 of 25 studies; however, pooled differences were statistically significant for streptococcal and *Staphylococcus aureus* IMIs and not for IMIs caused by CNS or coliforms. Significant findings in the majority of the studies could be due to the fact that contributions of streptococcal species and *Staph. aureus* represented more than 35% of IMIs in 50% of the studies included. One trial included in the meta-analysis, Berry and Hillerton (2002), found that while the overall new IMI risk was significantly higher in the untreated versus treated cows, the most frequently cultured organism contributing to new IMIs in low SCC herds was *S. uberis;* no significant differences were found in the prevalence of CNS post-calving between treated and untreated groups. In contrast, on our trial dairy, less than 2 percent of infections were caused by *Strep.* spp. with a majority of new infections (57.5%) described as CNS. A small percentage of infections were diagnosed as *Corynebacterium* spp. These 2 groups of minor pathogens are known to infect quarters at the end of the dry period, and in the current trial, we speculate that we do not observe larger differences in IMI postpartum between the treatment groups as the preventive action of the dry cow antimicrobial is no longer effective (Oliver and Jayarao, 1997; Bradley et al., 2003).

Internal teat sealant was not used in the current trial; use of an external teat sealant, rather, was a part of the existing protocol. Several studies show a benefit to the use of internal teat sealant, amounting in fewer infections and lower SCC in the subsequent lactation, whether used alone or in combination with antimicrobials (Godden et al., 2003; Rabiee and Lean, 2013). When introduced into healthy quarters, the application of an inert, internal teat sealant without

antibacterial properties is not risk-free; a case report indicates that improper administration could lead to outbreaks of mastitis pathogens (Milnes and Platter, 2003). While the efficacy of a one-time application of external teat sealant over the course of the dry period has been questioned, there are studies that show that some are effective at preventing new IMIs during the dry period when applied regularly or according to label (Hayton, 2003; Timms, 2004; McDougall et al., 2008). Although no differences were detected between groups for the new IMI outcome in the current trial, numerical reductions in new IMIs in the TS group could have occurred with use of an internal teat sealant versus an external teat sealant. Further research needs to be performed to determine how our algorithm performs with use of an internal teat sealant.

Cow-level outcomes such as the amount and quality of milk produced as well as clinical mastitis and culling risks, are more economical outcomes, as they are more visible to the producer than bacteriological cure and new IMI risk. When linear regression models were used to analyze first-test date milk production or longitudinal production over the first 30 DIM, no important differences were found between treatment groups. This agrees with findings from 2 studies that followed selectively treated cows for greater than 180 DIM (Rajala-Schultz et al., 2011; Cameron et al., 2015). Additionally, the referenced studies found no statistical differences in SCC or log transformation of SCC between the treatment groups, similar to the LS comparisons in current trial. These results differ from findings by Rajala-Shultz et al. (2011) and Scherpenzeel et al. (2014) who found that either next-lactational SCC or SCC at calving and 14 DIM, respectively, was significantly lower than that of untreated cows. Dissimilarities between findings could be due to the presence of higher levels of major pathogens on the included dairies, the lack of teat sealant use, or the inclusion of herds with BTSCC >250,000 cells/mL.

In the current trial, no important differences were found between treatment groups when assessing the risk of clinical mastitis during the trial period. Cameron et al. (2014) used culture as a screening tool in addition to individual SCC and found no statistical differences in clinical mastitis cases in the first 120 DIM between the selective and blanket treatment groups. In the current trial, the number of cows enrolled with a single mastitis event was not evenly distributed between the groups; more cows that had experienced a case of mastitis were allocated to the TS group. Despite enrollment distributions, a higher number of next-lactation mastitis cases were described in the ABXTS group. The number of clinical cases within 30 DIM described for enrolled animals corresponded with the monthly incidence rate at this dairy. The fact that only low-risk cows were represented in this figure might explain why this number was not as high as expected for fresh animals. Accordingly, the first studies on decreased use of dry cow therapy in herds with low BTSCC were not selective, treatment was omitted for all cows; cows were not separated based on risk or infection status. This often resulted in an increased incidence rate of clinical mastitis. For example, when no screening process was used in a low BTSCC herd (140,000 cells/mL; n = 50 cows) to treat 2 and leave 2 quarters untreated within the same cow, there was a 10-fold increased risk of clinical mastitis in the untreated quarters (Schukken et al., 1993). Similarly but more recently, Scherpenzeel et al. (2014) used a split udder design on 1,657 cows with individual low SCCs (<150,000 cells/mL for primiparous and <250,000 cells/mL for multiparous) and found that the incidence of clinical mastitis was 1.7 times higher in quarters dried without antimicrobials. Finally, our findings also differed from Berry and Hillerton (2002) in which untreated quarters at dry-off had significantly more cases of clinical mastitis over the dry period. In those quarters with IMIs at freshening, the incidence of a subsequent case of clinical mastitis was significantly greater for untreated cows. While all herds included in the

Berry and Hillerton trial (2002) had a BTSCC of 150,000 cells/mL, only cows with no IMI, CNS, or *Corynebacterium* spp. one week before dry-off could be randomized into treatment groups. Therefore, cows at risk due to historically higher SCC or multiple mastitis events were among the cows included in the non-treated group. Inclusion of more variables in our algorithm allowed us to capture cows with these characteristics. Berry and Hillerton also noted discrepancies in detection rates between farms and between pathogen types, and noted that one farm contributed to a large portion (>70%) of the clinical cases. Employees responsible for mastitis detection in the current trial were blinded to treatment and therefore could not be biased when detecting cases of clinical mastitis.

The few cases of clinical mastitis experienced over the trial period (n = 14) could explain why mastitis was not important in a model that evaluated culling. Culling was not statistically different between treatment groups. A limited number of SDCT trials evaluate or describe survival or culling risks between treatment groups. Østerås and Sandvik (1996) found a numerically shorter lifespan (30 d shorter) for cows in placebo-treated and untreated groups versus those cows receiving antibiotics in infected quarters when using Cox modeling over an entire lactation. However, Cox and logistic regression analysis did not produce a significant association with the dry cow therapy regimen in that study.

To our knowledge, this is the first clinical trial that used a computer-automated system that relied on DHIA test-day data and recorded mastitis events to determine dry cow antibiotic treatment. Using bacteriological culture is the current gold standard to diagnose an IMI. Accordingly, the accuracy measurements of the algorithm to identify IMI in this study are based on comparisons to this diagnostic tool. Nearly all (>90%) of the low-risk quarters were uninfected at dry-off, indicating that the method of using sequential test-day SCC (as an average

of the last 3) in addition to the use of last-test-day SCC and mastitis events was good at identifying cows that had mostly uninfected quarters on the trial dairy. At the apparent prevalence of IMI at the current dairy, the positive and negative predictive values of the algorithm were approximately 70%. These values indicate, based on initial quarter-level culture results, the probability that a cow identified as low-risk, was experiencing an IMI, and was not treated with antibiotics was 30% (1-NPV); the probability that a cow identified as high-risk, was not experiencing an IMI, and was treated was 30% (1-PPV). High prevalence of minor pathogens might explain suboptimal predictive values. When only cows with major pathogens were characterized as infected, the NPV and PPV became 92% and 38%, again suggesting that a large proportion cows designated by the algorithm as high-risk were not infected, but treated. More importantly, cows allocated by the algorithm to the low-risk group infrequently cultured with major pathogens. One must recognize that the algorithm was used not only to capture and treat cows with current infections (culture positive) but also those cows at risk for acquiring infections throughout the dry period, a characteristic that would be difficult to predict or include in these calculations. The PPV was higher than the 42.5 and found in a SDCT trial that used a SCC threshold of 200,000 cells/mL for 3 consecutive months before dry-off and no cases of mastitis to determine which cows to treat (Torres et al., 2008). Reasons for dissimilarities in predictive values could be due to the addition of criteria in the current algorithm or differences in IMI prevalence on the test dairies. For an increase in apparent prevalence of 5%, the PPV and NPV of the algorithm would be 74.5% and 66.2%; for a decrease of 5%, PPV and NPV would be 65.8% and 75.1%. The predictive values of our algorithm are impacted by study design. Treatment assignment was on the cow level while bacteriological sampling was on the quarter level. A discrepant quarter-level result within one cow could largely affect the predictive value

calculation. As an example, if a cow was defined as low-risk by the algorithm but had one bacteriologically positive quarter and 3 negative quarters, the overall NPV of the algorithm would decrease. Regardless, we elected to apply treatment at the cow level as other groups have shown an interdependence of quarters towards new infection during the dry period, particularly in herds with higher risk of infection (Browning et al., 1994; Robert et al., 2006a). Additionally, 72% of large US dairies use DHIA for milk recording data, and while the most accurate relationship exists between quarter-level SCC and IMI, producers only have access to cow composite information (USDA, 2016a; Schukken et al., 2003).

We used a threshold of 200,000 cells/mL as a component of our algorithm. Other groups have shown that this is the optimal level to decrease error related to detection of IMIs, with the highest sensitivities and specificities amounting to at best 0.75 and 0.90 (Dohoo and Leslie, 1991; Schepers et al., 1997). However, the sensitivity and specificity of using only last-test SCC as an indicator of IMI at a threshold of 200,000 cells/mL is approximately 0.4 and 0.8 (Pantoja et al., 2009). This likely is due to the slight increases in SCC experienced by a late-lactation cow as she approaches dry-off and is a reason why decisions should not be based exclusively on a late lactation SCC (Schepers et al., 1997; Bradley and Green, 2004). On the contrary, Green et al. (2007) found that at least one SCC ≥200,000 cells/mL within the last 90 d before drying off was associated with an increased rate of clinical mastitis in the periparturient period. The most efficient use of thresholds in a SDCT protocol was identifying cows using 3 months' SCC records with a threshold of 200,000 cells/mL for cows without clinical mastitis during the lactation and a threshold of 100,000 cells/mL during the rest of the lactation for cows with clinical mastitis during the first 90 DIM (Torres et al., 2008). These findings support the need to interpret SCC as well as other health data, e.g. mastitis events, when making treatment decisions.

Treatment decisions are also influenced by economics. A benefit of the algorithm used in this trial is the adaptability of the parameters used to define a low-risk cow. The SCC thresholds can be manipulated to increase sensitivity and decrease risk. Because no important differences were seen in milk production, LS, clinical mastitis cases, or culling, savings in labor and antimicrobial costs would result with use of SCC thresholds lower than those tested. Few SDCT studies provide economic analysis and the directionality of the impact is dependent upon accuracy, labor, and costs of the test method, cost of the antibiotic, and the prevalence of IMI at dry-off in the herd. Use of quarter-level culture results to drive selective treatment on a farm with a dry-off IMI prevalence of 34.8% resulted in a savings of $2.77 per cow using fixed costs for materials, labor, and IMM antibiotics (Patel et al., 2017). When a threshold composite SCC was employed in a SDCT protocol to model economic returns, only 2 out of 8 scenarios resulted in gains: use of a 50,000 cells/mL cutoff for both heifers and cows or use of a 150,000 cells/mL for heifers and a 50,000 cells/mL cutoff for cows (Scherpenzeel et al., 2016). The alternate 6 strategies resulted in economic losses over benefits due to differences observed in the incidence rates of clinical mastitis and subclinical mastitis between the treated and untreated animals. Another economic model was built to calculate farm-specific costs of mastitis related to the dry period using trial data and expert interviews. In it, SDCT was economically the best option as compared with BDCT or no antimicrobial use (Huijps and Hogeveen, 2007). However, the optimal decision was dependent upon the herd-specific rate of infection and other farm traits such as the attitude of the farmer toward risk. One should note that if regulations are placed on antimicrobial dry tube use for US dairies, economics might play less of a role on the selection of a SDCT protocol.

One weakness of the current trial is the inclusion of a single dairy rather than a probabilistic sample of all eligible dairies. Using a power of 80% and a Type I error risk of 0.05, a sensitivity analysis for each regression model indicated that we were able to detect odds ratios of 0.3 or lower and 1.3 or greater for bacteriological cure and new infection risks, respectively, odds ratios of 0.3 or lower for both clinical mastitis and culling risks, a difference in LS of 0.4 or greater, and a difference in longitudinal milk production of 1kg when comparing TS versus ABXTS. This herd represented dairy farms in the region that might wish to employ a SDCT program with respect to production, BTSCC, facilities, and herd size. As indicated from the large ranges of prevalence of quarter-level infection status at dry-off and postpartum, there is much variability between dairies. This variability could result in detectable differences in the outcomes investigated, but smaller, significant differences might not always be important. This is corroborated in the study by Rajala-Schultz et al. (2011) in which a SCC difference as small as 20,000 cells/mL was found to be significant when 4 dairies were included in a trial. Likewise, results could be largely influenced by one herd and the effects of SDCT can be opposite across herds (beneficial in some and detrimental in others). This dictates the need for farm-specific decisions when developing dry cow protocols. Overall, the willingness of producers to adopt a selective treatment strategy at dry-off relies on an accurate, easily accessible, and cost-effective method to identify cows, reserving antimicrobial treatment for cows that will benefit most.

CONCLUSIONS

Use of selective dry cow therapy protocols, such as the algorithm identified in this trial, protects aspects of public health by reducing the risk of bulk tank antimicrobial residues and minimizing the potential for antimicrobial resistance. Similar protocols at appropriate dairies can

reduce dry cow antimicrobial use by approximately 60% without negative consequences on new infection risk or cow-level production and health outcomes. More research is needed to determine the characteristics of farms that lead to successful implementation of SDCT algorithms, however, utilizing only on-farm data would obviate the need for microbiologic or cow-side methods that require labor, time, and money.

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CHAPTER SEVEN

SUMMARY OF THE RESULTS, OVERALL CONCLUSIONS, AND FUTURE DIRECTIONS

Mastitis is a prominent disease in the dairy industry, causing 2 billion dollars in yearly losses, attributed to consequential decreases in milk production, milk discard, treatment and labor costs, and decreases in reproductive efficiency. To combat these losses and to address and prevent clinical disease, traditionally, all clinical cases and all quarters prior to dry-off are treated with antimicrobials. Alternatively, "prudent use" involves administration of products only to animals that would benefit from their use. Reasons for prudent use include preserving the sustainability of veterinary and human formulations, decreased risk of antimicrobial resistance, decreased risk of residues in agricultural products, and potential economic returns. In many situations, antimicrobials are useful and their ability to resolve disease for appropriate cases should not be challenged; as such, approaches for implementation of protocols should rely on research findings. For mastitis, this includes the findings that a large proportion of clinical cases produce negative culture results and that a majority of quarters at dry-off are also culture-negative. Additionally, high spontaneous resolution of gram-negative cases has been reported, while the refractory or resistant nature of others to antimicrobials should also be considered. On the contrary, non-contagious, gram-positive pathogens are susceptible to antibiotics. To accomplish prudent use of antimicrobials for the treatment and prevention of mastitis, this research explored and successfully implemented strategies that acknowledge such findings.

Summary of the Results

The first objective was to evaluate if noninferiority could be established for a semi-synthetic penicillin, hetacillin, versus the 3rd generation cephalosporin, ceftiofur. Noninferiority, defined as hetacillin being comparative in outcomes within a margin of 15% relative to ceftiofur, was conclusive for bacteriological cure, pathogen cure, survival to day 30 and survival to day 60.

When logistic and linear models were used to explore outcomes, no statistical differences were found in bacteriological cure risk, pathogen cure risk, clinical cure risk, postevent milk production, and linear scores (LS). Cows treated with hetacillin were more likely to have a clinical cure than ceftiofur by day 4. No differences were observed between groups when Cox proportional hazards were performed for exit from the herd or in the risk for a subsequent mastitis event. Hospital days differed favorably for hetacillin, allowing milk from hetacillin-treated cows to contribute to the bulk tank 1.8 d sooner. These findings support the use of a narrow-spectrum antibiotic for clinical mastitis (CM) treatment as an alternative to a 3rd generation cephalosporin, which is a critically important drug class to human medicine according to the world health organization.

The second major objective was to assess a delayed pathogen-driven treatment protocol based on results received from a diagnostic laboratory within 1 d of the mastitis event. Results showed no statistical differences in days to clinical cure, postevent milk production, next test-day LS, and odds of survival 30 and 60 d post-enrollment between cases in the blanket therapy group and cases in the pathogen-based treatment group. However, pathogen-based cows experienced 3 fewer days in the hospital than blanket therapy cows. This protocol resulted in a reduction in antimicrobial use of 68.5% on the trial dairy.

Once successful implementation of a pathogen-based protocol with daily sample retrieval and prompt reporting of results was established, the ability to apply this strategy on moderate-sized dairies without access to a professional laboratory was investigated. Results indicate that when samples were picked up and results reported 5 d per week, or when farm employees were trained to perform on farm culture (OFC), compliance to the protocol and satisfaction with the experience was high. Reductions in antimicrobial usage ranged from 6.8% to 92.5% dependent

on farm protocol and pathogen profile of the herd. Application of the protocols did not contribute to large undesirable changes in bulk tank somatic cell count, milk production, or monthly mastitis incidence. Decreased antibiotic use resulted in savings between $2,200 and $4,600 per dairy ($3.00-$6.00 per cow), per year.

Selected milk samples from the pathogen-based trial were then used to explore the metagenomic profiles of CM cases to determine if these cases represent a “missed opportunity” for treatment. The data generated from metagenomic analysis suggested that there is not an inherent “mastitic microbiome” in culture-negative or *E.coli*-positive cases that indicates that a cow will experience negative outcomes, such as larger decreases in milk production or increases in linear scores.

The last major objective was to evaluate a culture-independent, data-driven, selective dry-cow therapy (SDCT) program. Results showed that when low-risk cows identified by the algorithm were not treated with antimicrobials at dry-off, they did not differ in new infection risk, milk production, LS, culling, or CM risk from low-risk cows that were treated. Bacteriological cure differed between the groups with 19/20 non-cured quarters experiencing CNS infections. The employed algorithm decreased dry-cow antibiotic use by approximately 60%.

Overall Conclusions and Future Directions

Based on the results generated during this Ph.D., implementation of a pathogen-based treatment protocol can be successful if results are supplied by a university diagnostic laboratory, veterinary laboratory, or OFC. However, access to a laboratory or training and guidance for OFC may not be feasible. The biggest needs to facilitate the implementation of the protocols presented

are diagnostic tools that provide prompt and accurate results. Many research groups and animal health companies are investigating cow-side or on-farm diagnostics such as PCR for rapid determination of etiology and application of selective treatment protocols. The creation of selective culture agar that facilitates accurate and quick diagnosis by untrained personnel is continuously being explored for on-farm use. Also, as we become a technology and computer-driven society, using these means as a way of teaching, communication, and diagnosis can enable and assist dairies in their use of pathogen-driven programs. For example, using OFC and a standardized photo-capture system to submit queries to diagnostic labs for generation of results would promote confidence in their own interpretation of bacterial growth.

In regards to research needs, in the field of metagenomics there are few longitudinal studies of mastitic quarters with permutations of treatments and pathogen types. Such research would elucidate pathogen-specific changes in the microbial profile of the mammary gland as well as the impacts of antimicrobial use or prudent use protocols on the microbiome. Additionally, as profiles can vary by farm and by animal, a large survey of the milk microbiome of animals across the country and in different management systems would be beneficial.

Defining the specifics of “appropriate” dairies for selective treatment programs is still underway. No research project accounts for the variability across dairies in respects to size, management, genetics, or pathogen profiles to permit the generalizability of the findings to all facilities. If provided the resources, it would be useful to assess how successful these protocols are in different management systems, in the US and around the world.

Importantly, knowledge attained from the research presented in this dissertation has been shared with practitioners, farmers, and industry professionals at conferences, meetings, and in magazines with hopes that protocols will be applied at appropriate dairies. However,

understanding dairy farmers' behavior, motivations, and barriers can influence how veterinarians translate research into practice, and can guide professionals to effective ways of implementing new protocols. To this end, collaborative work with Dr. Lorin Warnick's laboratory and with Dr. David Just, a behavioral economist at Cornell University, will employ the theory of planned behavior and structural equation modeling using survey answers from NY dairy owners to determine what exactly drives antibiotic use decisions. Results from this research may direct practitioners in approaches that create behavioral changes on dairies that currently employ blanket therapy.

Future directions for our laboratory for SDCT include collaborative work with Dr. Sandra Godden at the University of Minnesota and Dr. Alfonso Lago at Dairy Experts (Tulare, CA). A project will explore and compare options for SDCT in regions across the US using different protocols (algorithm-based and culture-based) and will investigate the ability of 2 rapid diagnostic tests for identifying quarters with an IMI at dry-off. Additionally we will describe the effect of antibiotic treatment of uninfected cows at dry off on the milk microbiome after calving, and on udder health and cow performance in early lactation. Sequencing will also be performed on colostrum samples from the SDCT trial included in this dissertation. More specifically, Dr. Carla Foditch from Dr. Lorin Warnick's laboratory will perform shotgun sequencing on colostrum samples to determine if antimicrobial residues and/or resistant organisms are differentially present in colostrum from animals that were treated versus those that were not. Additionally, cow-side residue tests are currently being optimized and validated for colostrum samples and again, samples in the SDCT trial will be investigated for the presence of residues.

In closing, implementation of protocols that promote prudent use of antimicrobials will not contribute to the reduction of mastitis on dairy farms; prevention of mastitis should be the

goal. Overall strategies should rely on the three points of the epidemiological triangle: the agent, the host, and the environment. To this end, dairies should optimize dry cow management and cow comfort, improve immune status via vaccination and provision of quality nutrition, and decrease infection pressure by maintaining cow and facility cleanliness. Additionally, post-milking teat disinfection, culling of chronic cases, and milking machine maintenance should also be considered. Knowledge of these many aspects of mastitis control and management can be acquired via review of additional publications.