THE APPLICATION OF NEXT GENERATION SEQUENCING TO FURTHER UNDERSTAND THE MICROBIAL DYNAMICS OF BOVINE CLINICAL MASTITIS

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THE APPLICATION OF NEXT GENERATION SEQUENCING TO FURTHER UNDERSTAND THE MICROBIAL DYNAMICS OF BOVINE CLINICAL MASTITIS

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Bovine mastitis is one of the greatest challenges faced by dairy farmers worldwide. In the United States, one in four cows experience at least one case of clinical mastitis throughout the lactation. This disease creates an enormous economic burden due to decreased milk production, treatment costs, and premature culling. Mastitis is also an important animal welfare issue. The main objective of the research carried out during this doctorate was to generate knowledge to better understand and offer directions for the proper treatment of bovine mastitis.

This dissertation comprises four research articles that are organized into two components: The applied portion of this research aimed to evaluate a novel method for diagnosis of mastitis pathogens and its's suitability for use in the field (chapter two). Next, we assessed the potential of trace mineral supplementation in helping animals with subclinical mastitis resolve the disease (chapter three). The basic component of these doctoral studies employed molecular techniques to further understand the dynamics of mastitis and antimicrobial treatment in naturally infected animals (chapter four) and experimentally infected animals (chapter five).

A novel chromogenic method for identification of mastitis-associated pathogens is suitable for use under field conditions. The color-based and straightforward interpretation allows

individuals with limited microbiology training to accurately diagnose the causative pathogen and provide basis for efficient selective antimicrobial therapy. Supplementation with trace minerals did not improve the overall cure of subclinical mastitis, nor decreased the overall incidence of clinical mastitis. However, we identified a tendency for improved subclinical mastitis cure in cows of third or greater lactation, and a decrease in the odds of having chronic clinical mastitis for primiparous cows.

Longitudinal profiling of the milk microbiome revealed astonishing differences between healthy and mastitic milk. A significant reduction in microbial diversity was associated with disease in both naturally infected and experimentally challenged animals. Extended intramammary treatment with ceftiofur significantly decreased bacterial load, however such effect could only be observed during treatment administration. No differences on the microbial profile of mastitic or healthy milk due to antimicrobial treatment were observed. The capability of the milk microbiome to return to a healthy status in a relatively short period independent of intramammary antimicrobial therapy was observed consistently in both studies. The results observed here indicate that the milk harbors a resilient microbiome capable of restoring itself after mild and moderate cases of mastitis, independent of antimicrobial therapy.

BIOGRAPHICAL SKETCH

Erika Korzune Ganda was born in Cacoal, state of Rondonia - Brazil, in 1990. Without any farming background, Erika decided to pursue a degree Veterinary Medicine in the Federal University of Goias, Brazil. During veterinary school Erika was fortunate to receive mentoring from the professionals of the Dairy Management Project, a partnership of Dairy Partners of America (DPA) and the Federal University of Goias that provided consultancy, training and assistance to small dairy farmers within a 150 km radius of Goiania, GO. From this experience, she became passionate about all aspects involved in the dairy industry: from animal health, to milk quality, safety, and overall farm management.

She received her degree of Doctor in Veterinary Medicine in 2012. After that, she worked as a research assistant for Dr. Rodrigo Bicalho at Cornell University, and participated in several research projects that investigated various aspects of dairy cow health and productivity.

In 2013 she received a Science without Borders scholarship (Ciencias Sem Fronteiras), from the Brazilian government. She was granted funding to work in conjunct with Dr. Bicalho on a project that aimed to use Next Generation Sequencing to study dairy cow mastitis and antimicrobial therapy.

She was accepted as a Ph.D. student at the Department of Animal Science at Cornell and began her doctoral studies in the fall of 2013. Her work focuses in basic and applied aspects of Bovine Mastitis, and has as objectives generate data that will support a better understanding and management of this important disease.

To those who believed in me.

And those that supported me.

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LIST OF ABBREVIATIONS

ANOSIM – analysis of similarities
ANOVA – analysis of variance
ATCC – american type culture collection
BCS – body condition score
CCM – chronic clinical mastitis
CFU – colony forming units
CI – confidence interval
CM – clinical mastitic
CNS – coagulase negative *staphylococci*
CTRL – control
DHIA – dairy herd improvement association
DIM – days in milk
DNA – deoxyribonucleic acid
EDTA – ethylenediaminetetraacetic acid
ITMS – injectable trace mineral supplementation
KOH – potassium hydroxide
LARTU – large animal teaching and research unit
LS – linear score
NMC – National Mastitis Council
NPV – negative predictive value
NRC – National Research Council
OTU – operational taxonomic unit
PBS – phosphate buffered saline
PcoA – principal coordinate analysis
PCR – polymerase chain reaction
PPV – positive predictive value
QMPS – Quality Milk Production Services
qPCR – quantitative polymerase chain reaction
RAPD – random amplification of polymorphic DNA
ROS – reactive oxygen species
rRNA – ribosomal ribonucleic acid
SCC – somatic cell count
Se – sensitivity
SE – standard error
SOD – superoxide dismutase
Sp – specificity
StdDev – standard deviation
K – Cohen's kappa coefficient

CHAPTER 1

INTRODUCTION

Bovine clinical mastitis is a highly prevalent disease, and creates an incontestable economic burden in the dairy industry (Heikkila et al., 2012, Rollin et al., 2015, Taponen et al., 2017). The main contributors to the cost of mastitis are decreased milk production, treatment costs, discarded milk, and premature culling (Cha et al., 2011, Heikkila et al., 2012, Rollin et al., 2015). Most recent reports indicate that 24.8% of the United States' dairy herd are affected with clinical mastitis (USDA, 2016), leading to the treatment of 21.7% of the US' 9.3 million dairy cows with intramammary antimicrobials. It is not surprising that the vast majority of antimicrobials used in the dairy industry are associated with the treatment or prevention of mastitis (Pol and Ruegg, 2007, Gomes and Henriques, 2015). The overall objective of this doctoral research is to enrich the body of literature and provide evidence that can be used for better and more efficient management, treatment, and understanding of bovine mastitis.

The etiology of bovine mastitis is complex. It is true that mammary infections caused by fungi, algae, and even viruses have been reported (Wellenberg et al., 2002, Dworecka-Kaszak et al., 2012, Ricchi et al., 2013); however, the vast majority of mastitis cases have been associated with bacteria (Hertl et al., 2014, Taponen et al., 2017). Due to the variety of pathogens that are causative of clinical mastitis, treatment based on knowledge of the organism responsible is an important practice, given that success of antimicrobial treatment is dependent on the pathogen involved (Royster and Wagner, 2015, Breen, 2016). On-farm culture allows for identification of mastitis pathogens as early as 24 hours after diagnosis of clinical mastitis, and became an invaluable tool (Royster et al., 2014, Viora et al., 2014). Equally important, studies have reported that selective antimicrobial therapy has the potential to decrease antibiotic usage by 50% without

significant differences in cow's health or production traits (Lago et al., 2011a, b). Nevertheless, the accuracy of on-farm culture is highly variable between different observers (McCarron et al., 2009a, b). On chapter two we evaluate a novel diagnostic tool for on-farm diagnosis of mastitis pathogens that is based on color. Chromogenic culture media has been employed in both human and animal samples (Perry and Freydiere, 2007, Kalchayanand et al., 2013) and allows rapid and accurate identification of specific pathogens. We compared the on farm use of Accumast®, a chromogenic based culture system, to results from the Cornell Quality Milk Production Services (QMPS) and molecular identification of cultured pathogens using 16S rRNA sequencing in two different studies. Results from these studies comprise the most applicable portion of this dissertation.

In the same way as clinical mastitis, subclinical mastitis is also a prevalent disease and the lack of abnormal milk makes routine testing and identification of animals affected an important management practice in dairy farms. Subclinical mastitis has been associated with decreased milk production (Green et al., 2006, Boland et al., 2013), impaired animal welfare (Peters et al., 2015), increased risk of premature culling (Barlow et al., 2009), and decreased milk products yield (Ma et al., 2000, Mazal et al., 2007, Bobbo et al., 2017). Although certain intramammary antibiotics are currently labeled for the treatment of subclinical mastitis, evidence suggests that this practice is not cost-effective (Swinkels et al., 2005, Steeneveld et al., 2007).

One possible alternative to reduce the impact of subclinical mastitis is ensuring adequate trace mineral availability. For instance, Selenium concentrations in blood have been associated with diminished intramammary infections, and low bulk tank somatic cell counts (Erskine et al., 1987, Weiss et al., 1990, Kommisrud et al., 2005). Previous studies have also reported that injectable trace mineral supplementation can maintain elevated serum concentrations of copper,

selenium and zinc for two weeks after treatment (Bicalho et al., 2014), and that trace mineral supplementation during the transition period reduced the incidence of clinical mastitis and somatic cell count (Machado et al., 2013). In chapter 3 we hypothesized that injectable trace mineral supplementation had the potential to improve udder health of cows diagnosed with subclinical mastitis by restoring endogenous pools of these minerals, and we report results of a randomized clinical trial that evaluated the effect of a single injection of trace mineral supplementation on cure of subclinical mastitis, incidence of chronic clinical mastitis, and milk composition.

In addition to the applied research conducted in chapters two and three, this dissertation delivers results from longitudinal profiling of the milk microbiome in chapters four and five. Extensive research has provided evidence that the milk harbors a plethora of bacteria, considerably larger than what can be observed using culture-dependent studies (Jost et al., 2013, Keane et al., 2013, Kuehn et al., 2013), highlighting the usefulness of molecular-based techniques on the investigation of mastitis (Oikonomou et al., 2012, Kuehn et al., 2013, Jimenez et al., 2015). Our objectives in chapter four were to apply state-of-the-art technologies to describe the microbiome of mastitic and healthy milk from ipsilateral quarters in animals naturally infected with a variety of mastitis pathogens and yielding negative results upon aerobic culture. Additionally, we evaluate the effect of third generation cephalosporins on cure of clinical mastitis.

In chapter five we characterize the milk microbial profile before, during, and after experimental infection with *Escherichia coli* and subsequent treatment with a third generation cephalosporin. About 53% of dairy operations identify coliforms in mastitis cases, and ceftiofur, the only third generation cephalosporin labeled for veterinary use in the United States, accounts

for over 50% of treatments of mastitis (USDA, 2016). Amongst gram-negative pathogens, *E. coli* is the most common pathogen and accounts for as much as 80% of those cases of mastitis, especially in well managed dairy herds (Bradley et al., 2007). Nevertheless, the efficacy of antimicrobial treatment of *E. coli* mastitis is still ambiguously discussed. Investigators have reported improved bacteriological cure when animals affected with nonsevere cases of gram-negative mastitis received extended intramammary treatment with a third generation cephalosporin (Schukken et al., 2011). On the other hand, after reviewing the available literature, Suojala (2013) recommends not treating animals affected with mild and moderate cases of *E. coli* mastitis (Suojala et al., 2013). Moreover, we assessed the effects of exposing the microbiome of healthy milk to ceftiofur. To date, no studies assessing the impact of these broad-spectrum antibiotics on the healthy milk microbiota have been carried out. Intramammary administration of a broad-spectrum antimicrobial might favor the overgrowth of specific organisms and incite a shift in the microbial profile, similar to what has been previously reported in other niches (Chambers et al., 2015). The evidence gathered in this chapter offers information that can be used to substantiate the proper management of *E. coli* mastitis and further the understanding on the impact of third generation cephalosporins on the milk microbiome.

In summary, the collection of studies that compose this dissertation explore a diagnostic tool for on-farm identification of pathogens associated with clinical mastitis, a non-antimicrobial alternative for the treatment of subclinical mastitis, and applies Next Generation Sequencing aiming to understand in depth the microbial dynamics of bovine clinical mastitis. The overall objective of the research conducted is to provide data that will facilitate effective management, treatment, and understanding of this disease.

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CHAPTER 2

EVALUATION OF AN ON-FARM CULTURE SYSTEM (ACCUMAST®) FOR FAST IDENTIFICATION OF MILK PATHOGENS ASSOCIATED WITH CLINICAL MASTITIS IN DAIRY COWS

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ABSTRACT

The present study aimed to evaluate an on-farm culture system for identification of milk pathogens associated with clinical mastitis in dairy cows using two different gold standard approaches: standard laboratory culture in study 1 and 16S rRNA sequencing in study 2. In study 1, milk from mastitic quarters (i.e. presence of flakes, clots, or serous milk; n = 538) was cultured on-farm using a single plate containing three selective chromogenic media (**Accumast®** - FERA Animal Health LCC, Ithaca, NY) and in a reference laboratory using standard culture methods, which was considered the gold standard. In study 2, mastitic milk was cultured on-farm and analyzed through 16S rRNA sequencing (n = 214). In both studies, plates were cultured aerobically at 37°C for 24 h and read by a single technician masked to gold standard results. Accuracy, sensitivity, specificity, positive (**PPV**) and negative predictive value (**NPV**) were calculated based on standard laboratory culture in study 1, and PPV was calculated based on sequencing results in study 2. Overall accuracy of Accumast® was 84.9%. Likewise, accuracy for identification of Gram-negative bacteria, *Staphylococcus* sp., and *Streptococcus* sp. was 96.4%, 93.8%, and 91.5%, respectively. Sensitivity, specificity, PPV, and NPV were 75.0%, 97.9%, 79.6%, and 97.3% for identification of *E. coli*, 100.0%, 99.8%, 87.5%, and 100.0% for *S. aureus*, 70.0%, 95.0%, 45.7%, and 98.1% for other *Staphylococcus* sp., and 90.0%, 92.9%, 91.8%, and 91.2% for *Streptococcus* sp. In study 2, Accumast® PPV was 96.7% for *E. coli*, 100.0% for *Enterococcus* sp., 100.0% for Other Gram-negatives, 88.2% for *Staphylococcus* sp., and 95.0% for *Streptococcus* sp., respectively. In conclusion, Accumast® is a unique approach for on-farm identification pathogens associated with mastitis, presenting overall sensitivity and specificity of 82.3% and 89.9% respectively.

Keywords: Accumast®, intramammary infection, *Staphylococcus*, *Streptococcus*, *E. coli*, antimicrobial therapy.

INTRODUCTION

Clinical mastitis remains an important animal health issue and leads to major economic losses to the dairy industry worldwide. From 20% to 30% of dairy cows are diagnosed with clinical mastitis at least once during lactation (Sargeant et al., 1998, Hertl et al., 2014a). Estimated costs per case of clinical mastitis range between $179 and $488 depending upon milk prices, level of production in affected cows, culling policies, and stage of lactation when the disease occurred (Bar et al., 2008, Hagnestam-Nielsen and Ostergaard, 2009). In fact, treatment and prevention of mastitis are considered the most common causes of antibiotic use in dairy herds (Erskine et al., 2003, Pol and Ruegg, 2007). Although fungi and algae have been observed in the milk of cows diagnosed with clinical mastitis, inflammation of the mammary gland is caused predominantly by bacterial infections. *Staphylococcus* sp., *Streptococcus* sp., and coliforms account for approximately 90% of isolates in the milk of mastitic cows (Sargeant et al., 1998, Olde Riekerink et al., 2008). Of particular importance for mastitis control programs, the success of antimicrobial therapy is dependent upon the causal pathogen associated with clinical mastitis. Intramammary antibiotic therapy improves the rate of cure in cows infected with coagulase-negative *staphylococc*i, *Staphylococcus* sp., and environmental *streptococci* (Roberson, 2012). On the other hand, the use of an intramammary antibiotic is not recommended for cows with mastitis associated with *E. coli* (Suojala et al., 2013). Rapid on-farm identification of milk pathogens is critical for targeted antimicrobial therapy, which helps avoid the indiscriminate use of antibiotics in livestock and reduces the economic burden of clinical mastitis.

Several on-farm culture systems have been developed to characterize milk pathogens and substantiate the decision to treat cows with clinical mastitis. Initial on-farm culture systems were based on blood and MacConkey agar plates, which allowed for categorization of microorganisms into Gram-positive, Gram-negative, or no growth within 24 to 32 h and at relative low cost compared to the use of referral diagnostic laboratories. Previously published studies indicate that the use of selective treatment based on on-farm culture systems results has the potential to reduce antibiotic usage by 50% with no changes in the risk of disease recurrence, bacteriological cure, somatic cell count, milk production, and survival throughout lactation (Lago et al., 2011a, b). More sophisticated on-farm culture systems allow for further genus classification of Gram-positive bacteria into *Staphylococcus* sp. and *Streptococcus* sp. and evaluation of *Staphylococcus aureus* presence in milk (Royster et al., 2014). Nevertheless, using these other techniques the assessment of colony appearance is required for detailed identification of milk pathogens and may reduce the predictive value of on-farm culture systems when conducted by farm personnel. In fact, the sensitivity of selective culture media to detect *S. aureus* in milk samples ranged from 43.2% to 59.1% when plates were read by individuals with only limited microbiology training (McCarron et al., 2009a).

Chromogenic media have been used for identification of microorganisms in both human and animal specimens (Perry and Freydiere, 2007, Kalchayanand et al., 2013). Chromogens incorporated in the culture media are cleaved by specific bacterial enzymes generating chromophores, which can be readily recognized with the naked-eye based on color change. The use of chromogenic culture media for identification of milk pathogens associated with mastitis has not been previously evaluated. However, this technology has the potential to increase the number of bacteria distinguishable using on-farm culture systems without requiring intensive

microbiology training by farm personnel. The main hypothesis of this study was that the use of chromogenic culture media allows for identification of milk pathogens associated with clinical mastitis in lactating dairy cows with satisfactory sensitivity and specificity. Therefore, specific objectives were to evaluate the use a of selective chromogenic on-farm culture system designed for identification of specific mastitis pathogens: *staphylococci*, *streptococci*, and Gram-negative bacteria, constituted by a single plate containing three selective chromogenic media (Accumast® - FERA Animal Health LCC, Ithaca, NY). Predictive values of this on-farm culture system were evaluated based on the results from an official diagnostic laboratory (study 1) and on through molecular identification of cultured pathogens using 16S rRNA sequencing (study 2) as gold standards.

MATERIALS AND METHODS

In-vitro Assessment of Colony Characteristics of Pure Bacterial Cultures Plated onto Chromogenic Media

The assessment of growth of pure ATCC strains of pathogens previously described to be associated with bovine mastitis was performed in laboratory using Accumast® for the purpose of evaluation of the growth characteristics. The following Gram-positive and Gram-negative ATCC strains (Species, catalog number) were used in the evaluation: (*Staphylococcus aureus*, 25923; *Staphylococcus epidermidis*, 12228; *Staphylococcus chromogenes*, 43764; *Streptococcus agalactiae*, 27956; *Streptococcus dysgalactiae*, 43078; *Streptococcus uberis*, 700407; *Enterococcus faecalis*, 29212; *Escherichia coli*, 25922; *Klebsiella oxytoca*, 49131; *Pseudomonas aeruginosa*, 15442). Bacteria stocks were activated in unselective tryptic soy agar plates supplemented with 5% sheep blood and 0.1% esculin (BioMerieux, Durhan, NC). Plates were

incubated aerobically at 37°C for 24 h to ensure the presence of live bacteria and absence of contamination. For each strain, a single colony was transferred to 5 mL of brain heart infusion broth (Bacto Brain Heart Infusion; Becton, Dickinson and Company, Franklin Lakes, NJ), homogenized, and incubated overnight aerobically at 37°C. Bacterial cultures were diluted to 1:1,000 in sterile PBS solution (Boston BioProducts, Ashland, MA). A sterile cotton swab was used to plate the diluted bacterial culture into each section of Accumast®, ensuring that the swab had been saturated in the bacterial sample between plating different sections of the plate. Plates were incubated aerobically at 37°C (**Figure 2.1**).

The threshold selected for considering a sample positive for bacterial growth using the Accumast® culturing system was the presence of five or more colonies in a single section of the plate. Presence of bacterial growth in each of two different sections of the plate was considered a mixed infection and counted as positive for both types of bacteria. Presence of bacterial growth in each of three sections was considered contamination.

Ethics Statement

The present study was carried out in agreement with the recommendations of The Animal Welfare Act of 1966 (P.L. 89-544) and its amendments of 1970 (P.L. 91-579), 1976 (P.L. 94-279), and 1985 (P.L. 99-1998) that regulates the transportation, purchase and treatment of animals used in research. The research protocol was reviewed and approved by the Institutional Animal Care and use Committee of the Cornell University (Protocol number: 2013-0056). Sampling animals that present abnormal milk during forestripping on milking preparation is also routine procedure at the study site.

Conflict of Interest Statement

The product evaluated in the present study was originally developed in Dr. Bicalho's research laboratory, in Cornell University, Ithaca, NY. Cornell University requires inventors to assign to the university or its designee all rights and titles of their inventions and related property rights that result from activity conducted in the course of an appointment with the university and/or using university resources, including those provided through an externally funded grant, contract, or other type of award or gift to the university. A U. S. Provisional Patent application (No. 62/212,482) was submitted by Cornell University on August 31, 2015 and listed Dr. Bicalho as the inventor. Dr. Bicalho founded the company FERA Animal Health, LLC and licensed the patent rights from Cornell University on February 25, 2016.

Farm and Management

On-farm evaluation of the use of Accumast® for identification of pathogens associated with clinical mastitis was performed in a single commercial dairy herd located in Venice Center, NY. During the study, approximately 2,800 cows were milked thrice daily in a double-52 milking parlor and the yearly rolling herd average for milk yield was 13,800 kg, with an average bulk tank somatic cell count of 360.000 cells/mL. Primiparous and multiparous cows were housed separately in free-stall barns equipped with sprinklers, fans, and concrete stalls bedded with manure solids. Cows were fed a total mixed ration to meet or exceed the nutrient requirements of a 650 kg lactating Holstein cow producing 45 kg/d of milk with 3.5% fat and 3.2% true protein when dry matter intake is 25 kg/d (NRC, 2001).

Sample Collection

Clinical mastitis was defined as the presence of abnormal milk (i.e. presence of flakes, clots, or serous milk) during forestripping performed at the milking parlor. Milk from affected quarters was sampled aseptically by trained farm personnel following recommendations of the National Mastitis Council. Briefly, teats were cleaned and disinfected using 70% ethanol (vol/vol). The initial three streams were discarded and approximately 5 mL of milk was collected into a sterile plastic tube without preservative (Corning Life Sciences, Tewksbury, MA). Milk samples were kept refrigerated at 4 ^{o}C in a designated office adjacent to the milking parlor and plated at the farm no longer than 12 h after sample collection.

On-farm Culture System

The on-farm culture system evaluated here was created to allow for selective growth and identification of specific mastitis pathogens (i.e. s*taphylococci*, *streptococci*, and Gram-negative bacteria), using a single plate containing three selective chromogenic media (Accumast®, FERA Animal Health LCC, Ithaca, NY).

Milk samples were plated onto Accumast® using a sterile cotton swab. Before application into each of the three sections of Accumast® the swab was immersed in the milk sample. Plates were incubated at 37^{o}C for 24 h and read on-farm by one of the investigators according to the flowchart provided for on-farm diagnosis of mastitis pathogens identifiable by Accumast® (**Figure 2.2**). Presence of bacterial growth, number of colonies, and color of colonies were recorded. Identification of milk pathogens was performed following instructions of the flowchart developed based on characteristics of growth of American Type Culture Collection (**ATCC**) strains described above.

Study 1: Identification of Milk Pathogens by Standard Laboratory Culture

A total of 538 milk samples obtained from cows affected with clinical mastitis were collected as described above, from April to July, 2014. Immediately after collection and plating onto Accumast®, milk samples were refrigerated at 4°C and transported to the Quality Milk Production Services (**QMPS**) laboratory (Cornell University, Ithaca, NY) on ice. Milk samples were cultured following standard procedures for mastitis associated pathogens identification (NMC, 1999) and standard laboratory culture results were used as gold standard for evaluation of on-farm culture system. In summary, milk samples were plated onto trypticase soy agar plates supplemented with 5% of sheep blood and 0.1% esculin using a sterile cotton swab. Plates were incubated aerobically at 35°C to 38°C for at least 24 h but no longer than 48 h. Culture characteristics evaluated included size, color, hemolytic pattern, and odor. Ancillary tests for further bacterial classification included Gram stain and wet mount microscopic evaluations. Biochemical tests comprised evaluation of the presence of catalase using 3% hydrogen peroxide, coagulase using EDTA rabbit plasma tubes, indole using SpotTest (Hardy Diagnostics), KOH string test using 3% potassium hydroxide, oxidase, lactose, sorbitol fermentation, and CAMP tests. Additionally, surface carbohydrates group typing (BactiStaph and PathoDx, Thermo Scientific) and selective differential agars such as MacConkey, Edwards, and bile esculin were used when needed. Samples were considered mixed infections when two clearly distinct bacterial types in a well distributed growth pattern were detected, and both pathogens were reported. Identification of more than two distinct colony types and no contagious pathogens such as *S. aureus* or *S. agalactiae* present was considered contamination of the sample. Samples were considered negative when no aerobic bacterial growth was observed in the first 48 h of incubation following guidelines for accredited diagnostic laboratories.

Study 2: Identification of Milk Pathogens by 16S rRNA Sequencing

To further evaluate the accuracy of Accumast® for on-farm identification of milk pathogens, a second study utilized bacterial 16S rRNA sequencing from pathogens isolated from mastitic milk samples cultured on-farm between October to December of 2014 using Accumast® plates as described above (n = 214) utilizing the Illumina platform.

On-farm identification of milk pathogens was performed as previously described. Plates were read at the farm and those with bacterial growth were transported at room temperature to the laboratory for bacterial isolation, DNA extraction, and sequencing. For each sample, one colony was selected from Accumast®, collected using a sterile inoculating loop, and plated onto unselective tryptic soy agar plates supplemented with 5% sheep blood and 0.1% esculin (BioMerieux, Durhan, NC). Plates were incubated aerobically at 37^{o}C for 24 h. This procedure was repeated twice using blood agar plates (Northeast Laboratory Services, Winslow, ME) to ensure that the colony isolated from Accumast® was free of contamination.

Genomic DNA was isolated using InstaGene matrix (Bio-Rad laboratories, Hercules, CA) according to manufacturer's recommendations. Concentration and purity of extracted DNA were evaluated based on optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). Amplification of the V4 hypervariable region of the bacterial 16S rRNA gene was performed from genomic DNA by PCR utilizing the primers 515F (AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCG GTAA) and barcoded 806R (CAAGCAGAAGACGGCATACGAGAT*XXXXXXXXXXXX* AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT) which have been optimized for the Illumina MiSeq platform (Caporaso et al., 2012). To allow for multiplex sample sequencing,

each sample was tagged with a unique 12-bp error-correcting Golay barcode for the 16S rRNA PCR selected using the earth microbiome project (http://www.earthmicrobiome.org/) as formerly described (Gilbert et al., 2010, Caporaso et al., 2012). Barcoded amplicons were generated in triplicate using 3 µL DNA template, 1X EconoTaq Plus Green Master Mix (Lucigen, Middleton, WI), and 5 µM of each primer. Thermocycler conditions were as follows: an initial denaturing step at 94°C for 3 min, 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 90 s, and a final elongation step of 72°C for 10 min. Blank controls were incorporated in each reaction batch to ensure the absence of bacterial DNA contamination. Replicate amplicons were pooled and and visualized by electrophoresis through 1.2% (wt/vol) agarose gels stained with 0.5 mg/mL ethidium bromide. Purification of amplicons was performed using Gel PCR DNA Fragment Extraction kit (IBI Scientific, Peosta, IA). The NanoDrop ND-1000 spectrophotometer was used for quantification.

Aliquots of 16S rRNA amplicons were standardized to the same concentration (i.e. 16 ng/µL) and sequentially diluted to 20 pM following DNA denaturation. Because bacterial DNA was harvested from a single purified colony, amplicons were combined with 70% of PhiX (Illumina Inc., San Diego, CA). Amplicons were pooled into a single run and final equimolar library was sequenced using the MiSeq reagent kit V2 Nano for 300 cycles on the MiSeq platform (Illumina Inc., San Diego, CA). The 16S rRNA gene sequences were processed using the MiSeq Reporter analysis software version 2.5. Indexed reads were demultiplexed for generation of individual FASTQ files and reads were aligned to the Illumina-curated version of Greengenes database for genus-level classification of milk pathogens.

Statistical Analyses

The predictive values of Accumast® for the identification of pathogens associated with mastitis were evaluated based on comparing Accumast® results with those from standard laboratory culture and results from 16S rRNA sequencing, which were considered the gold standards for comparison in study 1 and 2, respectively. Samples considered contaminated either by the reference laboratory (n = 3) or Accumast® (n = 6) were not included in the analysis. Results are presented as parameter estimates and 95% confidence intervals. Confidence intervals were calculated based on the standard error obtained from a binomial distribution following the formulas: SE= $\frac{\sqrt{p(1-\mathrm{p})}}{n}$ and CI = estimate ± 1.96 × SE.

Study 1: Standard Laboratory Culture as Gold Standard

Sensitivity, specificity, positive predictive value (**PPV**), and negative predictive value (**NPV**) were calculated based on true positives, true negatives, false positives and false negatives as stated by (Dohoo I, 2009) comparing results from on-farm culture of milk samples collected between April and July 2014 and reference laboratory results using the same milk samples. In addition, accuracy was calculated by dividing the number of true positives and true negatives by the total number of tests. The simple Cohen's kappa coefficient (κ) was calculated using the FREQ procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC). This parameter assumes that the two response variables (on-farm culture system and gold standard) are independent ratings, and the coefficient equals 1 when there is complete agreement between the two tests. The null hypothesis for this test is that if agreement happens due to chance the Kappa coefficient is equal to zero. Under this null hypothesis, *P*-values associated with this test equal or smaller than 0.05 were considered significant.

Study 2: 16S rRNA Sequencing as Gold Standard

Because only positive results from Accumast® plates were analyzed by 16S rRNA sequencing, the PPV and the simple Cohen's kappa coefficient between sequencing and Accumast® were assessed as previously described. Interpretation of Cohen's kappa coefficient was applied following description in (Dohoo I, 2009). A Kappa coefficient between 0.81 and 1.0 corresponded to almost perfect agreement, 0.61 to 0.80 represented substantial agreement, an estimate between 0.41 and 0.60 was considered moderate agreement and a value of 0.21 to 0.40 denoted fair agreements.

RESULTS

Study 1: Prevalence of Milk Pathogens Associated with Clinical Mastitis

The prevalence reported herein was calculated based on results from standard laboratory culture which was considered the gold standard and is known as the true prevalence. The most prevalent pathogens in the milk of cows diagnosed with clinical mastitis according to results from QMPS standard laboratory culture were *S. uberis*, *Streptococcus* sp., and *E. coli* (**Table 2.1**). Only a small proportion of quarters were diagnosed with mixed infections and 31.2% of milk samples did not result in growth of significant organisms. Three samples were characterized as contaminated by the reference laboratory and 6 samples were characterized as contaminated based on Accumast® and were excluded from subsequent analyses. Detailed information is provided in the supplementary tables 2.S1 and 2.S2.

Study 1: Test Characteristics of Accumast® Plates for Identification of Milk Pathogens Compared to Standard Laboratory Culture

The overall sensitivity, specificity, PPV, NPV, accuracy, and κ coefficient of Accumast® for identification of mastitis associated pathogens are presented on (**Table 2.2**). Among the Gram-negative bacteria observed in milk samples, the sensitivity and PPV were smaller for the detection of other Gram-negatives compared with *E. coli* and *Pseudomonas* sp. (**Table 2.3**). The overall sensitivity, specificity, and accuracy for detection of *Staphylococcus* sp. were 78.4%, 94.9%, and 93.8%, respectively (**Table 2.4**). Nevertheless, the accuracy for the identification of *S. aureus* was greater than for other *Staphylococcus* sp. Additionally, overall sensitivity, specificity and accuracy for identification of bacteria belonging to the *streptococci* group was high and the Cohen's kappa coefficient among Accumast® and standard laboratory culture for this bacterial group was denoted substantial.

Study 2: Test Characteristics of Accumast® Plates for Identification of Milk Pathogens Compared to 16S rRNA Gene Sequencing

Results from 16S rRNA gene sequencing confirmed the precision of Accumast® plates for identification of milk pathogens associated with clinical mastitis in dairy cows (**Table 2.5**). Cohen's kappa coefficient was above 85% for all bacterial groups evaluated. Likewise, PPV was above 88% across all groups.

Table 2.1. Prevalence of pathogens associated with clinical mastitis.

Bacteria identified by laboratory culture[1]	Number	Prevalence, %
Streptococcus uberis	134	24.9
Streptococcus sp.	56	10.4
Escherichia coli	49	9.1
Streptococcus dysgalactiae	40	7.8
Staphylococcus sp.	28	5.2
Klebsiella sp.	16	3.0
Mixed infection	14	2.2
Trueperella pyogenes	10	1.9
Staphylococcus aureus	7	1.3
Enterococcus sp.	7	1.3
Gram-negative bacilli	5	0.9
Pseudomonas sp.	1	0.2
No growth	168	31.2
Contamination	3	0.6
Total	538	100

[1]Results from standard laboratory culture performed by the Quality Milk Production Services laboratory at Cornell University (Ithaca, NY).

Table 2.2. Overall test characteristics of selective chromogenic culture plates to identify bacteria associated with clinical mastitis determined by standard laboratory culture.

Parameter	Accumast®	95% Confidence Interval
Number of Tests	529	
True Prevalence, % (n/n)	66.2 (350/529)	(66.0 – 66.3)
Sensitivity, %	82.3	(82.1 – 82.5)
Specificity, %	89.9	(89.6 – 90.3)
PPV[1], %	94.1	(94.0 – 94.3)
NPV[2], %	72.2	(71.8 – 72.6)
Accuracy, %	84.9	(84.7 – 85.0)
κ [3], %	0.68	(0.61 – 0.74)
κ *P*-value	<0.0001	

Each milk sample was cultured for identification of bacteria associated with clinical mastitis and culture results from the reference laboratory were considered the gold standard. Each plate was capable of identifying Gram-negative bacteria (*E. coli*, *Pseudomonas* sp., and other Gram-negatives), *Staphylococcus* sp. (*S. aureus* and *Staphylococcus* sp.), and *Streptococcus* sp. Only sections with more than five colonies were considered positive. Samples considered contaminated in either standard culture (n = 3) or on-farm culture (n = 6) were not included in the analysis (n = 9). Plates with no bacterial growth (n = 168) were considered in all calculations.

[1] Positive predictive value.

[2] Negative predictive value.

[3] Cohen's kappa coefficient. $\kappa \leq 0$ denotes poor agreement; 0.01 to 0.20 denotes slight agreement; 0.21 to 0.40 denotes fair agreement; 0.41 to 0.60 denotes moderate agreement; 0.61 to 0.80 denotes substantial agreement and 0.81 to 1.00 denotes almost perfect agreement.

Table 2.3. Test characteristics of Accumast® plates to identify Gram-negative bacteria associated with clinical mastitis determined by standard laboratory culture.

	Plate results			
Parameter	Overall Gram-negative	*E. coli*	*Pseudomonas* sp.	Other Gram-negatives
True Prevalence, % (CI[1]) n/n	14.4 (14.2 – 14.5) 76/529	9.8 (9.7 – 9.9) 52/529	0.2 (0.2 – 0.2) 1/529	4.3 (4.3 – 4.4) 23/529
Sensitivity, % (CI)	81.6 (80.6 – 82.6)	75.0 (73.4 – 76.6)	100.0 (100.0 – 100.0)	52.2 (47.9 – 56.4)
Specificity, % (CI)	98.9 (98.9 – 98.9)	97.9 (97.8 – 98.0)	99.8 (99.8 – 99.8)	99.2 (99.2 – 99.2)
PPV[2], % (CI)	92.5 (91.8 – 93.3)	79.6 (78.0 – 81.2)	50.0 (1.0 – 99.0)	75.0 (69.7 – 80.3)
NPV[3], % (CI)	97.0 (96.9 – 97.0)	97.3 (97.2 – 97.4)	100.0 (100.0 – 100.0)	97.9 (97.8 – 97.9)
Accuracy, % (CI)	96.4 (96.3 – 96.5)	95.7 (95.6 – 95.7)	99.8 (99.8 – 99.8)	97.2 (97.1 – 97.2)
κ[4], % (CI)	0.84 (0.77 – 0.91)	0.74 (0.65 – 0.84)	0.66 (0.04 – 1.0)	0.60 (0.41 – 0.78)
κ *P*-value	<0.0001	<0.0001	<0.0001	<0.0001

Each milk sample was cultured for identification of bacteria associated with clinical mastitis and culture results from the reference laboratory were considered the gold standard. Only sections with more than five colonies were considered positive. Samples considered contaminated in either standard culture (n = 3) or on-farm culture (n = 6) were not included in the analysis (n = 9). Plates with no bacterial growth (n = 168) were considered in all columns. Pure and mixed cultures with the species of interest were combined. Growth on Gram-negative section regardless of color of colonies was considered positive for overall calculations. Only correct identification of bacterial group based on color was considered positive for within group calculations.

[1] 95% confidence interval.

[2] Positive predictive value.

[3] Negative predictive value.

[4] Cohen's kappa coefficient. $\kappa \leq 0$ denotes poor agreement; 0.01 to 0.20 denotes slight agreement; 0.21 to 0.40 denotes fair agreement; 0.41 to 0.60 denotes moderate agreement; 0.61 to 0.80 denotes substantial agreement and 0.81 to 1.00 denotes almost perfect agreement.

Table 2.4. Test characteristics of Accumast® to identify Gram-positive bacteria associated with clinical mastitis determined by standard laboratory culture.

Parameter	Plate results			
	Overall Gram-positive *Streptococcus*	Overall Gram-positive *Staphylococcus*	*S. aureus*	*Staphylococcus* sp.
True Prevalence, % (CI[1]) n/n	47.1 (46.9 – 47.3) 249/529	7.0 (6.9 – 7.1) 37/529	1.3 (1.3 – 1.4) 7/529	5.7 (5.6 – 5.8) 30/529
Sensitivity, % (CI)	90.0 (89.7 – 92.7)	78.4 (76.2 – 80.6)	100.0 (100.0 – 100.0)	70.0 (67.0 – 73.0)
Specificity, % (CI)	92.9 (92.7 – 93.0)	94.9 (94.8 – 95.0)	99.8 (99.8 – 99.8)	95.0 (94.9 – 95.1)
PPV[2], % (CI)	91.8 (91.6 – 92.0)	53.7 (51.9 – 55.5)	87.5 (79.4 – 95.6)	45.7 (43.5 – 47.8)
NPV[3], % (CI)	91.2 (91.0 – 91.4)	98.3 (98.3 – 98.4)	100.0 (100.0 – 100.0)	98.1 (98.1 – 98.2)
Accuracy, % (CI)	91.5 (91.4 – 91.6)	93.8 (93.7 – 93.9)	99.8 (99.8 – 99.8)	93.6 (93.5 – 93.7)
κ[4], % (CI)	0.82 (0.78 – 0.87)	0.60 (0.48 – 0.72)	0.93 (0.80 – 1.00)	0.52 (0.37 – 0.66)
κ *P*-value	<0.0001	<0.0001	<0.0001	<0.0001

Each milk sample was cultured for identification of bacteria associated with clinical mastitis and culture results from the reference laboratory were considered the gold standard. Only sections with more than five colonies were considered positive. Samples considered contaminated in either standard culture (n = 3) or on-farm culture (n = 6) were not included in the analysis (n = 9). Plates with no bacterial growth (n = 168) were considered in all columns. Pure and mixed cultures with the species of interest were combined. Growth on Gram-positive section regardless of color of colonies was considered positive for overall calculations. Only correct identification of bacterial group based on color was considered positive for within group calculations.

[1] 95% confidence interval.

[2] Positive predictive value.

[3] Negative predictive value.

[4] Cohen's kappa coefficient. $\kappa \leq 0$ denotes poor agreement; 0.01 to 0.20 denotes slight agreement; 0.21 to 0.40 denotes fair agreement; 0.41 to 0.60 denotes moderate agreement; 0.61 to 0.80 denotes substantial agreement and 0.81 to 1.00 denotes almost perfect agreement.

Table 2.5. Test characteristics Accumast® plates to identify bacteria associated with clinical mastitis determined by 16S rRNA sequencing.

	Parameter			
Bacterial group	Tests, (n/n)	PPV[1], % (CI[2])	κ [3], %	κ *P*-value
Overall	214	95.3 (95.1 – 95.5)	0.89 (0.83 – 0.95)	<0.0001
Escherichia	30/214	96.7 (95.5 – 97.8)	0.85 (0.76 – 0.95)	<0.0001
Enterococcus	3/214	100.0 (100.0 – 100.0)	1.000 (1.0 – 1.00)	<0.0001
Other Gram-negatives	23/214	100.0 (100.0 – 100.0)	0.95 (0.88 –1.00)	<0.0001
Staphylococcus	17/214	88.2 (84.5 – 91.9)	0.93 (0.83 – 1.00)	<0.0001
Streptococcus	141/214	95.0 (94.7 – 95.3)	0.91 (0.86 – 0.97)	<0.0001

Isolates from cases of clinical mastitis cultured using Accumast® plates from October 2014 to December 2014 were subjected to 16S rRNA gene sequencing for genus level determination of bacterial growth. Only positive results were available for comparison, therefore only PPV and the Cohen's kappa coefficient between 16S rRNA sequencing and Accumast® were calculated.

[1] Positive predictive value.

[2] 95% confidence interval.

[3] Cohen's kappa coefficient. $\kappa \leq 0$ denotes poor agreement; 0.01 to 0.20 denotes slight agreement; 0.21 to 0.40 denotes fair agreement; 0.41 to 0.60 denotes moderate agreement; 0.61 to 0.80 denotes substantial agreement and 0.81 to 1.00 denotes almost perfect agreement.

Figure 2.1. Visual assessment of Gram-positive and Gram-negative bacterial growth on Accumast® plates performed in laboratory**.** Pictures were taken onto dark background. Plate without bacteria (panel A), *Staphylococcus aureus* (panel B), *Staphylococcus epidermidis* (panel C), *Staphylococcus chromogenes* (panel D), *Streptococcus agalactiae* (panel E), *Streptococcus dysgalactiae* (panel F), *Streptococcus uberis* (panel G), *Enterococcus faecalis* (panel H) *Escherichia coli* (panel I)*, *Klebsiella oxytoca* (panel J)*, and *Pseudomonas aeruginosa* (panel K). *Pictures were taken onto light background.

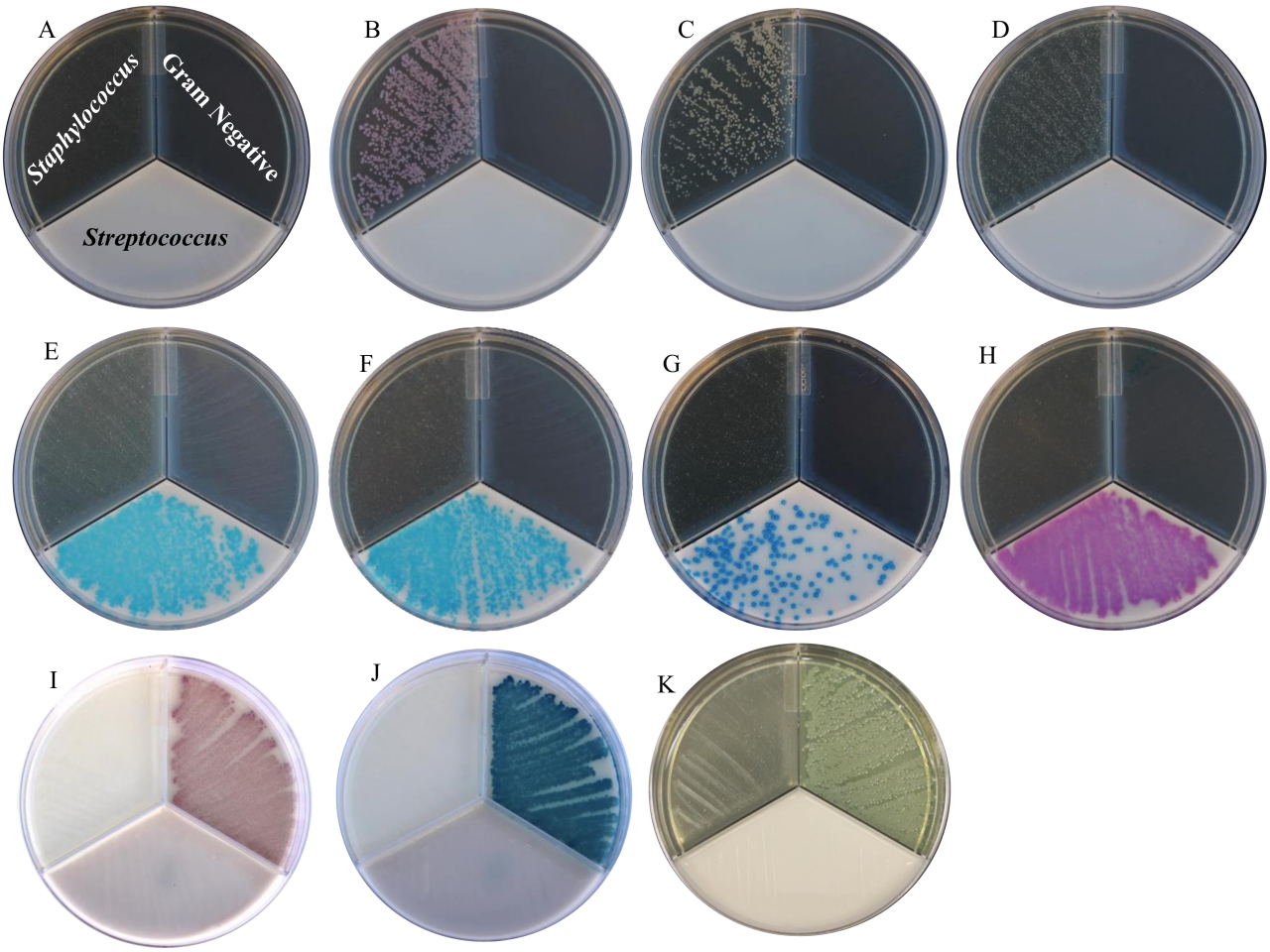
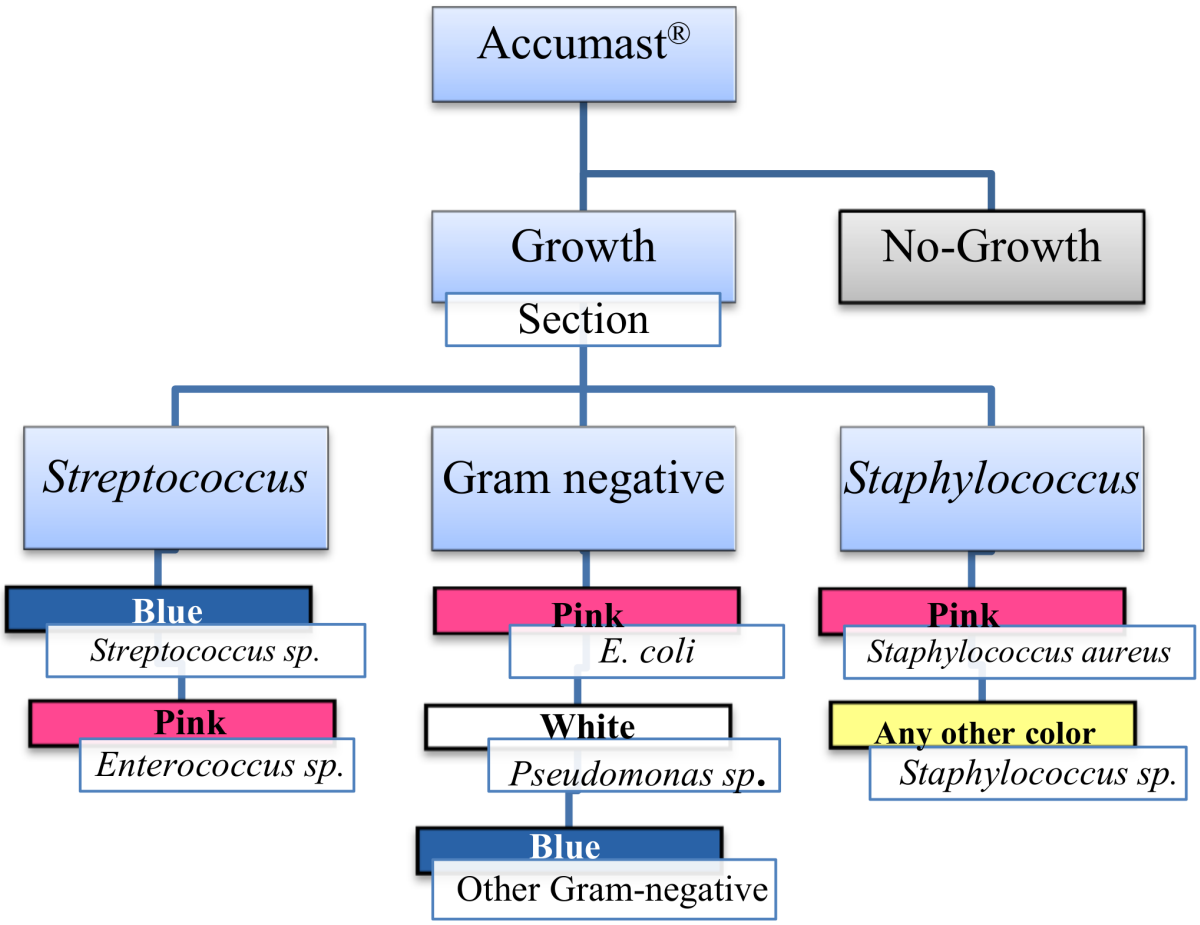


Figure 2.2. Flow-chart for on-farm diagnosis of mastitis pathogens based on Accumast®.



DISCUSSION

Knowing the etiology of mammary infections is extremely valuable for the development of strategies to control mastitis (Cha et al., 2014). Selective treatment of cows diagnosed with clinical mastitis present major advantages to dairy herds including smaller costs associated with antimicrobials, reduction in the number of animals managed in the hospital pen, less discarded milk, and a potential reduction in the rate of development of antibiotic resistance in livestock (Lago et al., 2011a, Oliver and Murinda, 2012). Targeted therapy relies on rapid and accurate identification of milk pathogens (McDougall et al., 2007, Cha et al., 2014, Hertl et al., 2014a, Royster and Wagner, 2015). The on-farm culture system evaluated in this study was designed to enable farm personnel to identify major bacteria associated with clinical mastitis in dairy cows in a straightforward manner. The spectrum of microorganisms identifiable using Accumast® encompasses both Gram-positive (i.e. *streptococci*, *staphylococci*, and *S. aureus*) and Gram-negative pathogens (i.e. *E. coli*, *Pseudomonas* sp. and other Gram-negative pathogens). These bacteria have been reported to represent 80% and 100% of all milk pathogens isolated from mastitic cows in the states of New York (Hertl et al., 2014b) and Wisconsin (Oliveira and Ruegg, 2014). When compared to a referral laboratory, the overall accuracy of Accumast® to identify milk pathogens was 84.9%; with individual accuracies of 96.4%, 93.8%, and 91.5% for Gram-negative bacteria, *staphylococci*, and *streptococci*, respectively. Additionally, when compared to 16S rRNA sequencing results, the overall positive predictive value of Accumast® was 95.3% and the Cohen's kappa coefficient was 0.89, which according to (Dohoo I, 2009), is considered almost perfect agreement. The present results support the use of this culture system for on-farm identification of pathogens associated with clinical mastitis, for decision making in targeted treatment protocols, and for pathogen prevalence surveillance. It is important to acknowledge

that certain pathogens were present in very low prevalence in our study (e. g. *S. aureus)* and further research should be conducted to validate current findings.

Major advances in the control of contagious pathogens implicated in mastitis have been accomplished through improvement of milking hygiene and management practices (Bushnell, 1984, Hogan et al., 1989, Hillerton and Berry, 2003). However, mammary infections with *S. aureus* remain a concern in dairy herds and require constant surveillance, aggressive antibiotic therapy, and segregation or culling of infected cows (Zecconi et al., 2005, Boss et al., 2011). On-farm culture systems have been used successfully to characterize bacteria present in the milk of mastitic cows as Gram-positive, Gram-negative, or no growth after 24 h to 32 h of incubation. Nonetheless, inconsistent results were observed when classification at the genus and/or species level was attempted by readers lacking extensive microbiology training. For instance, the sensitivity and specificity of University of Minnesota Triplate for identification of *S. aureus* by four readers with limited microbiology training ranged from 43.2% to 59.1% and 93.8% to 95.9%, respectively (McCarron et al., 2009a). In another study in which two readers without extensive microbiology training used the same system to identify *S. aureus*, sensitivity ranged from 52% to 78% and specificity from 92% to 98% (Royster et al., 2014). Likewise, other investigators used a different triplate for identification of Gram-negative bacteria, *staphylococci*, and other Gram-positive bacteria and achieved sensitivity and specificity for identification of *S. aureus* of 65% and 94% (Viora et al., 2014). In the present study, the use of Accumast® resulted in sensitivity and specificity of 100% and 99.8% for identification of *S. aureus* under field conditions when compared to standard laboratory culture. These results were confirmed by our *in vitro* studies, in which ATCC strains of *S. aureus*, *S. chromogenes*, and *S. epidermidis* were plated in all sections of the Accumast® plate and the growth of *S. aureus* colonies were of pink

coloration and markedly different from the other two species of *staphylococci*. The high predictive value of this system compared with other on-farm culture systems can be partially explained by the clear difference in color between *S. aureus* and other *staphylococci* in Accumast®, as opposed to the need of identification of more subtle differences in colony characteristics and β-hemolysis in other methods (McCarron et al., 2009a). In fact, hemolysin production by *S. aureus* has been shown to be variable (Boerlin et al., 2003). It is important to acknowledge that this particular species comprised only 1.3% of the pathogen prevalence in the study sample and further research is needed to confirm the findings presented here. Additionally, when compared to 16S rRNA, Accumast® presented PPV and a Cohen's kappa coefficient of 0.89, considered almost perfect agreement (Dohoo I, 2009) for all bacterial groups evaluated. Unfortunately the use of this technique as the gold standard does not allow for calculation of NPV, Sensitivity and Specificity, since only positive results from the test being evaluated are available for comparison. However, as mentioned before, bacterial characteristics often used for species identification such as hemolysin production have been shown to be variable. This inconsistency is not an issue when targeted sequencing of the 16S rRNA gene is performed, once this gene has been proved to be highly conserved among different phenotypes of the same bacterial species.

The specificity and NPV of Accumast® for identification of *Staphylococcus* sp. were above 95%; however, its sensitivity and PPV were much smaller compared to that of *S. aureus*, which can be a limitation of the on-farm culture system evaluated here. Similarly, previous reports also observed a reduced sensitivity for discrimination between *S. aureus* and other *staphylococci* (McCarron et al., 2009a, Royster et al., 2014). Although not ideal, the reduced capacity of on-farm culture system to identify other *Staphylococcus* sp. has a smaller impact on

its applicability in dairy herds when compared to the capacity of correctly identifying *S. aureus*. Coagulase-negative *staphylococci* (**CNS**) are considered pathogens of minor importance compared with other bacteria while *S. aureus* remain a major concern because of its contagious behavior (Pyorala and Taponen, 2009, Boss et al., 2016, da Costa et al., 2016). In fact, CNS has been associated with subclinical or moderate clinical mastitis and with high spontaneous cure rates (McDougall, 1998, Wilson et al., 1999, Taponen et al., 2006, Tomazi et al., 2015). Other studies argue that CNS are the main species responsible for mammary gland infection in ruminants, causing significant changes in milk metabolites that play an important role in the quality of dairy products (Silanikove et al., 2014a, b). Regardless of the effect of CNS in mammary infections, milk yield, and downstream milk quality, we acknowledge that improvement on the capability of correctly diagnosing *Staphylococcus* sp. would be advantageous for the on-farm culture system presented here.

Environmental *streptococci* were the most prevalent bacteria isolated in the present study, which is in agreement with previous studies (Olde Riekerink et al., 2008, McCarron et al., 2009a, b, Oliveira and Ruegg, 2014). Among environmental pathogens, *S. uberis* plays an important role in intramammary infections because of its invasive comportment and association with recurring infections (Hillerton and Berry, 2003, McDougall et al., 2007, Abureema et al., 2014). The ability to identify cows infected with *Streptococcus* sp. is critical for health management in dairy herds as these infections respond well to commercially available intramammary antimicrobials (Roberson, 2012). The use of Accumast® resulted in high overall sensitivity and specificity for identification of environmental *streptococci* independent of the species characterized by standard laboratory culture. The sensitivity and overall accuracy of Accumast® plates for identification of *Streptococcus* sp. were comparable to the ones reported for the methods evaluated by McCarron

et al. (2009) and Royster et al. (2014). Although the differentiation among *Streptococcus* species using Accumast® was not attempted, visual inspection of ATCC cultures indicate a lighter blue associated with *S. agalactiae* and *S. dysgalactiae* compared with that of *S. uberis*. Similar patterns were also observed during the field study; however, such nuances in tonality were not recorded and further research is necessary to evaluate the predictive values of Accumast® for differentiation of *Streptococcus* sp. in the species level.

Bovine mastitis associated with *E. coli* has been reported to have high self-cure rates. In an elegant review, Suojala et al., (2013) compiled data from studies that evaluated the treatment of *E. coli* caused bovine mastitis and concluded that intramammary antibiotic therapy should not be recommended in mild and moderate cases. For this reason, identifying mastitic cows infected primarily with *E. coli* is a critical step towards reducing the use of antibiotics in dairy herds. On the other hand, results reported by Schukken et al., (2011) support the use of intramammary antimicrobials for treatment of mild and moderate cases of Gram-negative mastitis. In that study, a randomized clinical trial was conducted and revealed a significant increase in the odds of clinical and bacteriological cure in treated animals when compared to non-treated controls. The accuracy of Accumast® to identify *E. coli* was 95.7% compared with standard laboratory culture, with sensitivity and specificity of 75.0% and 97.9%, respectively which are greater than the results from Viora et al. that reported a sensitivity and specificity of 67% and 92% for identification of *E. coli* in the milk of mastitic cows using a triplate containing selective culture media. Nevertheless, the use of systemic antimicrobial therapy combined with support therapy and anti-inflammatory drugs are recommended in severe cases of *E. coli* mastitis due to the high indices of bacteremia experienced in cows undergoing this presentation of the disease (Wenz et al., 2001, Erskine et al., 2002).

CONCLUSIONS

The on-farm culture system evaluated in the present study is suitable for use under field conditions and presented substantial overall accuracy for detection of common mastitis pathogens, which was confirmed by 16S rRNA gene sequencing. Accumast® provides a unique approach for on-farm identification of mastitis associated pathogens, mostly through its straightforward color-based classification of bacteria. Identification of bacteria based on color allows for easy interpretation by individuals with limited microbiological training; thus, providing the basis for selective antimicrobial therapy of mastitic cows based on causal microorganisms. Further research is warranted to evaluate test characteristics of Accumast® between multiple study sites with distinct mastitis pathogens prevalence profiles and among readers without microbiology experience.

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AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: RCB. Performed the experiments: EKG DHD. Analyzed the data: EKG RSB RCB. Wrote the paper: EKG RSB RCB.

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SUPPLEMENTARY INFORMATION

Table 2.S1. Results from Study 1: Standard laboratory culture was considered the gold standard

Accumast result	False Negatives	False Positives	True Negatives	True Positives
E. coli	4	2	0	23
E. coli, Staphylococcus	0	0	0	2
E. coli, Streptococcus	1	0	0	17
Enterococcus	1	0	0	0
Enterococcus, Staphylococcus	1	0	0	0
Negative	42	0	160	0
Other Gram-	1	1	0	9
Other Gram-, *Staphylococcus*	0	0	0	1
Other Gram-, *Streptococcus*	0	0	0	4
Staphylococcus	3	9	0	18
Pseudomonas	1	0	0	1
S. aureus	0	1	0	7
Streptococcus	8	5	1	194
Streptococcus, Staphylococcus	0	0	0	12
Total	62	18	161	288

Table 2.S2. Results from Study 1: Distribution of results between on-farm culture system and standard laboratory culture

	Standard laboratory culture performed by the Quality Milk Production Services laboratory																				
Accumast result[1]	*A pyogenes*	*E. coli*	*Enterococcus*	G- *bacillus*	*Klebsiella*	*Negative*	*Pseudomonas*	*S. aureus*	*Staph* sp	*S. dysgalactiae*	*S.dysgalactiae, S. uberis*	*Strep sp*	*Strep* sp, *E coli*	*Strep sp, G- bacillus*	*Strep sp, Klebsiella*	*Strep sp, Staph sp*	*Strep sp, Strep dysgalactiae*	*Strep uberis*	*Strep uberis, E coli*	*Strep uberis, G- bacillus*	Total
E. coli	0	23	0	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	29
E. coli Staphylococcus	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
E. coli Streptococcus	0	8	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	5	1	18
Enterococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Enterococcus Staphylococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Negative	9	11	0	1	1	151	0	0	8	5	0	5	0	0	0	0	0	11	0	0	202
Other Gram-	0	1	0	0	9	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
Other Gram- *Staphylococcus*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Other Gram- *Streptococcus*	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	4
Staphylococcus	0	0	0	0	0	9	0	0	17	0	0	3	0	0	0	1	0	0	0	0	30
Pseudomonas	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2
S. aureus	0	0	0	0	0	0	0	7	1	0	0	0	0	0	0	0	0	0	0	0	8
Streptococcus	1	1	7	0	0	5	0	0	0	31	1	45	0	0	0	0	1	116	0	0	208
Streptococcus Staphylococcus	0	0	0	0	0	0	0	0	1	3	0	3	0	0	0	1	0	4	0	0	12
All	10	46	7	5	15	168	1	7	28	39	1	56	1	1	1	2	1	134	5	1	529

[1]Only sections with more than five colonies were considered positive.

[2] Contaminated samples in either standard culture (n = 3) or on-farm culture (n = 6) were not included in the analysis (n = 9).

CHAPTER 3

EFFECTS OF INJECTABLE TRACE MINERAL SUPPLEMENTATION IN LACTATING DAIRY COWS WITH ELEVATED SOMATIC CELL COUNTS

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ABSTRACT

Objectives of this clinical trial were to evaluate the effects of injectable trace mineral supplementation (ITMS) on somatic cell count (SCC), linear score (LS), milk yield, milk fat and protein contents, subclinical mastitis cure, and incidence of clinical mastitis in cows with elevated SCC. Holstein cows from a commercial dairy farm in New York were evaluated for subclinical mastitis, defined as SCC $\geq 200 \times 10^3$ cells/mL on the test day preceding enrollment. Cows with a history of treatment for clinical mastitis in the current lactation and those pregnant for more than 150 d were not eligible for enrollment. Cows fitting inclusion criteria were randomly allocated to one of two treatment groups. Cows assigned to ITMS (n = 306) received one subcutaneous injection containing zinc (300 mg), manganese (50 mg), selenium (25 mg), and copper (75 mg) at enrollment (d 0). Control cows (CTRL; n = 314) received one subcutaneous injection of sterile saline solution. Following treatment, visual assessment of milk was performed daily and cows with abnormal milk (i.e. presence of flakes, clots, or serous milk) were diagnosed with clinical mastitis (CM). Chronic clinical mastitis (CCM) was defined as cows with three or more cases of CM. Milk yield, milk fat and protein contents, SCC, and LS were evaluated once monthly. Additionally, randomly selected animals were sampled to test serum concentrations of selected minerals on d 0 and d 30 (n = 30 cows/treatment). Treatment did not affect serum concentrations of calcium, magnesium, phosphorus, potassium, copper, iron, manganese, selenium, and zinc on d 30. Injectable supplementation with trace minerals did not improve overall cure of subclinical mastitis (CTRL = 42.8 vs. ITMS = 46.5%), although a tendency was observed in cows with three or more lactations (CTRL = 27.1 vs. ITMS = 40.0%). Supplementation did not reduce treatment incidence of CM (CTRL = 48.2 vs. ITMS = 41.7%); however, it tended to reduce the proportion of cows diagnosed with CCM (CTRL = 16.9 vs.

ITMS = 12.0%) particularly among first lactation cows (CTRL = 18.4 vs. ITMS = 7.6%). Cure of subclinical mastitis was associated with higher serum concentrations of phosphorus and selenium on d 30. Supplementing trace minerals to cows with elevated SCC had no effect on milk yield, milk fat and protein contents, SCC, and LS.

Keywords: mastitis, udder health, zinc, manganese, selenium, copper

INTRODUCTION

Mastitis remains a highly prevalent disease in dairy herds and leads to major economic losses to the dairy industry worldwide, with each clinical case being estimated to cost between $179 and $488 (Bar et al., 2008, Hagnestam-Nielsen and Ostergaard, 2009). Despite the absence of clinical signs and visible changes in milk, subclinical mastitis, commonly diagnosed through an increase in somatic cell count (**SCC**), is also extremely costly to dairy herds. Cows with subclinical mastitis produce up to 4.6 kg/d less milk compared with healthy herdmates (Green et al., 2006, Halasa et al., 2009, Boland et al., 2013), represent a reservoir of infection for other cows in the herd (White et al., 2006) and are at a greater risk of being culled or developing clinical mastitis (Deluyker et al., 1993, Barlow et al., 2009).

Ensuring adequate trace mineral availability is a potential strategy to reduce the impact of high SCC. Trace minerals are critical for proper immune response and play an important role in udder health. Selenium is a component of the antioxidant enzyme glutathione peroxidase and selenium-deficient dairy cows have impaired neutrophil function in blood (Hogan et al., 1990) and milk (Grasso et al., 1990). Elevated concentrations of selenium in blood are associated with reduced prevalence of intramammary infection and reduced bulk tank SCC (Erskine et al., 1987,

Weiss et al., 1990, Kommisrud et al., 2005). Additionally, copper and zinc are essential for bovine superoxide dismutases, enzymes responsible for catalyzing superoxide radicals into oxygen and hydrogen peroxide. Dietary copper has been associated with neutrophil killing activity in dairy cattle (Torre et al., 1996). Dairy heifers fed peripartum diets supplemented with copper had lower bacterial counts and SCCs in milk following an intramammary challenge with *E. coli* compared with counterparts fed a diet with marginal copper content (Scaletti et al., 2003).

Although diets formulated to meet the NRC recommendations are expected to provide adequate amounts of trace minerals, nutrient interactions within the rumen, reduced appetite, restricted access to the feed bunk, and absorptive disorders might limit their intake or bioavailability. Conversely, two subcutaneous injections of a mineral solution in prepartum dairy cows sustained elevated serum concentrations of copper, selenium and zinc for approximately 14 d following the last treatment (Bicalho et al., 2014). Interestingly, cows diagnosed with metritis had lower selenium and zinc concentrations in serum during early postpartum compared with healthy herdmates (Bicalho et al., 2014). Previous results indicate that the use of the same injectable trace mineral supplementation during the transition period improved udder health in dairy cows. Supplementation performed at 230 and 260 d of gestation and at 35 DIM reduced the incidence of clinical mastitis and SCC in multiparous cows (Machado et al., 2013). These results suggest that cows undergoing stress caused by infectious diseases have lower levels of selenium and zinc, and that restoring endogenous pools might be associated with cure. The effects of injectable trace minerals in cows previously diagnosed with subclinical mastitis remains to be determined. For that reason, determining to what extent each mineral is affected in cows with elevated SCC and whether or not increasing circulating concentrations of trace minerals is

associated with reduction of SCC is expected to provide valuable information for the treatment of subclinical mastitis.

The hypothesis of the present study was that injectable trace mineral supplementation would improve udder health in dairy cows diagnosed with subclinical mastitis. Objectives were: 1) to evaluate the effects of an injectable trace mineral supplementation containing selenium, copper, zinc, and manganese on SCC, linear score (**LS**), milk yield and composition, incidence of clinical mastitis, and subclinical mastitis cure in cows previously diagnosed with subclinical mastitis and 2) to investigate the extent in which minerals are affected in dairy cows with elevated SCC.

MATERIALS AND METHODS

Animal Care Statement

The experimental procedures were carried out according to the research protocol 2013-0056, that was reviewed and approved by the Institutional Animal Care and use Committee of the Cornell University.

Farm and Management

This study was conducted on a single commercial dairy farm located in upstate New York. During the experimental period, the farm milked approximately 3,000 cows. In total, 620 Holstein cows were enrolled in the study from January 10th to April 8th, 2014. Primiparous and multiparous cows were housed separately in free-stall barns with concrete stalls covered with mattresses and bedded with manure solids. Cows were fed a total mixed ration to meet or exceed the nutrient requirements of a 650 kg lactating Holstein cow producing 45 kg/d of milk with

3.5% fat and 3.2% true protein when DMI is 25 kg/d (NRC, 2001). Fifteen feed samples were collected at the feed bunk, pooled, and submitted for analysis at the DairyOne Laboratory (DairyOne Laboratories, Ithaca, NY). Diet composition is presented in (**Table 3.1**). Cows were milked thrice daily in a double-52 milking parlor. The farm participated on DHIA and all lactating cows were tested for SCC and milk composition once monthly.

Enrollment Criteria and Case Definitions

Lactating dairy cows that had SCC $\geq 200 \times 10^3$ cells/mL in the closest DHIA test to enrollment, were less than 150 d pregnant, and had not received intramammary antibiotic therapy in the current lactation were eligible for enrollment. Milk sampling was performed by DHIA personnel and assayed using flow cytometry (DairyOne Laboratories, Ithaca, NY). Subclinical mastitis was defined as SCC $\geq 200 \times 10^3$ cells/mL and subclinical mastitis cure was defined for cows with SCC $< 200 \times 10^3$ cells/mL on the first monthly DHIA test following enrollment. Clinical mastitis was defined based on visual evaluation of milk in a forestripping test performed every milking by trained farm personnel. Cows with presence of flakes, clots, or serous milk were diagnosed with clinical mastitis independent of systemic illness and signs of inflammation in the mammary gland. Cows diagnosed with clinical mastitis were moved to the hospital pen and treated according to the study site's standard procedures. Chronic clinical mastitis was defined as cows with three or more cases of clinical mastitis after enrollment.

Experimental Design and Treatments

This study was conducted as a complete randomized design and cows were enrolled on a monthly basis. A total of 620 cows were included in the study, from which 240 (38.7%) were in the first lactation, 215 (34.7%) were in the second lactation, and 165 (26.6%) were in the third or

later lactations. Cows diagnosed with subclinical mastitis were randomly allocated into one of two treatments based on a list generated using the RAND function of Excel (Microsoft, Redmond, WA). On the day of enrollment, henceforth defined as study d 0, cows assigned to receive injectable trace mineral supplementation (**ITMS**; n = 306) were treated once subcutaneously in the ischiorectal fossa with 5ml of an injectable trace mineral solution containing zinc (300 mg), manganese (50 mg), selenium (25 mg), and copper (75 mg) (Multimin 90, Multimin North America Inc., Fort Collins, CO). Cows assigned to the control group (**CTRL**; n = 314) received a subcutaneous injection of sterile saline solution of the same volume (5 mL).

Sampling and Data Collection

Blood samples were collected from a randomly selected subset of cows (n = 30/treatment) before injection of trace mineral supplementation on d 0 and again on d 30 for the analysis of mineral concentrations. Blood was sampled by puncture of coccygeal vessels into evacuated tubes without anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Blood tubes were placed on ice and centrifuged at 2,000 x *g* for 15 min for serum separation within 5 h of collection. Serum samples were frozen at -80ºC until assayed.

Individual milk production was measured using on-farm milk meters (ALPRO, DeLaval, Tumba, Sweden) once monthly during the DHIA test day. Milk fat and protein contents were measured using Fourier transform infrared spectroscopy (MilkoScan, Eden Prairie, MN). Somatic cell counts were determined by flow cytometry (Fossomatic FC, Eden Prairie, MN). Linear scores were calculated based on SCC as follows:

$$LS = [ln(SCC/100x10^3)/0.693147] + 3$$

Body condition score (**BCS**) was evaluated on d 0 using a 5-point scale with quarter-point increments as described by (Edmonson et al., 1989) for Holstein cows.

Follow up data were retrieved from the study site's farm management software (DC305, Valley Agricultural Software, Tulare, CA). Somatic cell count, milk production, milk composition, and clinical mastitis events were recorded for 10 months after enrollment (follow up period) or until culling or drying off.

Analysis of Mineral Concentrations in Serum

Serum concentrations of calcium, magnesium, phosphorus, copper, iron, potassium, manganese, molybdenum, selenium, and zinc were determined by mass spectrometry (Varian 820-MS, Varian Inc., Palo Alto, CA) at the Veterinary Diagnostic Laboratory (Iowa State University, Ames, IA).

Statistical Analyses

Sample size calculation was performed using the POWER procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC). A total of 300 cows per treatment was deemed necessary to detect a 10 percentage points increase in the proportion of cows cured from subclinical mastitis with $\alpha = 0.05$ and $\beta = 0.20$.

Descriptive statistical analyses were performed using JMP version 11 (SAS Institute Inc., Cary, NC) for all following variables at enrollment: BCS, DIM, lactation number, days from sampling to treatment, milk yield, milk fat percentage, milk protein percentage, SCC, and LS.

To evaluate the effect of treatment on the binary outcomes such as subclinical mastitis cure and incidences of clinical mastitis and chronic clinical mastitis, logistic regression was

performed using the LOGISTIC and GLIMMIX procedures of SAS. The fixed effects of treatment, lactation group (1, 2, and ≥ 3 lactations), and the interaction between treatment and lactation group were included as independent variables in the statistical models. Results were presented as proportions and adjusted odds ratios (**AOR**) relative to a reference group.

Continuous outcomes were analyzed by ANOVA fitting a normal distribution. Visual assessment of the distribution of studentized residuals and Q-Q plots was performed for each dependent variable to ensure normality of residuals and homogeneity of variances. Concentrations of minerals in serum on study d 0 and 30 were analyzed in separate models using the GLIMMIX procedure of SAS. The models included the fixed effects of treatment, lactation group, and the interaction between treatment and lactation group. For analyses of mineral concentrations on d 30, data from d 0 were used as covariate. Tukey-adjusted pairwise comparisons between lactation groups were performed for mean mineral concentrations at enrollment.

To evaluate the effect of treatment in milk yield, milk fat and protein contents, SCC, and linear scores, ANOVA for repeated measures was performed using the MIXED procedure of SAS. The fixed effects of treatment, lactation group, time, and the interactions between treatment and time, treatment and lactation group, lactation group and time, and treatment, lactation group, and time were included as independent variables into the statistical models. To control for repeated measurements, cow was considered a random effect. *P*-values were adjusted using the method of Tukey for pairwise comparisons. The first-order autoregressive structure of covariance was used in all analyses because it resulted in the lowest Schwarz's Bayesian information criterion value. Results are presented as LSM ± SEM.

The effect of injectable trace mineral supplementation on enrollment to removal from the herd interval was analyzed by Kaplan-Meier survival analysis using MedCalc version 12.2.1.0 (MedCalc Software, Ostend, Belgium). Treatment was included in the statistical model as an independent variable and *P*-values were calculated using the Logrank test. For cows that were removed from the herd, right censoring was performed based on date of removal, for all other animals, censoring was done based on follow up period (date of dry-off).

For all statistical models, $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.

RESULTS

Descriptive Statistics

Body condition score, DIM, lactation number, milk yield and composition, SCC, and LS at enrollment did not differ between treatments (**Table 3.2**).

Mineral Concentrations in Serum

With the exception of calcium and manganese concentrations, which were higher in CTRL compared with ITMS cows, mineral concentrations in serum did not differ between treatments on d 0 (**Table 3.3**). Serum concentrations of molybdenum tended to be higher ($P = 0.09$) in CTRL compared with ITMS cows on d 30 (**Table 3.3**). Nevertheless, the remaining macro and trace minerals evaluated on d 30 were not affected by treatment. Initial serum concentrations of copper, phosphorus and calcium differed between lactation groups (**Table 3.3**). First lactation cows had higher ($P \leq 0.05$) circulating concentrations of calcium and phosphorus

compared with multiparous cows. In addition, copper and calcium concentrations were lower ($P \leq 0.05$) in cows with three or more lactations.

Udder Health, Cure of Subclinical Mastitis, and Incidence of Clinical Mastitis

Injectable trace mineral supplementation did not affect SCC during the follow-up period. Somatic cell count was lower ($P < 0.01$) for first lactation cows, followed by cows in the second lactation and those with three or more lactations (551 ± 43, 800 ± 50, and 1,043 ± 55 x 10^3 cells/mL, respectively). The interaction between treatment and lactation group did not affect SCC. In a similar fashion, treatment and the interaction between treatment and lactation group did not affect LS, which were also lower ($P < 0.01$) for first lactation cows, followed by cows in the second lactation and those with three or more lactations (4.0 ± 0.1, 4.6 ± 0.1, and 5.1 ± 0.1, respectively).

The overall proportion of cows cured from subclinical mastitis was 46.3% (287/620), whereas the incidences of clinical mastitis and chronic clinical mastitis were 44.4% (275/620) and 14.4% (89/620), respectively. Supplementing cows with injectable trace minerals did not improve overall cure of subclinical mastitis compared with non-supplemented controls (**Table 3.4**). However, a significant effect of parity on cure of subclinical mastitis was observed; cure of subclinical mastitis was greater for first lactation cows compared with those in the second lactation and three or more lactations ($P < 0.01$). The analysis within lactation group depicted a tendency ($P = 0.08$) for injectable trace mineral supplementation to increase subclinical mastitis cure in cows with three or more lactations. Treatment, lactation group, and their interaction did not affect the incidence of clinical mastitis (**Table 3.5**). Nevertheless, injectable trace mineral supplementation tended to reduce ($P = 0.09$) the overall incidence of chronic clinical mastitis

(**Table 3.6**). Although lactation group had no effect on the proportion of cows diagnosed with chronic clinical mastitis, the interaction between treatment and lactation group tended to affect ($P = 0.06$) the incidence of the disease. The proportion of cows diagnosed with chronic clinical mastitis during the follow-up period was reduced ($P = 0.02$) by injectable trace mineral supplementation in first lactation cows, but not in cows with two or more lactations. Based on survival analyses, treatment had no effect on interval to removal from the herd (**Figure 3.1**).

Mineral Concentrations in Serum According to Disease Status

When animals were compared according to disease status regardless of treatment group, cows that recovered from SCM within 30 d of study had higher ($P = 0.04$) concentrations of calcium and tended to have lower ($P = 0.10$) concentrations of zinc in serum at enrollment compared with herdmates that did not recover from the disease (**Table 3.7**). Cure of SCM was associated with higher ($P \leq 0.01$) serum concentrations of phosphorus and selenium on d 30. Serum concentrations of the remaining minerals did not differ between cows that recovered from SCM and herdmates that did not recover from the disease. There was no difference in serum mineral concentrations between cows that developed CM and herdmates without clinical signs of the disease (**Table 3.8**).

Milk Yield and Composition

Injectable trace mineral supplementation did not improve milk production during the follow-up period, which averaged 35.1 ± 0.5 kg for CTRL and 34.5 ± 0.5 kg for ITMS cows. Furthermore, milk yield was not affected by lactation group or the interaction between treatment and lactation group. Treatment and the interaction between treatment and lactation group had no effect on milk fat (CTRL = 3.91 ± 0.03 vs. ITMS = 3.93 ± 0.03%) and protein content (CTRL =

3.20 ± 0.02 vs. ITMS = 3.20 ± 0.02%). Milk fat content was greater ($P < 0.03$) for first as well as second lactation cows compared with those with three or more lactations (3.97 ± 0.03, 3.96 ± 0.03, and 3.82 ± 0.04%, respectively). Milk protein content was also affected by lactation group because second lactation (3.25 ± 0.02%) cows had higher ($P < 0.02$) concentrations compared with first lactation cows and those with three or more lactations (3.19 ± 0.02 and 3.15 ± 0.02%, respectively).

Table 3.1: Total Mixed Ration (TMR) analysis.

		Dry Matter
Crude Protein	%	15.6
Soluble Protein	%	52.0
Acid Detergent Fiber	%	22.4
Amylase-Treated Neutral Detergent Fiber	%	36.4
Lignin	%	2.90
Non-Fibrous Carbohydrates	%	37.0
Starch	%	27.6
Crude Fat	%	4.00
Ash	%	6.99
Total Digestible Nutrients	%	72.0
NEL	Mcal/Kg	1.67
Calcium	%	0.71
Phosphorus	%	0.42
Magnesium	%	0.24
Potassium	%	1.58
Sodium	%	0.40
Iron	mg/kg	234
Zinc	mg/kg	60.0
Copper	mg/kg	12.0
Manganese	mg/kg	40.0
Molybdenum	mg/kg	1.00
Selenium	mg/kg	0.49

Table 3.2. Descriptive statistics for cows enrolled in the study by treatment group. CTRL = cows received no trace mineral supplementation; ITMS = cows received a single injectable trace mineral supplementation containing zinc (300 mg), manganese (50 mg), selenium (25 mg), and copper (75 mg) at enrollment. Results presented as LSM ± SEM.

	Treatment		
Variable	CTRL (N=314)	ITMS (N=306)	*P*
Enrolled Animals lactation = 1, N	109	131	
Enrolled Animals lactation = 2, N	120	95	0.08
Enrolled Animals lactation ≥ 3, N	85	80	
Body condition score	3 ± 0.2	3 ± 0.2	0.52
Days in milk[1]	144.4 ± 7.1	152.0 ± 7.2	0.45
Days from sampling to Treatment	7.34 ± 0.1	7.45 ± 0.1	0.57
Milk yield[1], kg	37.8 ± 0.5	37.1 ± 0.5	0.35
Lactation = 1	33.4 ± 0.8	33.7 ± 0.7	0.77
Lactation = 2	39.4 ± 0.8	38.9 ± 0.9	0.75
Lactation ≥ 3	40.7 ± 0.9	40.0 ± 0.9	0.58
Milk fat[1], %	4.08 ± 0.04	4.00 ± 0.04	0.18
Lactation = 1	4.19 ± 0.07	4.00 ± 0.06	0.05
Lactation = 2	4.10 ± 0.08	4.08 ± 0.09	0.89
Lactation ≥ 3	3.93 ± 0.07	3.90 ± 0.07	0.73
Milk protein[1], %	3.17 ± 0.01	3.15 ± 0.02	0.48
Lactation = 1	3.18 ± 0.03	3.14 ± 0.03	0.40
Lactation = 2	3.22 ± 0.03	3.22 ± 0.03	0.96
Lactation ≥ 3	3.11 ± 0.03	3.11 ± 0.03	0.94
Somatic cell count[1], $x10^3$ cells/mL	922.0 ± 67.3	885.0 ± 68.2	0.69
Lactation = 1	852.7 ± 99.4	689.3 ± 90.6	0.22
Lactation = 2	855.1 ± 110.7	1,096.5 ± 124.5	0.14
Lactation ≥ 3	1,105.3 ± 146.2	954.0 ± 150.8	0.47
Linear score[1]	5.5 ± 0.1	5.4 ± 0.1	0.34
Lactation = 1	5.5 ± 0.1	5.2 ± 0.1	0.09
Lactation = 2	5.4 ± 0.1	5.6 ± 0.1	0.31
Lactation ≥ 3	5.7 ± 0.1	5.5 ± 0.1	0.36

[1] Evaluated at the test day preceding enrollment

Table 3.3. Mineral concentrations in serum of cows diagnosed with subclinical mastitis (SCC ≥ 200 × 10^3 cells/mL) based on the test day preceding enrollment by treatment group. CTRL = cows received no trace mineral supplementation (n = 30); ITMS = cows received a single injectable trace mineral supplementation containing zinc (300 mg), manganese (50 mg), selenium (25 mg), and copper (75 mg) at enrollment (Lactation = 1: CTRL n=18 and ITMS n=12; Lactation = 2: CTRL n=6 and ITMS n=10; Lactation ≥ 3: CTRL n=6 and ITMS n=8). Results presented as LSM ± SEM.

	Enrollment (d 0)			d 30		
Mineral	CTRL	ITMS	*P*	CTRL	ITMS	*P*
Macrominerals						
Calcium, mg/L	94.8 ± 1.1	91.8 ± 1.1	0.07	87.2 ± 0.9	87.2 ± 0.9	0.99
Lactation = 1	97.4 ± 0.9	97.2 ± 1.1	0.86	87.9 ± 0.9	87.9 ± 1.1	0.95
Lactation = 2	95.9 ± 1.9	88.6 ± 1.4	0.01	89.0 ± 1.4	84.9 ± 1.1	0.05
Lactation ≥ 3	85.5 ± 1.9	87.5 ± 1.7	0.46	82.9 ± 2.8	88.9 ± 2.4	0.14
Magnesium, mg/L	20.0 ± 0.4	19.2 ± 0.4	0.19	19.7 ± 0.4	19.4 ± 0.4	0.66
Lactation = 1	20.1 ± 0.6	19.6 ± 0.8	0.63	19.4 ± 0.6	18.3 ± 0.8	0.28
Lactation = 2	20.6 ± 0.6	18.8 ± 0.4	0.04	20.6 ± 0.7	19.5 ± 0.5	0.26
Lactation ≥ 3	18.8 ± 0.6	18.9 ± 0.5	0.86	19.4 ± 0.6	20.9 ± 0.5	0.12
Phosphorus, ppm	74.0 ± 2.4	63.4 ± 2.5	0.11	67.3 ± 1.6	64.3 ± 1.6	0.20
Lactation = 1	79.3 ± 3.1	76.4 ± 3.8	0.55	69.4 ± 2.2	67.9 ± 2.6	0.66
Lactation = 2	71.9 ± 4.0	66.6 ± 3.1	0.31	64.9 ± 2.7	60.9 ± 2.1	0.27
Lactation ≥ 3	60.1 ± 3.5	58.4 ± 3.1	0.72	62.9 ± 3.9	62.8 ± 3.4	0.99
Potassium, mg/L	179.9 ± 2.6	177.1 ± 2.6	0.44	175.5 ± 2.9	176.5 ± 2.9	0.81
Lactation = 1	180.7 ± 3.2	179.5 ± 4.0	0.81	172.4 ± 3.3	169.1 ± 4.0	0.53
Lactation = 2	185.7 ± 6.9	176.2 ± 5.3	0.29	178.8 ± 6.4	179.7 ± 5.0	0.91
Lactation ≥ 3	172.0 ± 5.3	174.5 ± 4.6	0.72	181.5 ± 7.7	183.4 ± 6.6	0.85

Table 3.3. (continued)

Mineral	Enrollment (d 0)		*P*	d 30		*P*
	CTRL	ITMS		CTRL	ITMS	
Trace minerals						
Copper, mg/L	0.92 ± 0.03	0.85 ± 0.03	0.12	0.82 ± 0.03	0.77 ± 0.03	0.25
Lactation = 1	0.99 ± 0.03	0.91 ± 0.04	0.17	0.85 ± 0.02	0.76 ± 0.03	0.06
Lactation = 2	0.93 ± 0.04	0.84 ± 0.03	0.13	0.88 ± 0.07	0.81 ± 0.05	0.46
Lactation ≥ 3	0.66 ± 0.04	0.76 ± 0.03	0.11	0.65 ± 0.05	0.73 ± 0.05	0.28
Iron, mg/L	2.5 ± 0.2	2.3 ± 0.2	0.40	2.3 ± 0.2	2.4 ± 0.2	0.71
Lactation = 1	2.38 ± 0.26	2.36 ± 0.32	0.95	2.3 ± 0.2	2.26 ± 0.25	0.92
Lactation = 2	2.86 ± 0.48	2.66 ± 0.37	0.74	2.48 ± 0.35	2.28 ± 0.27	0.65
Lactation ≥ 3	2.70 ± 0.5	1.72 ± 0.43	0.16	2.08 ± 0.56	2.71 ± 0.49	0.41
Manganese, ug/L	4.3 ± 0.2	3.5 ± 0.2	0.05	3.3 ± 0.8	4.8 ± 0.8	0.23
Lactation = 1	4.27 ± 0.38	3.41 ± 0.47	0.16	2.83 ± 0.35	3.66 ± 0.43	0.14
Lactation = 2	4.33 ± 0.54	4.0 ± 0.42	0.63	2.66 ± 0.26	3.5 ± 0.2	0.02
Lactation ≥ 3	4.16 ± 0.43	3.12 ± 0.37	0.09	5.33 ± 3.85	8 ± 3.33	0.61
Molybdenum, ug/L	8.70 ± 0.40	8.10 ± 0.40	0.26	9.7 ± 0.4	8.7 ± 0.4	0.09
Lactation = 1	8.38 ± 0.53	7.83 ± 0.65	0.51	9.22 ± 0.56	8.50 ± 0.68	0.42
Lactation = 2	8.66 ± 0.99	8.50 ± 0.77	0.89	10.5 ± 1.0	8.1 ± 0.8	0.08
Lactation ≥ 3	9.83 ± 0.65	8.00 ± 0.56	0.05	10.5 ± 0.9	9.62 ± 0.86	0.51
Selenium, ug/L	132 ± 3.	129 ± 3	0.51	126 ± 3	127 ± 3	0.94
Lactation = 1	130 ± 3	130 ± 4	0.94	125 ± 3	124 ± 4	0.84
Lactation = 2	131 ± 5	127 ± 4	0.51	133 ± 8	121 ± 6	0.23
Lactation ≥ 3	138 ± 7	132 ± 6	0.57	123 ± 7	138 ± 6	0.16
Zinc, mg/L	0.83 ± 0.03	0.83 ± 0.03	1.00	0.87 ± 0.03	0.89 ± 0.03	0.65
Lactation = 1	0.85 ± 0.03	0.87 ± 0.04	0.75	0.85 ± 0.04	0.91 ± 0.05	0.31
Lactation = 2	0.81 ± 0.07	0.87 ± 0.05	0.57	0.93 ± 0.06	0.91 ± 0.05	0.78
Lactation ≥ 3	0.78 ± 0.04	0.72 ± 0.03	0.30	0.86 ± 0.06	0.82 ± 0.06	0.65

Table 3.4. Logistic regression model for the effect of injectable trace mineral supplementation on subclinical mastitis cure (SCC < 200 × 10^3 cells/mL) of cows diagnosed with subclinical mastitis (SCC ≥ 200 × 10^3 cells/mL) on the test day preceding enrollment. CTRL = cows received no trace mineral supplementation (n = 314); ITMS = cows received a single injectable trace mineral supplementation containing zinc (300 mg), manganese (50 mg), selenium (25 mg), and copper (75 mg) at enrollment (n = 306).

Variable	Level	Cured[1], % (n)	OR[2]	*P*
Treatment	CTRL	42.8 (314)	Reference	0.38
	ITMS	46.5 (306)	1.16	
Parity	Lactation = 1	58.5 (240)	Reference	< 0.001
	Lactation = 2	42.8 (215)	0.53	
	Lactation ≥ 3	33.2 (165)	0.35	
Parity * Treatment	Lactation = 1 - CTRL	60.6 (109)	Reference	0.53
	Lactation = 1 - ITMS	56.5 (131)	0.85	
	Lactation = 2 - CTRL	42.5 (120)	Reference	0.92
	Lactation = 2 - ITMS	43.2 (95)	1.03	
	Lactation ≥ 3 - CTRL	27.1 (85)	Reference	0.08
	Lactation ≥ 3 - ITMS	40.0 (80)	1.80	

[1] Data presented as proportions adjusted by the logistic regression model.

[2] Odds ratios (OR) from the logistic regression model.

Table 3.5. Logistic regression model for the effect of injectable trace mineral supplementation on the incidence of clinical mastitis in cows diagnosed with subclinical mastitis (SCC ≥ 200 × 10^3 cells/mL) on the test day preceding enrollment. CTRL = cows received no trace mineral supplementation (n = 314); ITMS = cows received a single injectable trace mineral supplementation containing zinc (300 mg), manganese (50 mg), selenium (25 mg), and copper (75 mg) at enrollment (n = 306).

Variable	Level	CM[1], % (n)	OR[2]	*P*
Treatment	CTRL	48.2 (314)	Reference	0.11
	ITMS	41.7 (306)	0.77	
Parity	Lactation = 1	42.0 (240)	Reference	0.30
	Lactation = 2	43.3 (215)	1.05	
	Lactation ≥ 3	49.5 (165)	1.36	
Parity * Treatment	Lactation = 1 - CTRL	45.9 (109)	Reference	0.23
	Lactation = 1 - ITMS	38.2 (131)	0.73	
	Lactation = 2 - CTRL	43.3 (120)	Reference	0.98
	Lactation =2 - ITMS	43.2 (95)	0.99	
	Lactation ≥ 3 - CTRL	55.3 (85)	Reference	0.14
	Lactation ≥ 3 - ITMS	43.8 (80)	0.63	

[1] Clinical mastitis (CM) was defined as abnormal milk with clots in a forestripping test. Adjusted proportions according to the logistic regression model.
[2] Odds ratios (OR) from the logistic regression model.

Table 3.6. Logistic regression model for the effect of injectable trace mineral supplementation on the incidence of chronic clinical mastitis in cows diagnosed with subclinical mastitis (SCC ≥ 200 × 10^3 cells/mL) on the test day preceding enrollment. CTRL = cows received no trace mineral supplementation (n = 314); ITMS = cows received a single injectable trace mineral supplementation containing zinc (300 mg), manganese (50 mg), selenium (25 mg), and copper (75 mg) at enrollment (n = 306).

Variable	Level	CCM[1], % (n)	OR[2]	*P*
Treatment	CTRL	16.9 (314)	Reference	0.09
	ITMS	12.0 (306)	0.67	
Parity	Lactation = 1	12.0 (240)	Reference	0.13
	Lactation = 2	12.7 (215)	1.06	
	Lactation ≥ 3	18.9 (165)	1.71	
Parity * Treatment	Lactation = 1 - CTRL	18.4 (109)	Reference	0.02
	Lactation = 1 - ITMS	7.6 (131)	0.37	
	Lactation = 2 - CTRL	10.8 (120)	Reference	0.39
	Lactation = 2 - ITMS	14.7 (95)	1.42	
	Lactation ≥ 3 - CTRL	23.5 (85)	Reference	0.17
	Lactation ≥ 3 - ITMS	15.0 (80)	0.57	

[1] Chronic clinical mastitis (CCM) was defined as three or more cases of clinical mastitis. All cows included in the study were diagnosed with subclinical mastitis (SCC ≥ 200 × 10^3 cells/mL) on the test day preceding enrollment. Adjusted proportions from the logistic regression model.
[2] Odds ratios (OR) from the logistic regression model.

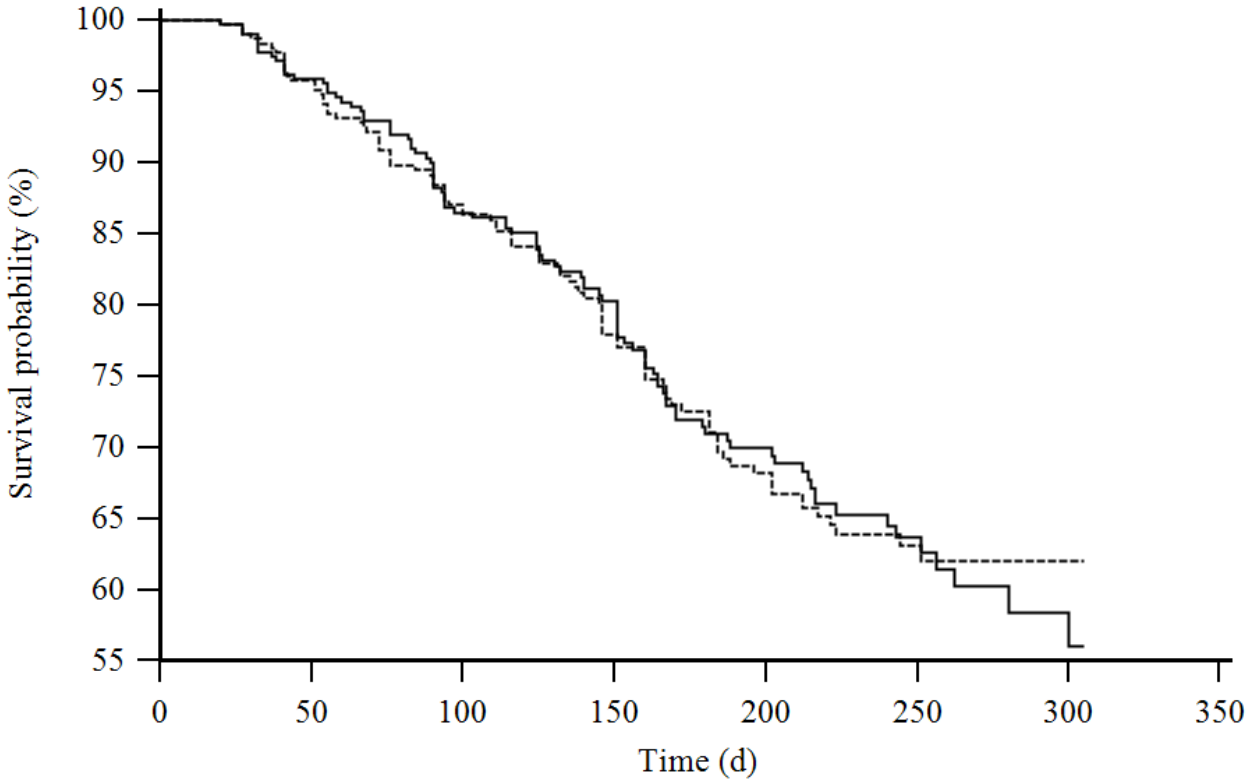
Table 3.7. Mineral concentrations in serum according to cure of subclinical mastitis (SCM). Cured = SCC < 200 × 10^3 cells/mL (n = 27). SCM= SCC ≥ 200 × 10^3 cells/mL (n = 33). All cows were diagnosed with subclinical mastitis (SCC ≥ 200 × 10^3 cells/mL) on the test day preceding enrollment. Results presented as LSM ± SEM.

	Enrollment (d 0)			d 30		
Mineral	SCM	Cured	*P*	SCM	Cured	*P*
Macrominerals						
Calcium, mg/L	91.8 ± 1.1	95.1 ± 1.2	0.04	86.8 ± 0.9	87.7 ± 1.0	0.45
Magnesium, mg/L	19.6 ± 0.4	19.6 ± 0.4	0.93	19.7 ± 0.4	19.4 ± 0.5	0.71
Phosphorus, mg/L	69.5 ± 2.4	73.3 ± 2.6	0.30	62.3 ± 1.4	69.9 ± 1.6	< 0.01
Potassium, mg/L	176.0 ± 2.5	181.6 ± 2.8	0.14	176.6 ± 2.8	175.3 ± 3.1	0.74
Trace minerals						
Copper, mg/L	0.87 ± 0.03	0.90 ± 0.03	0.39	0.80 ± 0.03	0.79 ± 0.03	0.73
Iron, mg/L	2.3 ± 0.2	2.5 ± 0.2	0.57	2.3 ± 0.2	2.4 ± 0.20	0.65
Manganese, ug/L	3.8 ± 0.3	4.0 ± 0.3	0.63	4.7 ± 0.8	3.3 ± 0.9	0.25
Molybdenum, ug/L	8.8 ± 0.4	8.0 ± 0.4	0.14	9.3 ± 0.4	9.1 ± 0.5	0.72
Selenium, ug/L	130 ± 3	133 ± 3	0.30	122 ± 3	132 ± 3	0.01
Zinc, mg/L	0.86 ± 0.03	0.80 ± 0.03	0.10	0.88 ± 0.03	0.88 ± 0.03	0.95

Table 3.8. Mineral concentrations in serum according to occurrence of clinical mastitis (CM) in cows diagnosed with subclinical mastitis (SCC ≥ 200 × 10^3 cells/mL) on the test day preceding enrollment. CM = animals with abnormal milk during a forestripping test (n = 26) Normal = cows that had normal milk at every test during the follow-up period (n = 34). Results presented as LSM ± SEM.

	Enrollment (d 0)			d 30		
Mineral	Normal	CM	*P*	Normal	CM	*P*
Macrominerals						
Calcium, mg/L	93.0 ± 1.1	93.7 ± 1.3	0.69	87.1 ± 0.9	87.4 ± 1.0	0.83
Magnesium, mg/L	19.4 ± 0.4	19.8 ± 0.5	0.54	19.5 ± 0.4	19.6 ± 0.5	0.85
Phosphorus, mg/L	69.5 ± 2.3	73.4 ± 2.7	0.28	65.5 ± 1.6	66.1 ± 1.8	0.79
Potassium, mg/L	178.3 ± 2.5	178.9 ± 2.9	0.86	175.3 ± 2.8	176.9 ± 3.1	0.70
Trace minerals						
Copper, mg/L	0.87 ± 0.02	0.90 ± 0.03	0.40	0.78 ± 0.03	0.82 ± 0.03	0.34
Iron, mg/L	2.4 ± 0.2	2.5 ± 0.2	0.81	2.5 ± 0.2	2.2 ± 0.2	0.20
Manganese, ug/L	4.1 ± 0.3	3.7 ± 0.3	0.34	3.4 ± 0.8	4.9 ± 0.9	0.22
Molybdenum, ug/L	8.0 ± 2.4	8.9 ± 0.4	0.12	9.2 ± 0.4	9.2 ± 0.5	0.90
Selenium, ug/L	128 ± 2	134 ± 3	0.11	126 ± 3	127 ± 3	0.99
Zinc, mg/L	0.84 ± 0.03	0.83 ± 0.03	0.79	0.91 ± 0.03	0.85 ± 0.03	0.17

Figure 3.1. Kaplan-Meier survival analysis of time from enrollment to removal from the herd. CTRL = cows with subclinical mastitis that received no mineral supplementation (n = 314; solid line); ITMS = cows with subclinical mastitis treated with an injectable trace mineral supplement (zinc, 300 mg; manganese, 50 mg; selenium, 25 mg; copper,75 mg) on d 0 (n = 306; dashed line). The mean enrollment to removal from the herd interval were 239.6 and 239.3 d for CTRL and ITMS, respectively ($P = 0.94$).



DISCUSSION

Although injectable trace mineral supplementation containing selenium, copper, zinc, and manganese during the prepartum period has been shown to reduce SCC throughout the first five months of lactation (Machado et al., 2013), its effects on health and productive performance of lactating dairy cows diagnosed with subclinical mastitis remains elusive. The requirements for trace minerals in lactating dairy cow diets have been calculated based on studies that focused in reproductive performance, milk production, and stability of skeletal system, rather than targeting optimum immune function. To the best of our knowledge, only copper and selenium had aspects of the immune function taken into consideration during requirement calculation. Therefore, it is possible that the trace minerals needed for optimum udder health have been underestimated. It is important to highlight that in the present study, mineral status of the study population was assessed through blood mineral panel analysis in a subset of animals at enrollment and all blood concentrations of minerals evaluated were in accordance with previously reported normal values (Dargatz and Ross, 1996, Kincaid, 2000, NRC, 2001). Mineral supplementation in lactating dairy cows with normal levels of blood minerals and with SCC $\geq 200 \times 103$ cells/mL significantly reduced the incidence of chronic clinical mastitis in primiparous cows, tended to reduce overall incidence of chronic clinical mastitis, and tended to improve subclinical mastitis cure in cows with three or more lactations. Nevertheless, ITMS did not significantly affect survival, milk yield, or milk composition in cows diagnosed with subclinical mastitis

Genther and Hansen (2014) supplemented beef steers with the same mineral combination used in the present study and reported increases in circulating concentrations of manganese and selenium for 1 and 15 days following injection, respectively. Similar to our results, that group did not observe differences in serum mineral concentrations between treatment and control

groups on d 29 post-injection; however, liver copper and selenium contents were higher in supplemented steers. One explanation for the similar blood mineral concentrations between ITMS and CTRL groups in the current trial could be the timing of sample collection. Supplemental minerals in ITMS could have been cleared through homeostatic processes or have been stored in body reserves. Given the circumstances of the present study, it was not possible to perform liver biopsies on d 30 to assess the effect of ITMS on other body reserves.

Our results indicate that the administration of ITMS can be used as a strategy to lower the incidence of chronic clinical mastitis in primiparous cows diagnosed with elevated SCC. The importance of trace minerals in the immune system and mammary health has been reported in several studies (Andrieu, 2008, O'Rourke, 2009, Overton and Yasui, 2014). Elevated SCC has been associated with greater odds of developing clinical mastitis (Peeler et al., 2003). Previous work from our group reported significantly lower odds of developing clinical mastitis in multiparous animals that received injectable trace mineral supplementation containing selenium, copper, zinc, and manganese during the dry period (Machado et al., 2013). The differences found between the results presented here and data reported by Machado et al. (2013) might be due to dissimilarities in the target population and the frequency of supplementation between studies. We supplemented lactating animals experiencing subclinical mastitis with a single dose of ITMS while the former work aimed to evaluate the effect of a three doses supplementation of healthy cows during the transition period. In that study, injectable trace mineral supplementation containing selenium, copper, zinc, and manganese was given at approximately 230 and 260 days of gestation and again at 35 DIM.

In the present study, injectable trace mineral supplementation of selenium, copper, zinc, and manganese also tended to increase subclinical mastitis cure in cows of third or greater

lactation. We observed that this group of animals had the lowest initial serum concentrations of copper, calcium and phosphorus. Cows of third or greater lactation were also the highest milk producing group; therefore, mineral requirements for those animals could possibly have been underestimated, and might explain why the supplementation with ITMS had an effect of subclinical mastitis cure in this group.

When animals were compared across treatments based on disease status, serum concentration of selenium on d 30 was lower in animals that remained affected with subclinical mastitis. Selenium is an essential element of the antioxidant enzymes glutathione peroxidase and thioredoxin reductase, playing a role in regulation and host cell resistance to reactive oxygen species (**ROS**) (McKenzie et al., 2002). Nonetheless, all animals in the present study had adequate selenium levels in both time points. In dairy cows, selenium supplementation has been associated with better neutrophil function (Ibeagha et al., 2009), decreased prevalence of mammary infections (Erskine et al., 1987, Jukola et al., 1996), and improved antioxidant status and immune function (Hall et al., 2014). Studies that used supplementation of bovine mammary cells in vitro suggested that selenium supplementation plays a role in the optimization of udder health in via increased resistance to oxidative stress (Miranda et al., 2011).

In addition to differences in selenium status between cows that underwent subclinical mastitis cure and those that remained with SCC above 200×10^3 cells/mL, associations between macro mineral concentrations and mammary gland health were observed. We identified that cows that experienced subclinical mastitis cure had higher concentrations of serum calcium on d 0. Circulating calcium levels have been associated with bovine immune function and disease status in other studies (Curtis et al., 1983, Kimura et al., 2006, Martinez et al., 2012). A study conducted in Florida reported that neutrophils isolated from cows with lower serum calcium in

the transition period had impaired *in vitro* phagocytosis and bacterial killing capacity, fewer circulating leucocytes, a more pronounced decrease in neutrophil count postpartum, and increased incidence of metritis (Martinez et al., 2012). That group evaluated the effects of subclinical hypocalcemia in dairy cows; however it is important to highlight that the study population herein had levels of calcium above of the cutoff of 8.59 mg/dl in which a cow is considered subclinically hypocalcemic. Nevertheless, circulating calcium levels have been directly correlated with amounts of releasable calcium in the endoplasmic reticulum (Kimura et al., 2006), playing a role in bovine neutrophil activation, ROS production, and degranulation, all of which are critical for proper immune function (Tintinger et al., 2005, Burgos et al., 2011).

In the present study, higher phosphorus concentrations were associated with subclinical mastitis cure. Cows that recovered from the disease by d 30 had higher serum concentrations of phosphorus on d 30 compared with those that remained with SCC $\geq 200 \times 10^3$ cells/mL. Limited research associating phosphorus and bovine immune function is available. Mullarky et al. (2009) found that supplementing lactating dairy cows with no (0.34%, no supplementary P), medium (0.43%), or high (0.52%) levels of phosphorus did not influence lymphocyte proliferation and neutrophil killing ability *in vitro*. Further research is necessary to evaluate the effect of phosphorus supplementation beyond required levels in dairy cows' immune function.

Although copper and zinc levels were not different between treatment groups nor associated with SCM cure or decreased odds of chronic CM; it is well known that these minerals are components of the enzyme superoxide dismutase (**SOD**), which has been previously associated with udder health (Machado et al., 2014). Additionally, Blanco-Penedo et al. (2014) reported that herds with low manganese had increased odds of CM as compared to herds with adequate levels of manganese, and that low copper levels were associated with increased odds of

CM cases for second lactation cows. Nevertheless, one cannot exclude the possibility of multiple factors, other than a specific mineral level, play a role in associations observed in survey-type studies such as the one conducted by Blanco-Penedo and colleagues in 2014. Further research is necessary to evaluate repeated ITMS supplementation to lactating dairy cows with elevated somatic cell counts belonging to various parity groups. Perhaps the administration of sequential doses of ITMS to animals with subclinical mastitis would be effective in providing supplemental trace minerals for improved udder health.

CONCLUSIONS

A single subcutaneous injection containing zinc, manganese, selenium, and copper administered to dairy cows with elevated SCC reduced the incidence of chronic clinical mastitis, particularly in primiparous cows, and tended to increase subclinical mastitis cure in cows with three or more lactations. Supplementation had no effect on milk production and composition in cows with elevated SCC. Regardless of supplementation, cows that were cured of subclinical mastitis had lower serum concentrations of zinc at diagnosis and higher selenium and phosphorus concentrations 30 d later compared with herdmates that maintained SCC above 200 $\times$ 10^3 cells/mL; thereby, supporting a possible role for availability and utilization of trace minerals for maintenance of udder health.

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CHAPTER 4

LONGITUDINAL METAGENOMIC PROFILING OF BOVINE MILK TO ASSESS THE IMPACT OF INTRAMAMMARY TREATMENT USING A THIRD-GENERATION CEPHALOSPORIN

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ABSTRACT

Antimicrobial usage in food animals has a direct impact on human health, and approximately 80% of the antibiotics prescribed in the dairy industry are used to treat bovine mastitis. Here, we provide a longitudinal description of the changes in the microbiome of milk that are associated with mastitis and antimicrobial therapy. Next-generation sequencing, 16S rRNA gene quantitative real-time PCR, and aerobic culturing were applied to assess the effect of disease and antibiotic therapy on the milk microbiome. Cows diagnosed with clinical mastitis associated with Gram-negative pathogens or negative aerobic culture were randomly allocated into 5 days of Ceftiofur intramammary treatment or remained as untreated controls. Serial milk samples were collected from the affected quarter and the ipsilateral healthy quarter of the same animal. Milk from the mastitic quarter had a higher bacterial load and reduced microbial diversity compared to healthy milk. Resolution of the disease was accompanied by increases in diversity indexes and a decrease in pathogen relative abundance. *Escherichia coli*-associated mastitic milk samples had a remarkably distinct bacterial profile, dominated by *Enterobacteriaceae*, when compared to healthy milk. However, no differences were observed in culture-negative mastitis samples when compared to healthy milk. Antimicrobial treatment had no significant effect on clinical cure, bacteriological cure, pathogen clearance rate or bacterial load.

INTRODUCTION

Production of animal protein to support the world's growing human population is one of the main challenges facing humankind. Concerns related to food safety and development of antimicrobial resistance may lead to decreased availability of antibiotics for use in food animals

and thereby limit our ability to control disease in agricultural animal species. Such a change in antibiotic usage in food animals could also alter perspectives on food security as it relates to public health concerns regarding antibiotic use in food animals. Thus, in-depth understanding of disease mechanisms is critical to promote animal health and at the same time encourage judicious use of antibiotics in livestock. Mastitis is one of the most common diseases in dairy herds, and approximately 20% to 30% of dairy cows develop clinical mastitis at least once during lactation (Sargeant et al., 1998, Hertl et al., 2014). Not surprisingly, prophylaxis and treatment of mastitic cows are the major reasons for antibiotic usage in dairy farms (Erskine et al., 2003, Pol and Ruegg, 2007).

Maternal milk harbors a rich microbial community that is vital for establishment of the gut microbiome and immune tolerance in neonates (Martin et al., 2003, Bertelsen et al., 2014). The same microbial community in the mammary gland may provide an environment that aids the host in preventing mammary infections. For instance, commensal bacteria present in human milk inhibit proliferation of *Staphylococcus aureus* (Heikkila and Saris, 2003), which is also a pathogen commonly associated with mastitis in dairy cows (Olde Riekerink et al., 2008). Considering that mastitis possibly reflects a dysbiosis within the mammary gland (Oikonomou et al., 2012, Kuehn et al., 2013, Oikonomou et al., 2014), a detailed assessment of the milk microbiome during active stages of clinical disease, spontaneous recovery, treatment and post-treatment is essential to further elucidate this pathological condition.

The multifactorial etiology of mastitis presents a major challenge for disease prevention and treatment of affected animals. Implementation of programs for mastitis control has reduced the prevalence of important contagious pathogens, and approximately 40% of clinical cases of mastitis are associated with opportunistic Gram-negative bacteria such as *Escherichia coli*,

Klebsiella spp., *Pseudomonas* spp., and *Pasteurella* spp. (Erskine et al., 1988, Olde Riekerink et al., 2008, Botrel et al., 2010). Although current guidelines do not recommend the use of intramammary antibiotics for cows diagnosed with Gram-negative mastitis (Erskine et al., 2003, Suojala et al., 2013), improved bacteriological and clinical outcomes have been shown in mastitic cows treated with third-generation cephalosporins compared with other antimicrobials or untreated controls (Schukken et al., 2011a, Schukken et al., 2013). However, the impact of these broad-spectrum antibiotics on the milk microbiota (other than major pathogens) remains unknown. In fact, currently no data concerning the effect of antibiotic therapy on the mammary microbiota are available in either humans or animals. Routine methods used to assess responses to intramammary treatments overlook numerous microorganisms, which is supported by the fact that 40% of milk samples collected from cows with clinical mastitis yield negative results by aerobic culture (Botrel et al., 2010). Later-generation cephalosporins have broad-spectrum antibacterial activity, so their use could unintentionally disrupt general mammary microbial populations and also increase the risk of antimicrobial resistance if not used in a judicious manner (Dolejska et al., 2012). Understanding the dynamics of the mammary microbiota upon antibiotic therapy is essential not only for development of effective treatment strategies, but also to facilitate the process of restoring a healthy mammary microbiota.

State-of-the-art technologies have allowed the investigation of microbial communities in milk without some of the limitations imposed by culture methods (Hunt et al., 2011, Cabrera-Rubio et al., 2012, Oikonomou et al., 2012). Therefore, the specific objectives of the present study were: 1) to use high-throughput DNA sequencing to describe the microbiome of milk in dairy cows affected by clinical mastitis associated with Gram-negative bacteria or negative culture; 2) to determine the bacterial load based on PCR quantification of 16S rRNA gene

copies, and compare microbial populations of affected and healthy quarters; and 3) to assess the effect of intramammary treatment with ceftiofur hydrochloride on the milk microbiome, bacterial load, and clinical cure in quarters affected with clinical mastitis.

MATERIALS AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2013-0056). The methods were carried out in accordance with the approved guidelines.

Animals, Enrollment Criteria, and Treatments

Milk samples were collected from lactating Holstein cows diagnosed with clinical mastitis between April and June, 2014. All cows were housed in a single herd located in upstate New York which milked approximately 2,800 cows thrice daily during the experimental period. Clinical mastitis was defined as the presence of at least visually abnormal milk (i.e. presence of flakes, clots, or serous milk) during forestripping performed at the milking parlor. Once mastitis was diagnosed, the initial milk sample for mastitis pathogen identification was collected by trained farm personnel according to National Mastitis Council guidelines (NMC, 1999). These samples were defined as day 0 samples. Teats were cleaned and disinfected using 70% ethanol (v/v), the initial three streams were discarded, and approximately 5 mL of milk was collected into a sterile plastic tube without preservative (Corning Life Sciences, Tewksbury, MA). Milk samples were cultured using an on-farm chromogenic culture system for fast identification of causal agents (Accumast®, FERA Animal Health LCC, Ithaca, NY) according to the manufacturer's recommendations, and then submitted for analysis at the Quality Milk Production

Services laboratory (**QMPS**; Cornell University, Ithaca, NY) to ensure the accuracy of on-farm culture. Disagreement between methods was observed in only two samples, which were excluded from further analyses after the results from QMPS were received.

Cows diagnosed with clinical mastitis associated with Gram-negative bacteria or negative on-farm culture and that had not been treated with intramammary antimicrobials in the 14 days preceding diagnosis were deemed eligible for enrollment. On study day 1, eligible cows were randomly allocated into one of two treatments based on a list of numbers generated using the RAND function of Excel (Microsoft, Redmond, WA), blocked by aerobic culture results. Clinical score was assessed on days 1, 8, 10 and 14 according to Wenz et al (Wenz et al., 2001, Wenz et al., 2006). Milk appearance, mammary gland appearance and systemic signs of disease (i.e. rectal temperature ≥ 39.5 ºC, dehydration and depression) were evaluated for classification of clinical score. A clinical score of 'mild' was assigned if only abnormal milk was observed. A 'moderate' score was assigned when abnormal milk and inflammation of the mammary gland were present. A 'severe' score was assigned if abnormal milk, local inflammation and one or more of the systemic signs of the disease were observed.

Cows assigned to the treatment group received daily intramammary infusions containing 125 mg of ceftiofur equivalents (as ceftiofur hydrochloride; Spectramast LC®, Zoetis, Florham Park, NJ) only on the mastitic quarter for five consecutive days, whereas those assigned to the control group did not receive intramammary therapy.

Sample and Data Collection

Serial milk samples were collected by a trained veterinarian member of the research team from each cow on study days 1, 2, 3, 4, 5, 8, 10, and 14, from both the mastitic quarter and the

ipsilateral healthy quarter of the same cow. For cows in the treated group, sampling on days 1 through 5 was performed after milk out of the quarter in untreated cows, whereas treated cows were sampled immediately before intramammary treatments were applied. Teats were disinfected as described above and 10 mL of milk was harvested from each quarter into a sterile plastic tube without preservative (Corning Life Sciences, Tewksbury, MA). Samples were immediately refrigerated at 4 ºC, transported to the laboratory on ice, and frozen at -20 ºC until assayed. Milk samples collected from mastitic quarters on days 10 and 14 were submitted to the QMPS laboratory for bacterial identification using standard aerobic culture.

Clinical cure was defined as cows without any clinical signs on both day 10 and day 14. Bacteriological cure was defined as both the samples taken on day 10 and day 14 being negative for the organism present on day 0. In all other cases the quarter was considered to be a non-cure or treatment failure. Bacteriological cure can only be evaluated in quarters that were culture positive on day 0.

DNA Isolation and Purification

Milk samples were thawed, homogenized by inverting the tubes, and a 6-mL aliquot was taken for DNA isolation and purification. Milk samples were centrifuged at 4 ºC and 16,100 × *g* for 3 minutes and the supernatant was discarded. Genomic DNA was isolated from the remaining pellet using a commercially available kit (PowerFood DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA) as described previously (Quigley et al., 2012). Concentration and purity of isolated DNA were evaluated based on optical density at 230, 260 and 280 nm wavelengths (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE).

Amplification of the V4 Hypervariable Region of the Bacterial 16S rRNA Gene, Library Preparation, and 16S rRNA Gene Sequencing

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from genomic DNA by PCR utilizing the primers 515F and 806R optimized for the Illumina MiSeq platform (Illumina Inc., San Diego, CA) (Caporaso et al., 2012) as described previously (Foditsch et al., 2015).

Equimolar libraries were sequenced in six runs using the MiSeq reagent kit V2 for 300 cycles on the MiSeq platform (Illumina). Each run included 279 samples and a sequencing control that consisted of the purified barcoded PCR product of DNA extracted from *Staphylococcus aureus* (ATCC 25923). Gene sequences were processed using the 16S Metagenomics workflow in the MiSeq Reporter analysis software version 2.5 based on quality scores generated by real-time analysis during the sequencing run. Quality-filtered indexed reads were demultiplexed for generation of individual FASTQ files and aligned using the banded Smith-Waterman method of the Illumina-curated version of the Greengenes database for taxonomic classification of milk microbes. Resulting FASTQ files were uploaded into the open-source pipeline Quantitative Insights into Microbial Ecology (**QIIME**) version 1.9.1 (Caporaso et al., 2010b). Sequences were filtered based on quality as described previously (Bokulich et al., 2013) and assigned to operational taxonomic units (**OTUs**) with 97% identity using UCLUST (Edgar, 2010). The OTU database was rarefied using the command single_rarefaction.py from QIIME and the number of OTUs, as well as Chao1 and Shannon indexes, was calculated for each sample at a rarefication level of 5,000 reads per sample.

Quantification of 16S rRNA Copies by qPCR

The number of 16S rRNA copies was used as a proxy to determine bacterial load in milk samples collected on days 1, 3, 8 and 14. 16S rRNA gene copies were quantified by qPCR as described previously (Boutin et al., 2015). Reactions were performed using Unibac primers (forward: 5′-TGG AGC ATG TGG TTT AAT TCG A-3′; reverse: 5′-TGC GGG ACT TAA CCC AAC A-3′; 50 pmol/reaction), 1X iQ™ SYBR® Green Mastermix (Bio-Rad Laboratories, Hercules, CA), and 1.5 µL of sample DNA. A standard curve was built using plasmid DNA quantified by spectrophotometry. All samples were assayed in duplicate using an iQ™5 Real-time PCR system (Bio-Rad Laboratories, Hercules, CA) set to perform denaturation at 95 ºC for 3 minutes, then 40 cycles of amplification (95 ºC for 10 seconds and 55 ºC for 30 seconds), one cycle at 95 ºC for 60 seconds, one cycle at 55 ºC for 60 seconds, and a melting curve determination.

Statistical Analyses

Descriptive analyses on sequencing results were performed using the UNIVARIATE procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Differences in the relative abundance of bacteria between quarters with clinical mastitis and healthy counterparts were evaluated at the phylum and family levels using JMP Pro 11 (SAS Institute Inc., Cary, NC). Cows were categorized according to the main pathogen identified on samples taken on study day 0 through standard culture methods into four groups, namely *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., and negative culture. Within each group, the effect of clinical mastitis on the relative abundance of each of the ten most prevalent phyla was evaluated by ANOVA. The prevalences of all remaining phyla were combined into a single cluster. The fixed effect of

disease (healthy vs. mastitic quarters) was included in the statistical models as an independent variable. Response screening was performed to assess the effect of clinical mastitis on the relative abundance of the 100 most prevalent families in each pathogen group. *P*-values were adjusted for false discovery rate (**FDR**) (Benjamini and Hochberg, 1995), and presented as FDR LogWorth (i.e. $-\log_{10}P$). The mean relative abundance for each family observed across all healthy quarters was used as a reference for calculation of fold-changes.

Microbiome changes occurring over time and in response to intramammary antibiotic therapy were described for the 25 most prevalent families in each pathogen group using the tabulate function of JMP Pro 11. Relative abundances of all remaining families were combined into a single cluster. The magnitude of change was scaled uniformly within health status (healthy vs. mastitic quarters). The relative abundances of major pathogens associated with clinical mastitis were evaluated within pathogen groups by ANOVA for repeated measures using the GLIMMIX procedure of SAS. Outcomes were the relative abundance of each pathogen and the explanatory variables were treatment, time, health status (healthy vs mastitic quarter) and their two- and three-way interactions. Cow was considered a random effect in all statistical models. Time changes in the number of OTUs, Chao1 index, and Shannon index were analyzed by ANOVA for repeated measures using the GLIMMIX procedure of SAS. Within each pathogen group, two statistical models were built to evaluate the effects of mastitis (i.e. fixed effects of mastitis, time, and interaction between mastitis and time) and treatment (i.e. fixed effects of treatment, mastitis, time, and all two- and three-way interactions).

The effect of cure on the relative abundance of *Enterobacteriaceae* family members and the Shannon diversity index was evaluated between cured and non-cured cows with clinical mastitis associated with *E. coli* by ANOVA for repeated measures using the Fit Model function

on JMP Pro 11. Tests for normality of residuals and homogeneity of variances were conducted for each dependent variable, and data that did not fulfill ANOVA assumptions were transformed accordingly (i.e. 16S rRNA gene copy numbers). The covariance structure with the smallest Schwarz's Bayesian information criterion value was selected for each analysis. Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies. Results are presented as average and standard deviation (i.e. descriptive analyses of sequencing results) or least square means followed by the respective standard error of the mean.

Multivariate analysis of microbiome data was carried out in R (R Core Team, Vienna, Austria) and QIIME. Beta diversity was analyzed through analysis of similarities (**ANOSIM**) using non-rarefied data normalized employing the packages metagenomeSeq (Paulson et al., 2013) and vegan (Jari Oksanen, 2016) in R. Principal coordinate analysis (**PCoA**) was performed using weighted Unifrac distances calculated in QIIME and visualized through EMPeror (Vazquez-Baeza et al., 2013).

RESULTS

Clinical and Bacteriological Cure

Intramammary treatment with ceftiofur hydrochloride did not significantly improve clinical and bacteriological cures of clinical mastitis compared with untreated controls (**Table 4.1**). Of the 40 cows enrolled in the control group, 75% (n=30) experienced clinical cure, whereas of the 40 cows that received intramammary antibiotic therapy, 77.5% (n=31) experienced clinical cure (*P*-value = 0.79). Clinical cures for cows affected with Gram-negative intramammary infections also did not differ between the treated (75% cure rate) and control cows (73.9% cure rate) (*P*-value = 0.93). Bacteriological cure followed the same pattern as for

clinical cure, with 82.6% of the milk samples collected from non-treated mastitic quarters being negative on days 10 and 14 for the organism present on day 0, whereas in the treated group, 79.2% of the samples were considered to be bacteriological cures (*P*-value = 1.00). Bacteriological cure was not altered by treatment when the data were stratified and analyzed by each pathogen group (**Table 4.1**).

Real-time PCR Results

Cows diagnosed with clinical mastitis caused by *E. coli* had a significantly (*P*=0.008) lower number of 16S rRNA gene copies in healthy quarters compared to mastitic ones on day 3 post diagnosis; however, no difference was observed in the bacterial load as measured in 16S rRNA gene copies between healthy and mastitic quarters at days 8 and 14. Intramammary treatment with Ceftiofur caused a significant decrease in the bacterial load of mastitic quarters on day 3 (*P* = 0.01) compared to non-treated mastitic quarters. Nonetheless, a treatment effect was no longer observed at study day 8 (**Figure 4.1a**).

In animals diagnosed with clinical mastitis yielding no bacterial growth upon aerobic culture, the number of 16S rRNA gene copies was higher in mastitic quarters compared to healthy ones. No treatment effect was observed on the bacterial load in this group of animals. Mastitic and healthy quarters exhibited the same bacterial load by study day 14 (**Figure 4.1c**).

Sequencing Results

Quality-filtered reads were demultiplexed and a total of 67,413,334 sequences was used for downstream analyses (mean=47,241.3 ± SD=32,625.0 reads/sample). The median length for all reads was 301 bp.

Microbiome Changes Associated with Clinical Mastitis

The mean relative abundance of bacteria from the phylum Proteobacteria was greater ($P < 0.01$) in the milk from mastitic quarters infected by *E. coli* and *Pseudomonas* spp. compared with that of healthy quarters (**Figure 4.2a-b**). This was driven mostly by greater abundances of *Enterobacteriaceae* ($P < 0.001$; **Figure 4.3**) and *Pseudomonadaceae* ($P = 0.03$; **Figure 4.S1**). On the other hand, the average abundance of Firmicutes, Actinobacteria, Bacteroidetes, Tenericutes, Chlorobi, and the combination of all remaining phyla was greater ($P < 0.05$) in healthy compared with mastitic quarters infected by *E. coli* (**Figure 4.2a**). A similar pattern was observed in cows with clinical mastitis associated with *Pseudomonas* spp. (n = 2 quarters), in which the abundance of Actinobacteria and Bacteroidetes was greater ($P < 0.05$) and that of Chlorobi tended to be greater ($P = 0.08$) in healthy compared with mastitic quarters (**Figure 4.2b**). The diversity of milk microbial populations was reduced ($P < 0.0001$) in *E. coli* mastitis compared with healthy quarters (**Figure 4.1b**). The Shannon index was also influenced ($P < 0.0001$) by the interaction between mastitis and time, as values increased from day 1 through 14 in mastitic quarters, whereas no change was observed in healthy counterparts (**Figure 4.1b**). Likewise, the richness of microbial communities was reduced in *E. coli* mastitis compared with healthy quarters (**Figure 4.S2 a**).

The relative abundances of Firmicutes ($P = 0.06$) and the remaining phyla ($P = 0.02$) were greater in healthy quarters compared with those infected by *Klebsiella* spp. (**Figure 4.2c**). Nevertheless, mastitis did not affect the relative abundances of other phyla or individual families (**Figure 4.S3**). Clinical mastitis associated with *Klebsiella* spp. had reduced ($P = 0.05$) Shannon values shortly after diagnosis but had no effect on the Chao1 index (**Figure 4.S4 a-b**).

Shifts in the milk microbiome were less pronounced in cases of clinical mastitis associated with a negative aerobic culture (**Figure 4.2d**). The relative abundance of Firmicutes was higher (P = 0.08), whereas those of Bacteroidetes (P = 0.06), Tenericutes (P = 0.05), Spirochaetes (P = 0.01), and the combined remaining phyla (P = 0.08) were lower in mastitic quarters. Fluctuations in bacterial communities were not associated with specific families (**Figure 4.4**). Nevertheless, diversity was reduced ($P < 0.01$) in mastitic compared with healthy quarters (**Figure 4.1d**). The same trend was observed in the Chao1 richness index on the first two days after diagnosis of clinical mastitis (**Figure 4.S2 b**).

Effect of Intramammary Antibiotic Therapy on the Milk Microbiome

In cows diagnosed with clinical mastitis caused by *E. coli*, microbiome dynamics in healthy quarters did not change over time (**Figure 4.5a**). On the other hand, the relative abundance of *Enterobacteriaceae* decreased from study day 1 to 14 (62.6% vs. 9.7%), whereas the relative abundances of other families increased in mastitic quarters (**Figure 4.5b**). Changes in milk bacterial populations were not affected by intramammary therapy with ceftiofur hydrochloride (**Figure 4.5b**). Treatment and the interaction between treatment and time did not affect the relative abundance of *Enterobacteriaceae* (**Figure 4.6a**), or the Shannon (**Figure 4.1c**) and Chao1 (**Figure 4.S2 a**) indexes in mastitic quarters infected by *E. coli*.

Similar patterns were observed in cows with clinical mastitis caused by *Klebsiella* spp., as intramammary therapy did not impact the milk microbiome or the relative abundance of *Enterobacteriaceae* (**Figure 4.S5 and 4.S6**). The only two cows diagnosed with clinical mastitis associated with *Pseudomonas* spp. presented an elevated abundance of *Pseudomonadaceae* on day 1 (44.3%), which was reduced until day 8 (3.3%) and then returned to initial values on day

14 (46.1%). Because both cows were assigned to receive intramammary infusion with ceftiofur hydrochloride, the effect of treatment on abundance of *Pseudomonas* spp. could not be assessed.

Changes in the milk microbiome over time were not observed in cows affected by clinical mastitis associated with negative aerobic culture (**Figure 4.7a**). Moreover, intramammary treatment with ceftiofur hydrochloride in these quarters did not affect the milk microbiome (**Figure 4.7b**) or the measures of microbial diversity and richness (**Figure 4.1d**), (**Figure 4.S2 b**).

Microbiome Changes Associated With Clinical Mastitis Cure on the Mastitic Quarters of Cows with Mastitis Caused by Escherichia coli

In cows diagnosed with clinical mastitis caused by *E. coli*, microbiome dynamics in mastitic quarters exhibited remarkable changes over time. Quarters that experienced clinical cure by the end of the study period had significantly lower abundances of *Enterobacteriaceae* family members in both control (**Figure 4.6c**) and treated animals (**Figure 4.6e**). Nevertheless, microbial diversity at diagnosis of clinical mastitis did not differ between quarters that eventually became cured or not (**Figure 4.6b**). However, microbial diversity of quarters that eventually were cured showed increasing microbial diversities in both the control (**Figure 4.6d**) and treated groups (**Figure 4.6f**) relative to quarters that did not show bacteriological cure during the study period. Similar patterns were observed for bacteriological cure (**Figure 4.S7**).

Multivariate Analysis of Microbiome Data from Healthy and Mastitic Quarters

Analysis of similarities revealed that mastitic quarters were significantly different from healthy quarters at the first day after diagnosis of clinical mastitis in cows with clinical mastitis associated with *E. coli* (**Figure 4.8a**), and negative culture (**Figure 4.8b**). A clear separation

between mastitic and healthy quarters was observed in the principal coordinate analysis of Unifrac distances in animals with clinical mastitis associated with *E. coli* (**Figure 4.8a**); however the same could not be observed in animals with mastitis associated with negative culture (**Figure 4.8b**). At the end of the study, namely day 14 after diagnosis of clinical mastitis, the microbiome of quarters that had been cured from clinical mastitis did not differ from the one of healthy quarters in either ANOSIM or Unifrac PCoA in cows previously identified with clinical mastitis associated with *E. coli* (**Figure 4.8e**) nor in animals with clinical mastitis yielding negative aerobic culture (**Figure 4.8f**). Interestingly, when the microbiome of quarters that remained with abnormal milk by the end of the study was included in the analysis, a significant difference could be observed in both ANOSIM and Unifrac PCoA on the microbiome of milk from cows identified with clinical mastitis associated with *E. coli* (**Figure 4.8c**). No separation between mastitic, healthy, and cured quarters could be observed when the first three components of Unifrac PCoA were plotted in animals with mastitis associated with negative culture (**Figure 4.8d**).

Table 4.1. Effects of intramammary treatment with ceftiofur hydrochloride on clinical mastitis cure in lactating dairy cows

Parameter	Clinical cure			Bacteriological cure[1]		
	Control	Ceftiofur	*P*	Control	Ceftiofur	*P*
	---------- % (n/n) ----------			---------- % (n/n) ----------		
Cure on day 10 and 14						
Gram negative	73.9 (17/23)	75.0 (18/24)	0.93	82.6 (19/23)	79.2 (19/24)	0.76
Escherichia coli	75.0 (15/20)	70.0 (14/20)	0.72	85.0 (17/20)	80.0 (16/20)	0.67
Klebsiella spp.	66.7 (2/3)	100.0 (2/2)	0.36	66.7 (2/3)	100.0 (2/2)	0.36
Pseudomonas spp.[2]	---	100.0 (2/2)	---	---	50.0 (1/2)	---
Negative culture[3]	76.5 (13/17)	81.3 (13/16)	0.73	NA	NA	NA
Overall	75.0 (30/40)	77.5 (31/40)	0.79	82.6 (19/23)	79.2 (19/24)	1.00

[1] Based on standard laboratory culture methods for identification of milk pathogens.

[2] All cows diagnosed with *Pseudomonas* spp. by laboratory culture were assigned to the Ceftiofur group; thus, evaluation of treatment effect was not possible.

[3] Evaluation of bacteriological cure is not applicable to cows with negative culture. NA = non-applicable.

Figure 4.1. Effect of clinical mastitis and intramammary treatment with ceftiofur hydrochloride (days 1-5) on the number of 16S rRNA gene copies in cows with clinical mastitis associated with *Escherichia coli* (a) or negative culture (c), and microbial diversity in cows with clinical mastitis associated with *Escherichia coli* (b) or negative culture (d). Bars represent standard error of the mean. Asterisks represent significant differences at $\alpha = 0.05$ between groups within the same study day. (a) Mastitic-Control had a significantly greater bacterial load than Mastitic-Ceftiofur and healthy quarters on day 3. (c) On day 1, both mastitic quarters had a significantly greater bacterial load when compared to healthy quarters. On day 8: Mastitic-Control had a significantly greater bacterial load than Mastitic-Ceftiofur and healthy quarters.

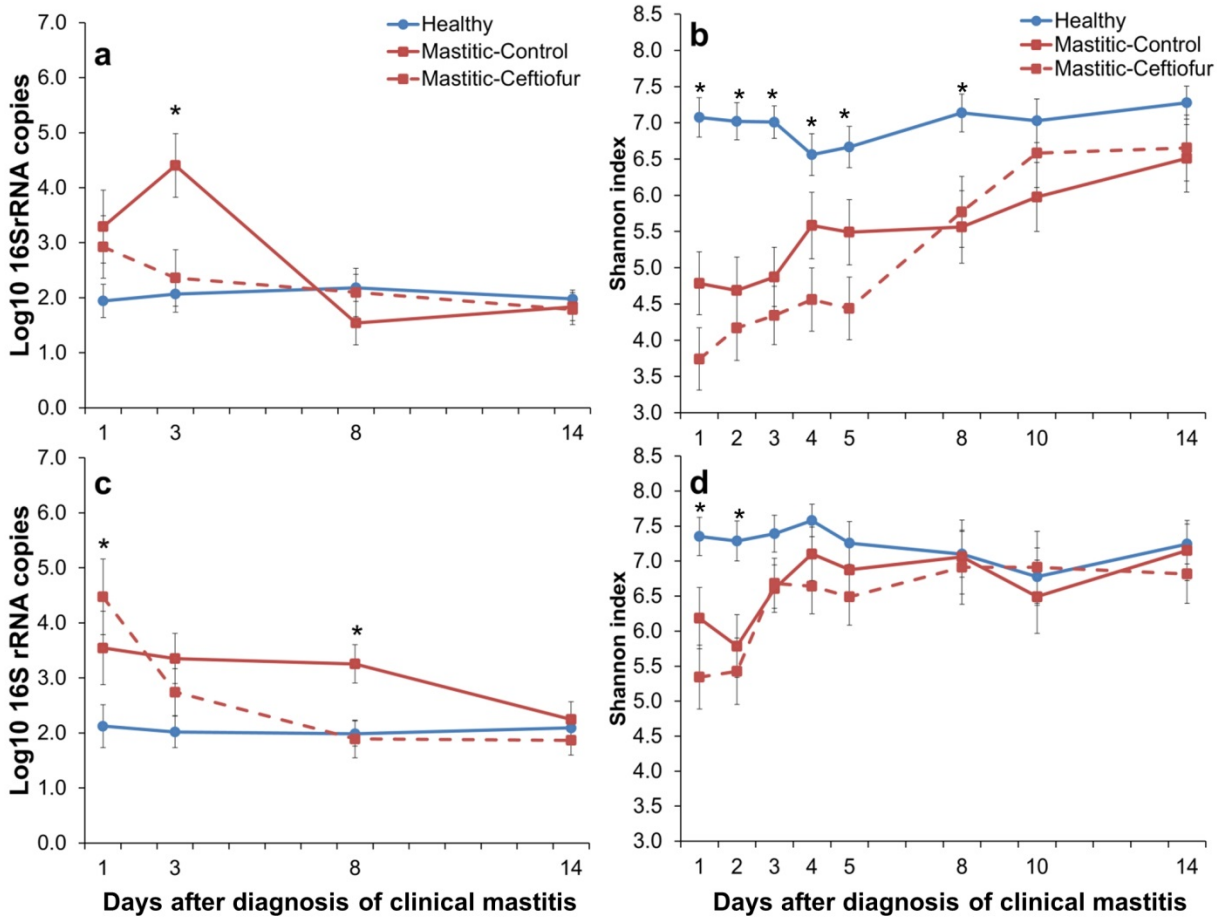


Figure 4.2. Relative abundance of phyla in quarters diagnosed with clinical mastitis (red bars) and healthy quarters (blue bars) according to identification of milk pathogens by laboratory culture. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $^{\dagger}P \leq 0.10$. Bars represent standard error of the mean.

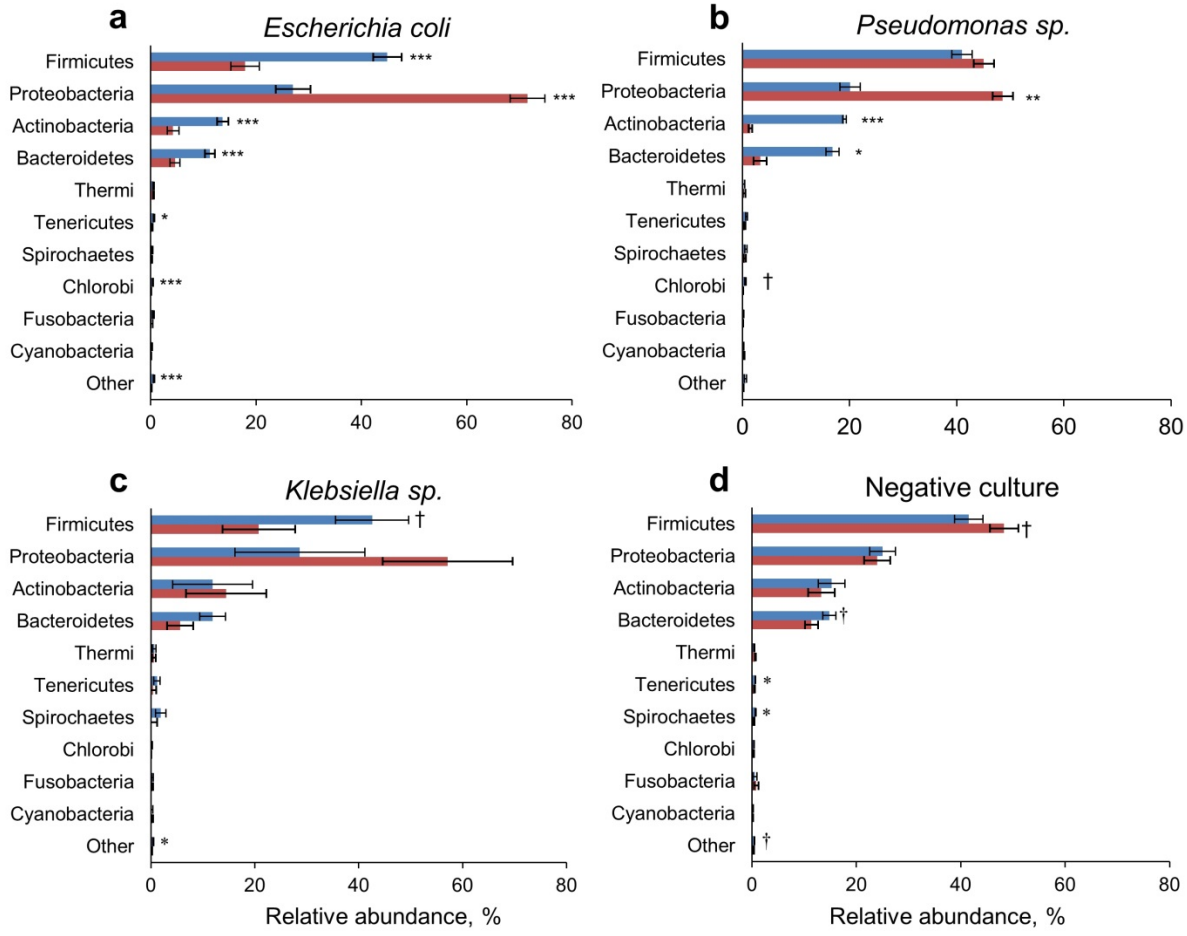


Figure 4.3. Comparison of the microbiome from quarters with clinical mastitis associated with *Escherichia coli* and healthy quarters (i.e. reference for calculation of fold change). Size of the circle is proportional to the overall prevalence of each family. Color of the circle is associated with effect size. The graph plots log fold change in 16S rRNA gene abundance in mastitic relative to healthy control quarters versus false discovery rate (FDR) logWorth (i.e. -log10P). P-values are adjusted for FDR. The dashed line represents the adjusted *P*-value = 0.01.

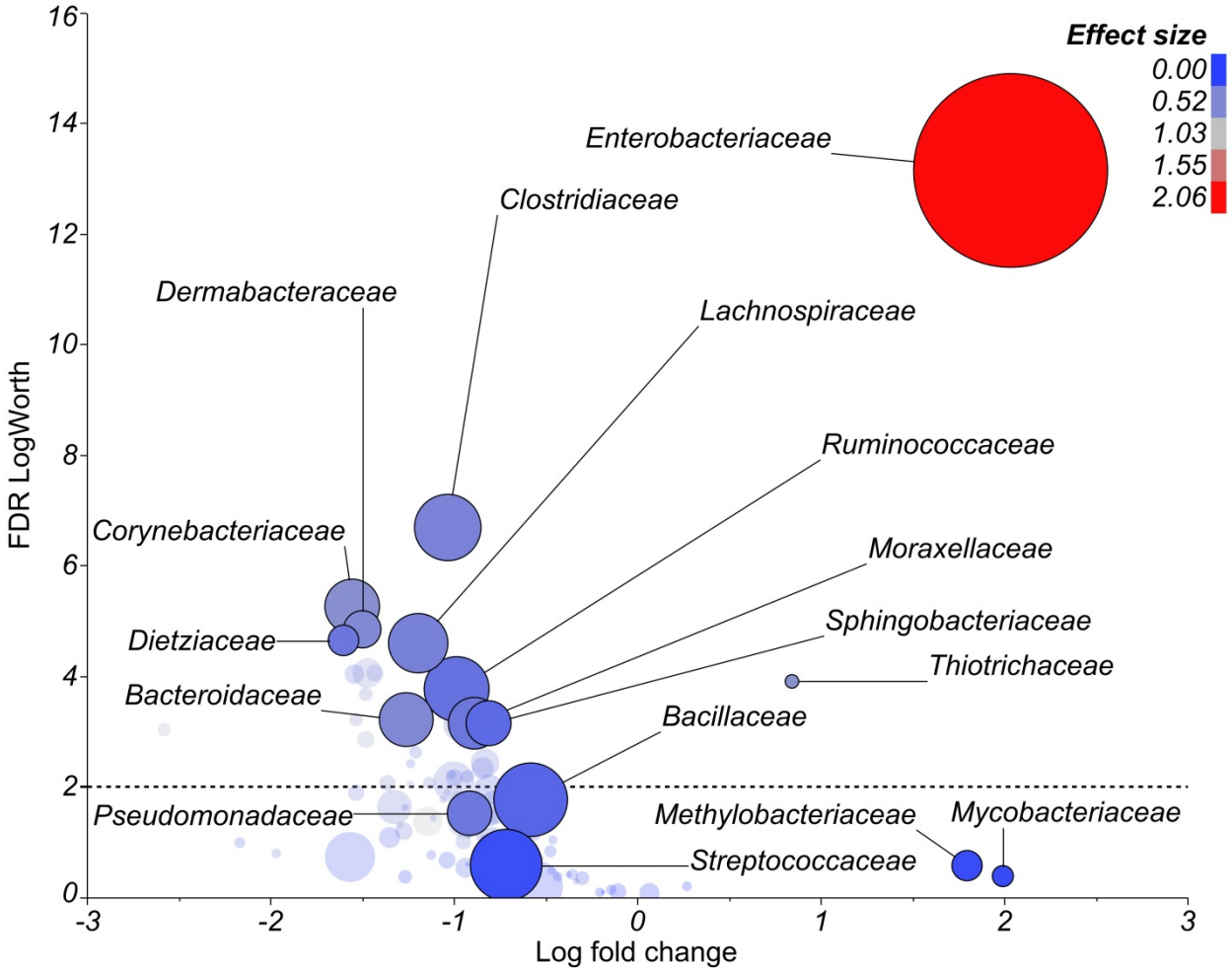


Figure 4.4. Comparison of the microbiome from quarters with clinical mastitis associated with negative culture and healthy quarters (i.e. reference for calculation of fold change) on day 0. Size of the circle is proportional to the overall prevalence of each family. Color of the circle is associated with effect size. The graph plots log fold change in 16S rRNA gene abundance in mastitic relative to healthy control quarters versus false discovery rate (FDR) logWorth (i.e. -log10P). P-values are adjusted for FDR. The dashed line represents the adjusted *P*-value = 0.05.

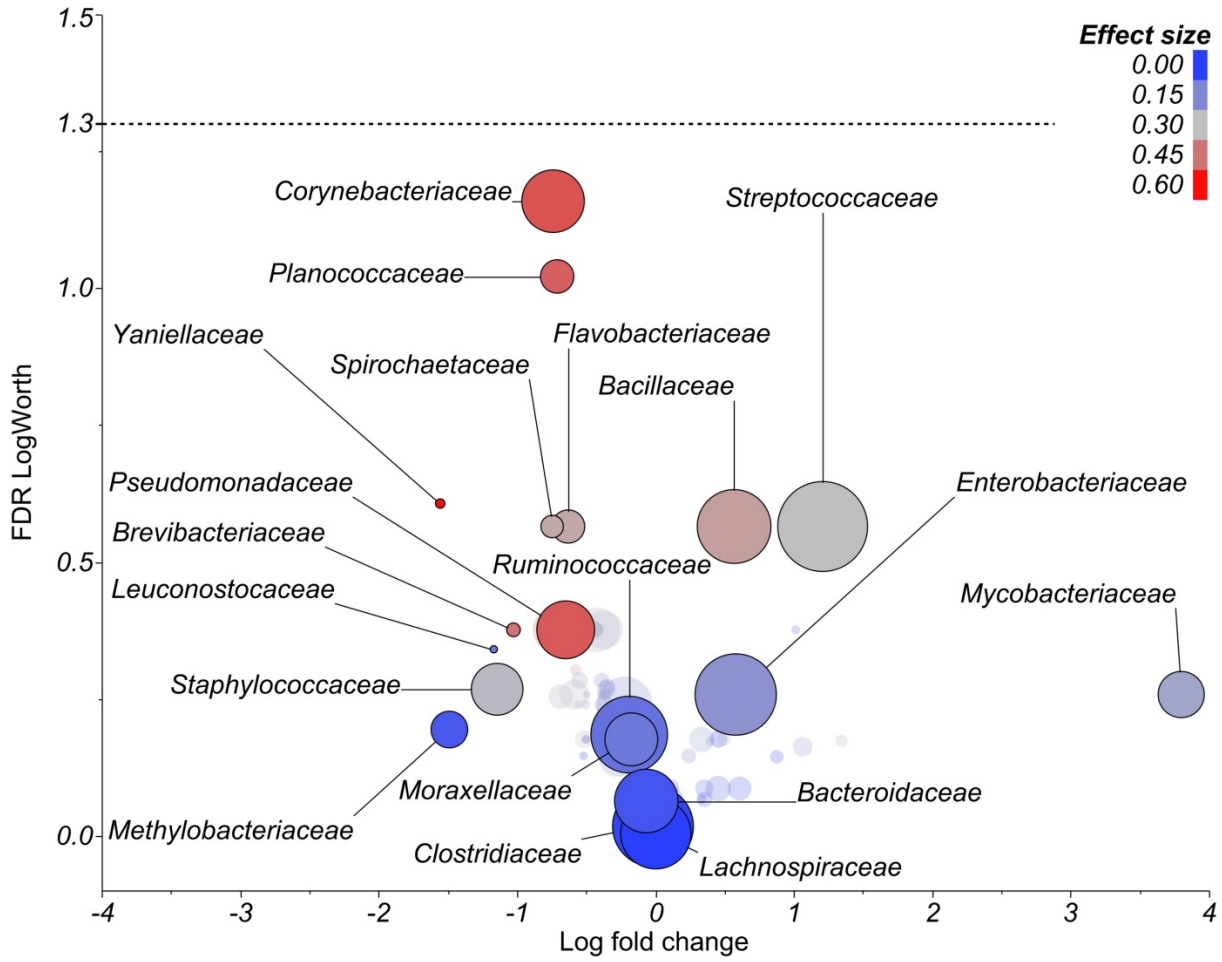


Figure 4.5. Effect of intramammary treatment with ceftiofur hydrochloride on relative abundance of the 25 most prevalent families in milk from quarters with clinical mastitis associated with *Escherichia coli*. Numbers indicate day after diagnosis of clinical mastitis.

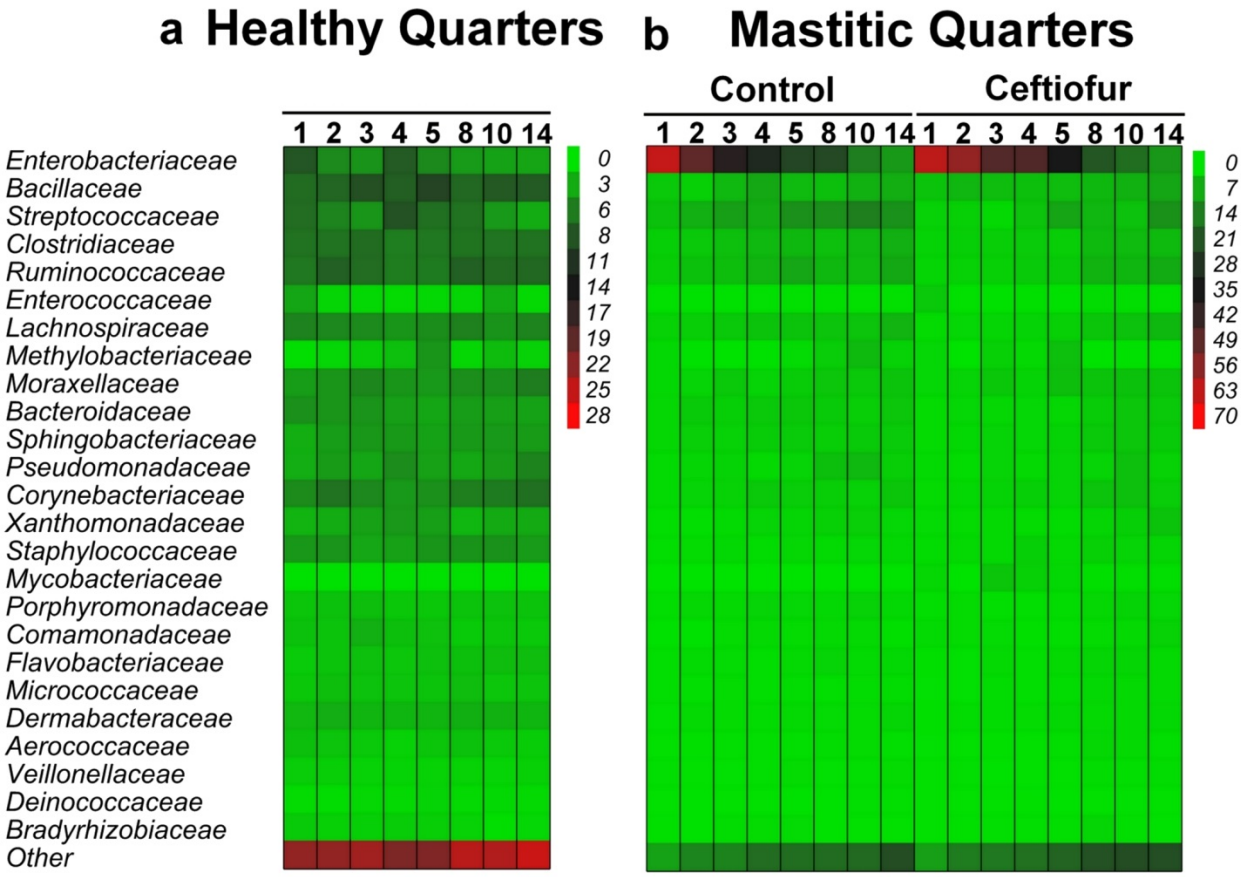


Figure 4.6. Effect of intramammary treatment with ceftiofur hydrochloride (day 1) or cure on the relative abundance of *Enterobacteriaceae* and Shannon diversity index in cows with clinical mastitis associated with *Escherichia coli*. (a) Effect of intramammary treatment with ceftiofur hydrochloride (day 1) on the relative abundance of *Enterobacteriaceae* in cows with clinical mastitis associated with *E. coli*. Effect of eventual clinical cure on the relative abundance of *Enterobacteriaceae* in cows with clinical mastitis associated with *E. coli* on control cows (c) and treated cows (e). Effect of cure on the Shannon index in cows with clinical mastitis associated with *E. coli* (b), on control cows (d) and treated cows (f). Asterisks represent significant differences at $\alpha = 0.05$ between groups within the same study day.

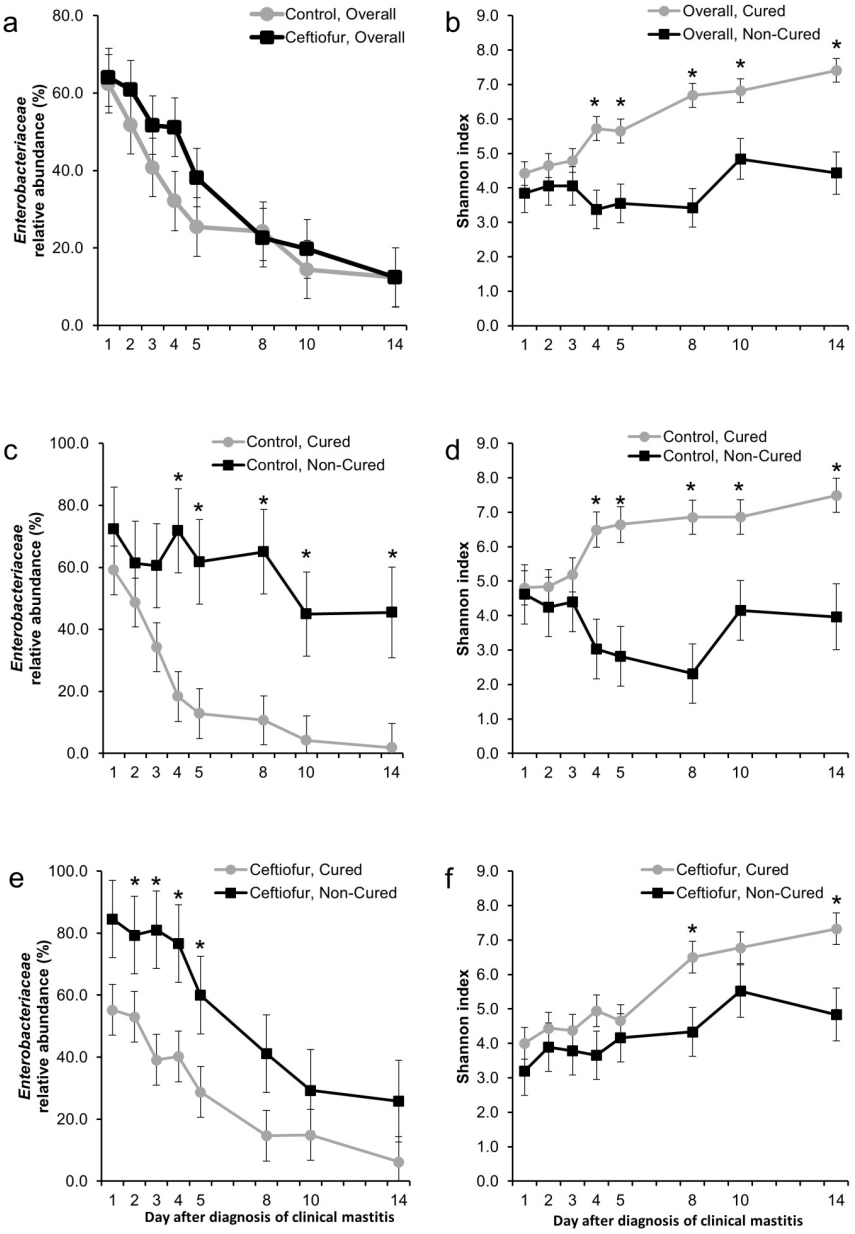


Figure 4.7. Effect of intramammary treatment with ceftiofur hydrochloride on relative abundance of the 25 most prevalent families in milk from quarters with clinical mastitis associated with negative culture. Numbers indicate day after diagnosis of clinical mastitis.

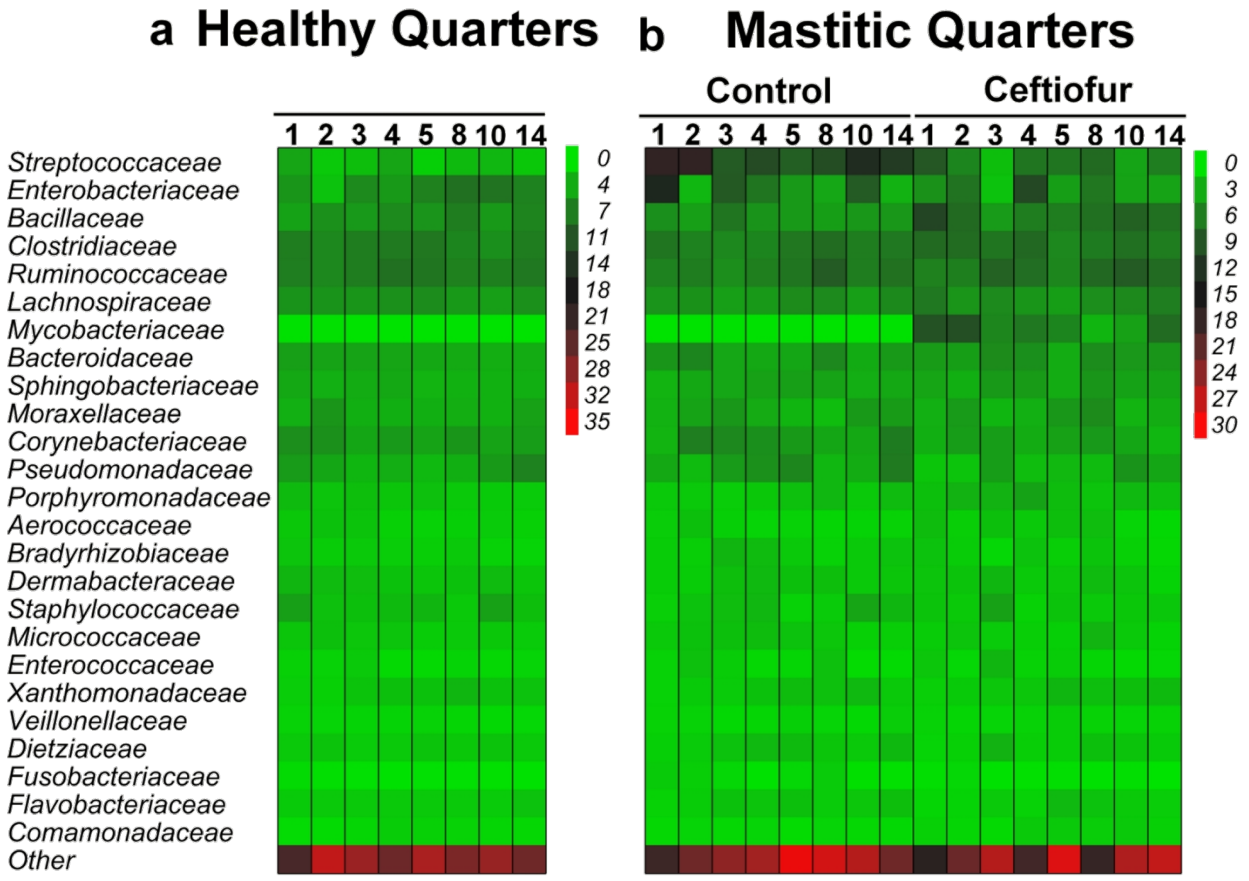
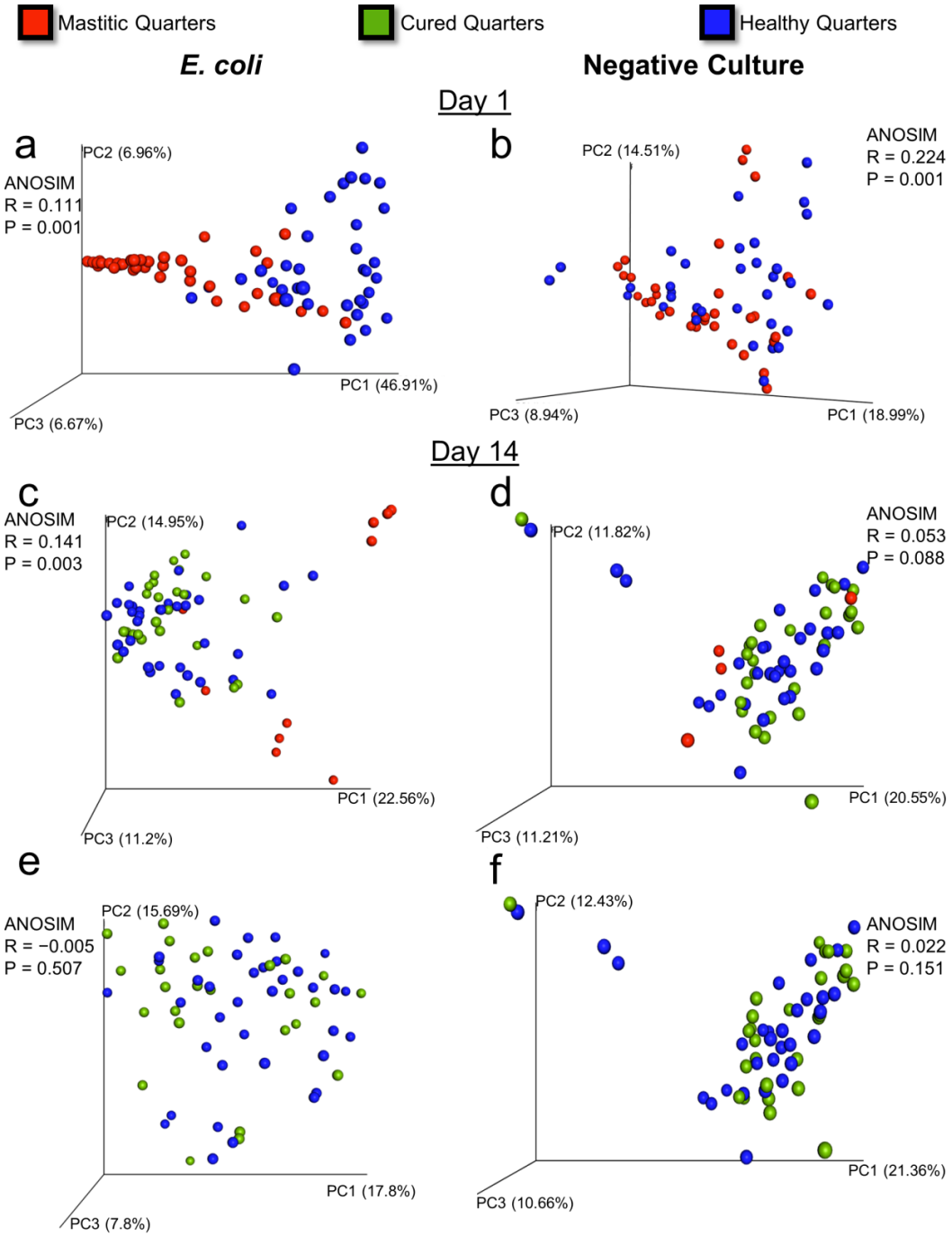


Figure 4.8. Principal coordinate analysis of weighted Unifrac distances and ANOSIM analysis comparing the microbiome data of samples from healthy and mastitic quarters on day 1 (a and b) and day 14 (c, d, e, and f). Samples from quarters with clinical mastitis associated with *E coli* are depicted in sections a, c and e. Samples from quarters with clinical mastitis associated with negative culture are shown in sections b, d, and f.



DISCUSSION

In an endeavor to better understand the effect of a third-generation cephalosporin (ceftiofur) in Gram-negative and culture-negative bovine mammary infections, we used high-throughput DNA sequencing to assess longitudinal changes in the microbiome of mastitic and healthy milk in a randomized clinical trial. Our data demonstrate that antimicrobial treatment did not significantly affect total bacterial load in the infected quarters by the end of the treatment period, nor was it able to increase the rate of pathogen clearance within the mammary gland. Moreover, this is the first study to document in depth the dynamics of the milk microbiota longitudinally using state-of-the art technology.

Treatment with ceftiofur did not affect clinical or bacteriological cure and did not have long-lasting effects on the milk microbiome. Our results are in disagreement with those of Schukken et al. (2011), who reported 38% bacteriological cure in non-treated cows and 73% bacteriological cure in treated animals. Their 5-day intramammary treatment regime with ceftiofur resulted in a significant increase in bacteriological cure, particularly in animals infected with *E. coli*, whereas our results demonstrate no difference between treated and untreated animals in this aspect. However, our results are in agreement with those of a landmark study conducted by Lago et al. (2011), which demonstrated that selective antimicrobial treatment of mastitic cows can lead to a considerable reduction in antimicrobial use without any significant differences in days to clinical cure, bacteriological cure risk, new intramammary infection risk or treatment failure. In that study, cows diagnosed with mastitis associated with *E. coli* either received two intramammary doses of cephapirin sodium 12 hours apart or did not receive antimicrobial treatment. The investigators were not able to detect any differences between

treated and untreated animals in either clinical or bacteriological cures. It is important to acknowledge that the treatment applied in that study utilized a first-generation cephalosporin, which has a lower effectiveness against Gram-negative pathogens compared to ceftiofur (Schukken et al., 2013).

Despite the observed effect of intramammary infusion of ceftiofur in reducing the total bacterial load measured by qPCR of the V4 region of the 16sRNA gene in the affected quarter on day 3, we failed to detect any differences between treated and untreated quarters at days 8 and 14 post diagnosis. Furthermore, our data revealed that ceftiofur therapy had no effect on total bacterial load 3 days after cessation of treatment. We also assessed the longitudinal effect of antibiotic therapy on the relative abundance of the causal mastitis pathogens between treated and untreated cows; again, no differences were observed between the treatment and control groups. The observation that pathogen load was not affected by antimicrobial treatment is substantiated by a consistent decrease in the relative abundance of *Enterobacteriaceae* at 14 days post diagnosis in both treatment arms. Lastly, regardless of the treatment group, milk samples obtained on day 14 from all quarters deemed as mastitic on day 0 and that had normal milk on day 14 all presented a similar, more diverse bacterial profile, one remarkably comparable to that in healthy milk. Our data demonstrate that antimicrobial therapy does not improve cure rates for mastitis caused by *E. coli*, given the similar patterns of reduction in the percentage of pathogens over time in treated versus non-treated animals, which is in line with the results of Leininger et al. (2003) and the recommendations of Suojala et al. (2013).

We have demonstrated how the microbiome of mastitic quarters associated with Gram-negative pathogens dynamically changes over time. More interestingly, quarters that were not

cured by the end of the study period had diverging abundances of *Enterobacteriaceae* and microbial diversities over time when compared to mastitic quarters that became healthy by the end of the study period. Reduced bacterial diversity has also been reported in other studies comparing samples derived from healthy and diseased mammary environments (Oikonomou et al., 2012, Oikonomou et al., 2014, Jimenez et al., 2015). Although, most mastitis cases caused by *E. coli* are of an acute/peracute nature and have a high self-cure rate, chronic cases have been reported in the literature (Dogan et al., 2006, Fairbrother et al., 2015). Further research is needed to understand the host and pathogen idiosyncrasies that are associated with the chronification of these *E. coli*-related mastitis cases.

Elucidating the milk microbiome has been a daunting task (Addis et al., 2016), particularly in clinical mastitis with negative culture results (Kuehn et al., 2013). Various reasons could explain why a negative result might be obtained from a mastitic milk culture: the microorganisms associated with the infection might be shed intermittently; or the number of viable bacterial cells are small; finally, the cow's immune system might have eliminated the pathogen, and the observation of abnormal milk could be a consequence of the inflammatory process that occurred during destruction of the pathogen (Britten, 2012). Nevertheless, mastitis has also been reported to be caused by mechanical or chemical injury, as well as by non-bacterial infectious agents such as viruses (Wellenberg et al., 2002) and yeasts (Dworecka-Kaszak et al., 2012). Although infrequent, it is important to acknowledge that a portion of these culture negative mastitis cases can be result of a viral infection playing a role in clinical mastitis. In fact, the historical role of viruses in mastitis might have been underestimated, given that the practice of laboratory diagnosis of viruses in mastitis cases is unusual (Wellenberg et al., 2002).

In our study, mastitic quarters yielding a negative aerobic result differed in bacterial load compared to their healthy counterparts. This is a very interesting finding, as we were not able to identify in the microbial profiles any specific bacterial family that could be associated with these mastitis cases. The identification of a higher bacterial load not linked to a specific group of pathogens might indicate that dysbiosis occurs not only with changes in the composition of the mammary microbiota, but also with a simple nonspecific increase of intramammary bacterial load, leading to clinical signs of mastitis. It is true that the number of 16S rRNA copies in the genome is variable, which can impact bacterial community analysis (Vetrovsky and Baldrian, 2013); however, the quantification of 16S rRNA gene has been proved to be useful as a proxy for estimating bacterial load (Nadkarni et al., 2002). In our study, mastitic quarters exhibited significantly lower microbial diversity upon diagnosis compared to healthy quarters, which could indicate that fewer microbes were dominating the milk microbiome. Our results are in line with those of Kuehn et al. (2013), who identified that the microbiome of mastitic quarters is less diverse than healthy ones in culture-negative mastitis cases. Recent work by Falentin et. al. (2016) has raised an interesting discussion when it comes to microbial diversity, dysbiosis and disease. The investigators demonstrated that animals presenting normal milk at sampling, but with different histories in regards of clinical and subclinical mastitis had remarkably different bacterial diversity, as well as an altered microbial profile far from an episode of clinical mastitis. Research is warranted to determine the relationship between changes of the mammary microbiota and timing of clinical mastitis, and elucidate if a shift in the microbial profile predisposes to clinical mastitis, or if an active colonization of a rather normal microbiome is to be held accountable for both the clinical episode and the lasting effect on the alteration of the

milk microbiome. Koskinen et al. (2010) evaluated the use of a pathogen-specific real-time PCR assay for identification of mastitis bacteria and reported that 76% of culture-negative clinical mastitis samples were positive for various mastitis pathogens, including members of the *Streptococci*, *Staphylococci* and *Enterobacteriaceae* families. However, it has previously been reported that such bacteria are found in the microbiome of healthy milk of both humans and cows (Martin et al., 2007, Hunt et al., 2011, Oikonomou et al., 2014). Although infrequent, mastitis caused by different species of *Mycobacterium* has been reported in bovines, alpacas and dogs (Menard et al., 1983, Schultze and Brasso, 1987, Richey et al., 2011, Murai et al., 2013). *Mycobacterium* is often misidentified as a negative culture due to its slow growth characteristics and because it is a facultative anaerobic microbe (Menard et al., 1983, Franco et al., 2013). In our results, we observed a non-significant increase in the relative abundance of *Mycobacteriaceae* in mastitic animals yielding negative aerobic culture results. Identifying which microorganisms are associated with culture-negative mastitis does not justify the use of antimicrobial treatment; however, it does shed light on the bacterial etiology of the disease, facilitating decision-making regarding mastitis prevention strategies.

Differences in the microbiome of healthy and mastitic milk samples have also been reported for cows (Oikonomou et al., 2012, Kuehn et al., 2013, Oikonomou et al., 2014) and humans (Hunt et al., 2011, Jimenez et al., 2015). However, a unique feature of the research presented here is that we used a controlled randomized clinical trial approach to longitudinally describe the differences between milk from mastitic mammary glands and from healthy ones and the impact of antibiotic therapy on the microbiome from the onset of disease until its resolution. To our knowledge, this is the first study to longitudinally evaluate the effect of antimicrobial

therapy using the combination of quantitative PCR and next-generation DNA sequencing in dairy cows. Bovine milk, similarly to human milk, exhibits a complex and dynamic microbial ecology (Hunt et al., 2011, Bhatt et al., 2012, Oikonomou et al., 2012, Kuehn et al., 2013, Oikonomou et al., 2014, Jimenez et al., 2015). Nevertheless, significant efforts have been recently undertaken using culture-independent techniques to evaluate the effects of antibiotic therapy in swine (Kim et al., 2016), horses (Costa et al., 2015), gorillas (Vlckova et al., 2016), and humans (Stressmann et al., 2012, Zhao et al., 2012, IHMP Research Network Consortium, 2014, Lax et al., 2014, Zhou et al., 2015).

Antimicrobial use in the food industry could potentially impact human health, warranting its judicious use (Wittum, 2012, van Helden et al., 2013). Ceftiofur is the only FDA-approved third-generation cephalosporin for use in food-producing animals and has been classified by the World Health Organization as one of the critically important antimicrobials for human medicine (WHO, 2012). In summary, our work corroborates the existing literature and also provides novel evidence that the use of intramammary ceftiofur therapy for the treatment of mild and moderate cases of *E. coli*-caused and culture-negative mastitis is ineffective. More importantly, it suggests that antimicrobial stewardship in food animals can be achieved in certain situations without compromising the health of the animals. Additionally, the combined use of quantitative PCR and sequencing of the 16s rRNA gene is an effective approach to evaluate the usefulness of antibiotic therapy.

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AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: RCB, GO, YHS. Performed the experiments: EKG, SFL, KK, DHD. Analyzed the data: EKG RSB RCB. Wrote the paper: EKG RSB RCB, Revised the manuscript: YHS, GO. All authors reviewed the manuscript.

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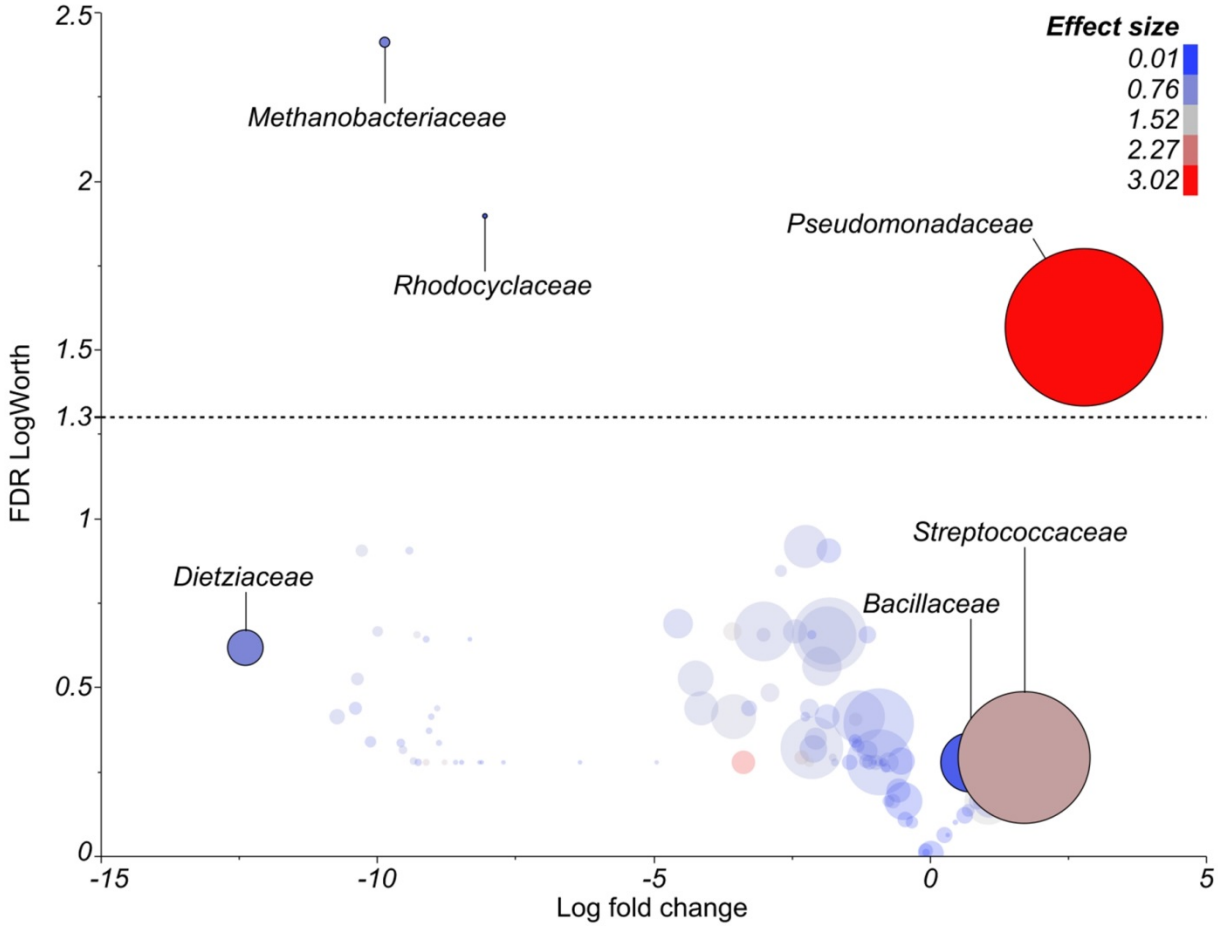
SUPPLEMENTARY INFORMATION

Figure 4.S1. Comparison of the microbiome from quarters with clinical mastitis associated with *Pseudomonas* spp. and healthy quarters (i.e. reference for calculation of fold change). Size of the circle is proportional to the overall prevalence of each family. Color of the circle is associated with effect size. The graph plots log fold change in 16S rRNA gene abundance in mastitic relative to healthy control quarters versus false discovery rate (FDR) logWorth (i.e. -log10P). P-



values are adjusted for FDR. The dashed line represents adjusted *P*-value = 0.05.

Figure 4.S2. Effect of clinical mastitis and intramammary treatment with ceftiofur hydrochloride on richness of the milk microbiome in cows with clinical mastitis associated with *Escherichia coli* (a) and in cows with clinical mastitis associated with a negative culture (b).

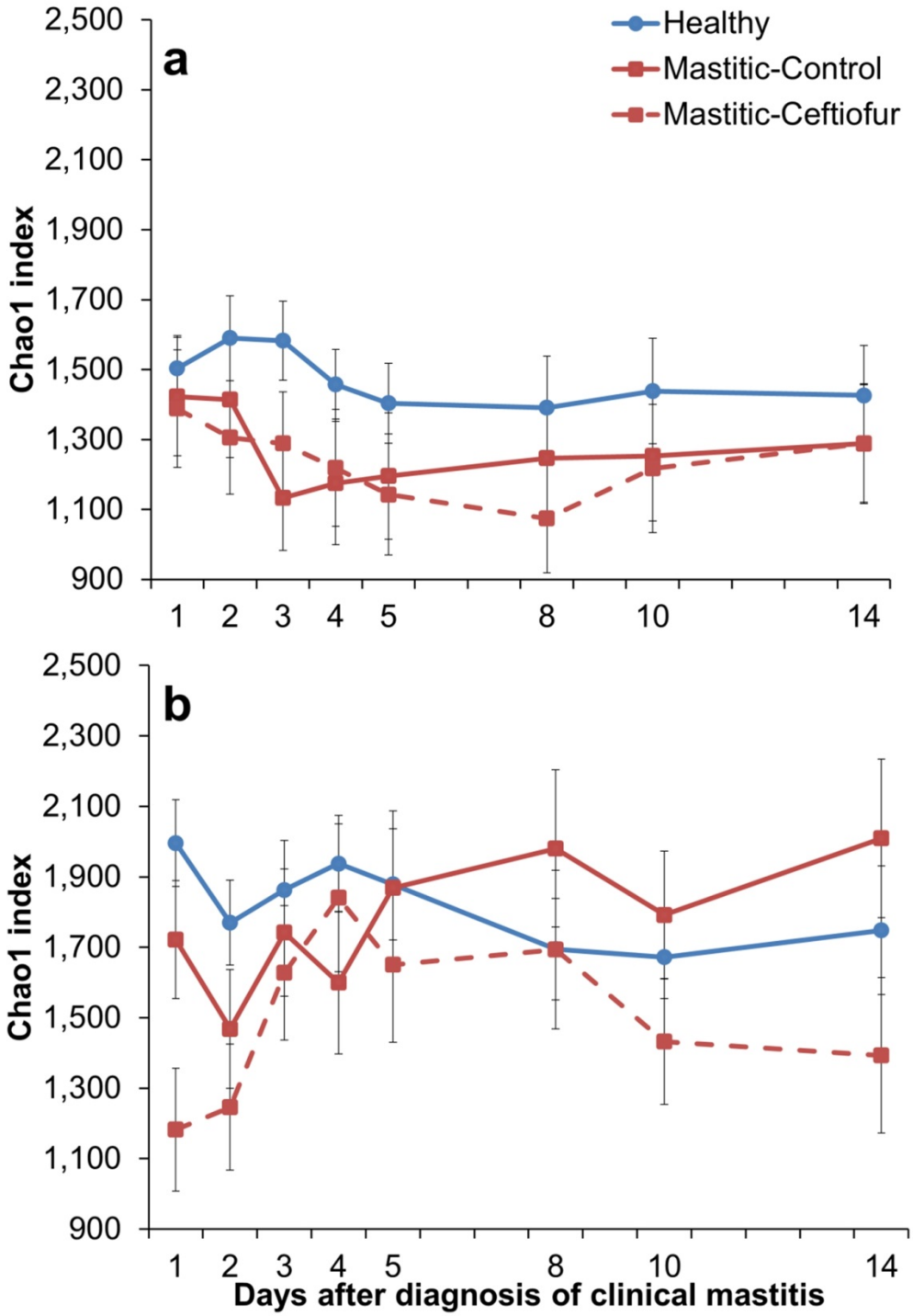


Figure 4.S3. Comparison of the microbiome from quarters with clinical mastitis associated with *Klebsiella* spp. and healthy quarters (i.e. reference for calculation of fold change). Size of the circle is proportional to the overall prevalence of each family. Color of the circle is associated with effect size. The graph plots log fold change in 16S rRNA gene abundance in mastitic relative to healthy control quarters versus false discovery rate (FDR) logWorth (i.e. -log10P). P-values are adjusted for FDR.

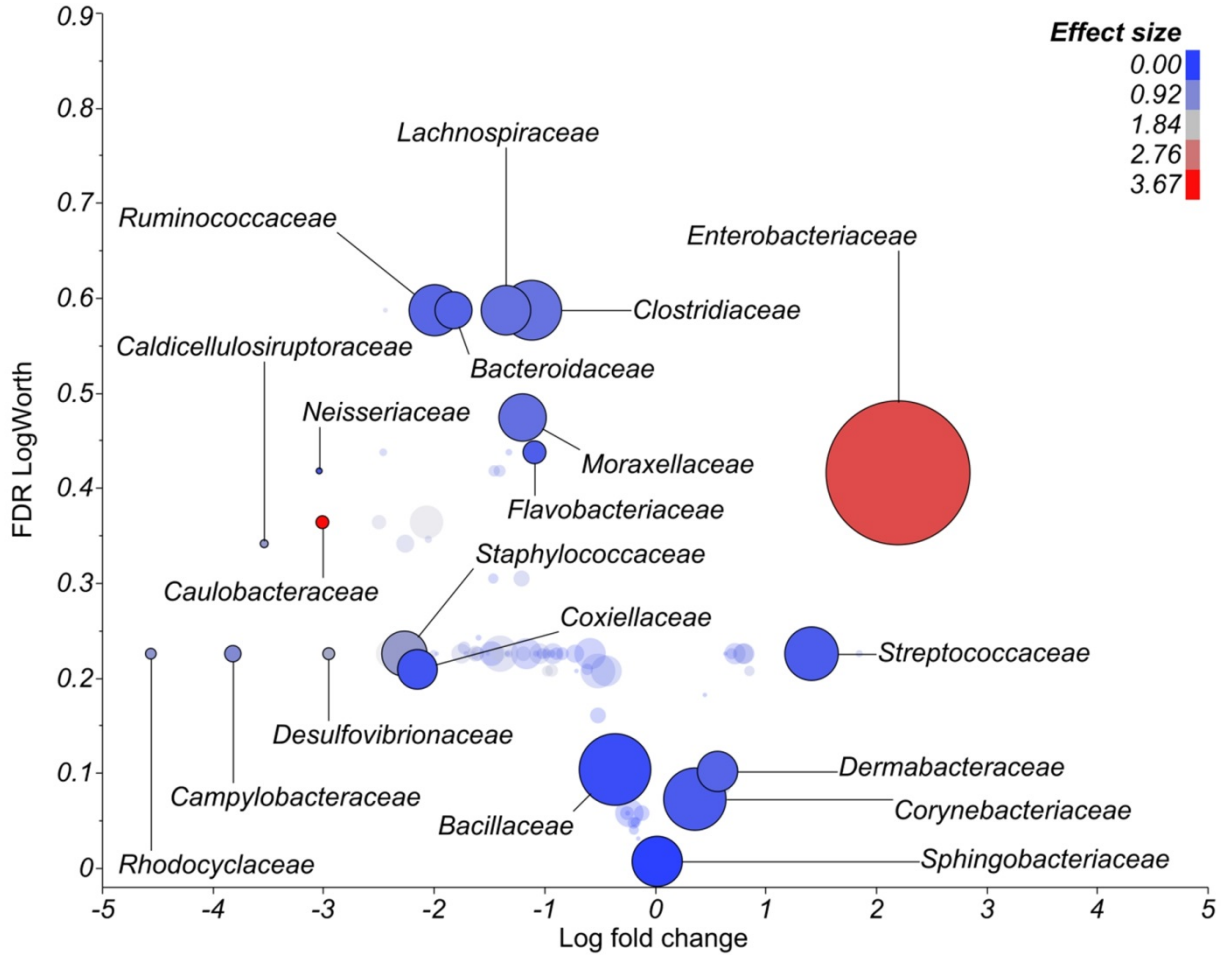


Figure 4.S4. Effect of clinical mastitis and intramammary treatment with ceftiofur hydrochloride on diversity (a) and richness (b) of the milk microbiome in cows with clinical mastitis associated with *Klebsiella* spp.

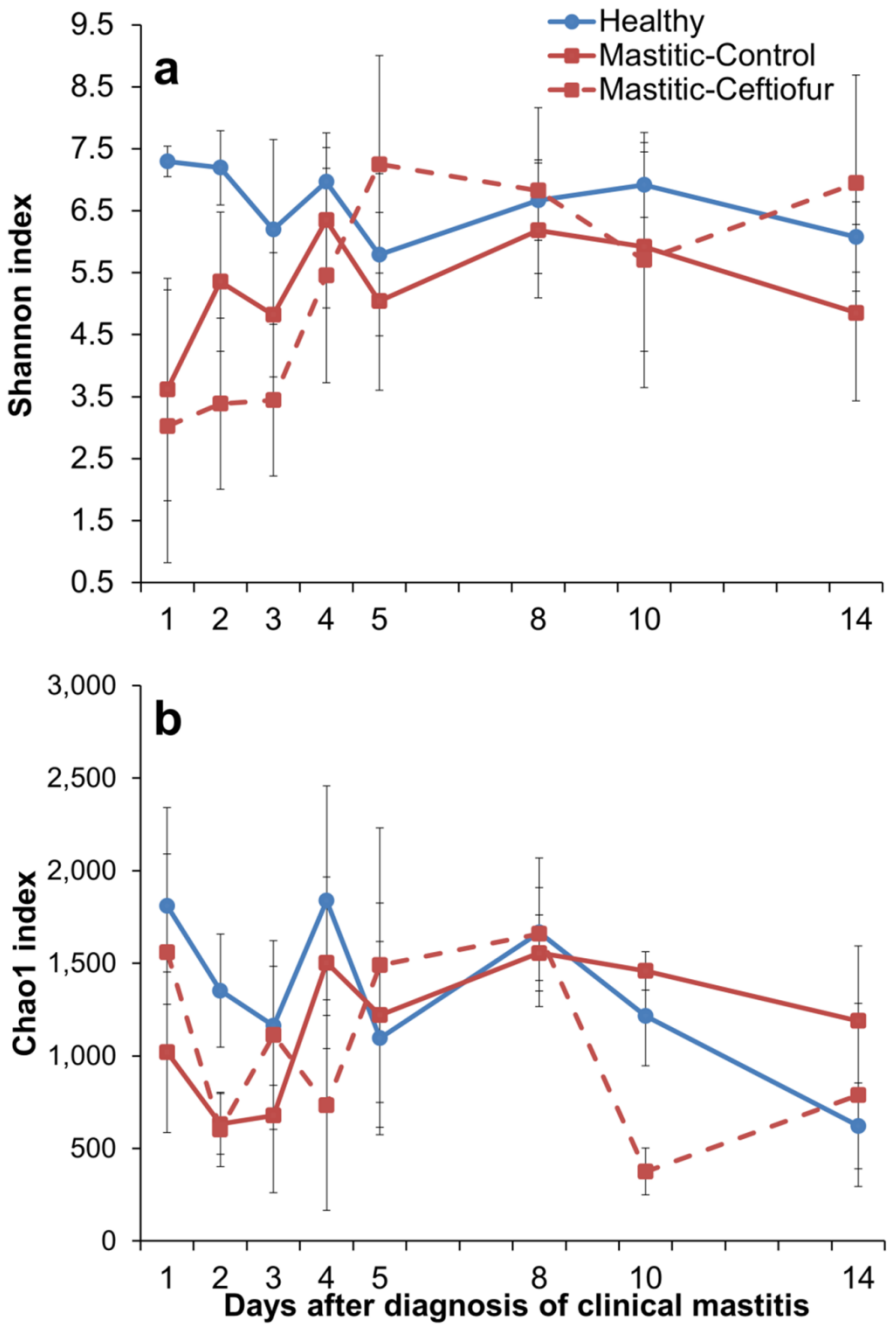


Figure 4.S5. Changes in relative abundance of the 25 most prevalent families in milk from quarters with clinical mastitis associated with *Klebsiella* spp. and healthy quarters. Numbers indicate day after diagnosis of clinical mastitis.

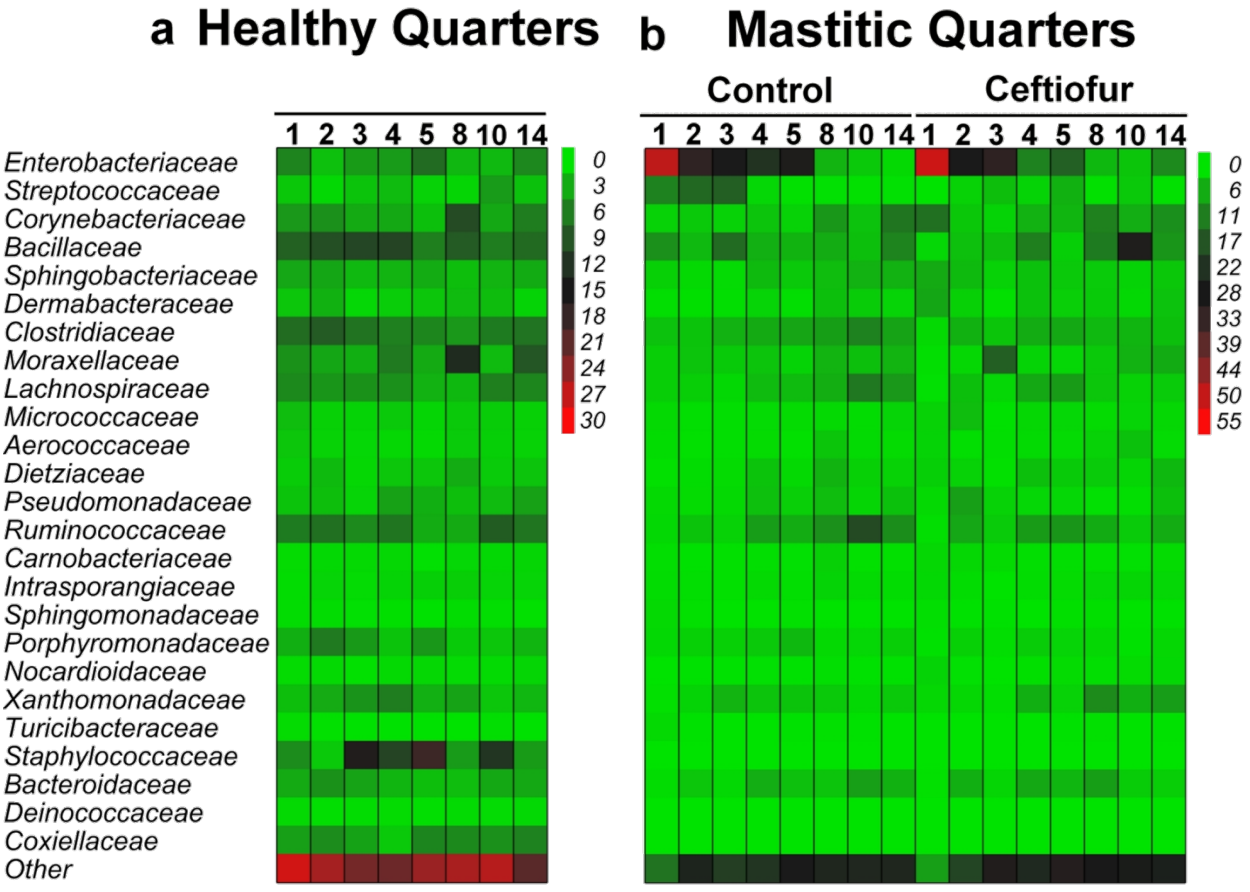


Figure 4.S6. Effect of intramammary treatment with ceftiofur hydrochloride (day 1) on the relative abundance of *Enterobacteriaceae* in cows with clinical mastitis associated with *Klebsiella* spp. TRT = effect of treatment; Day = effect of time; T×D = interaction between treatment and day.

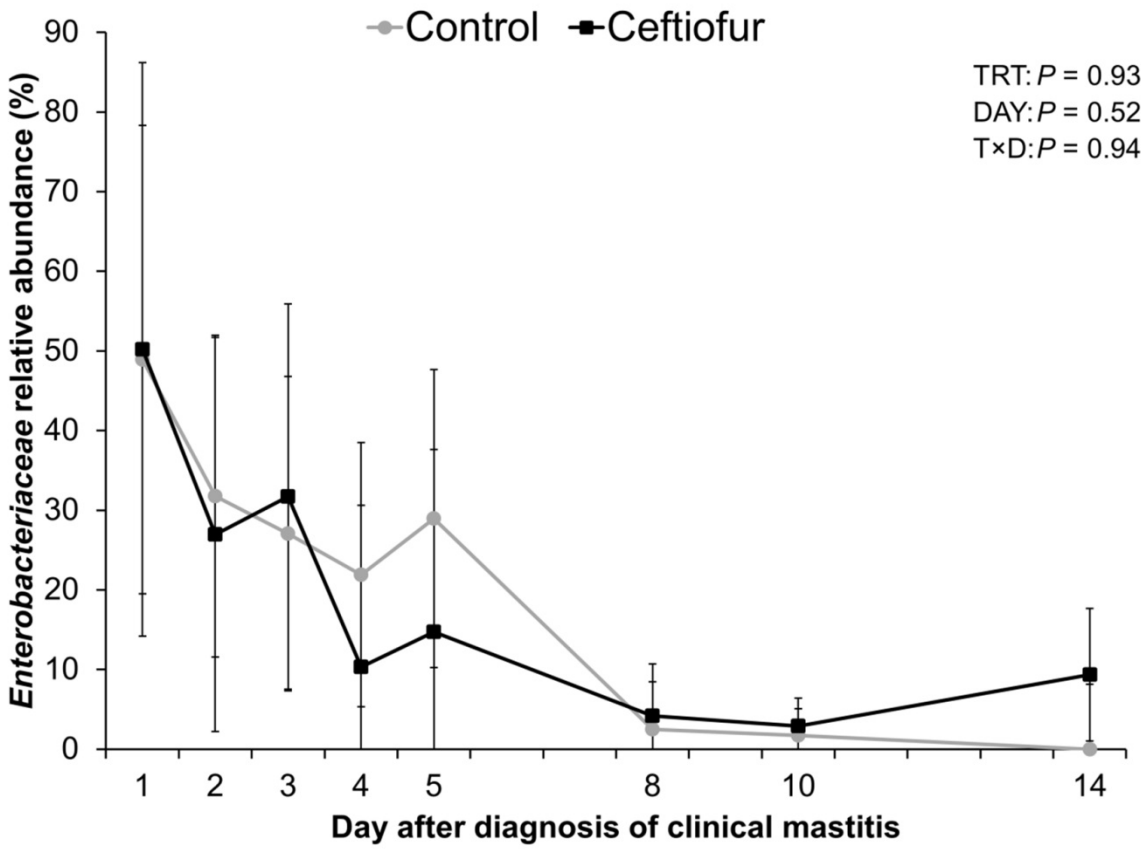
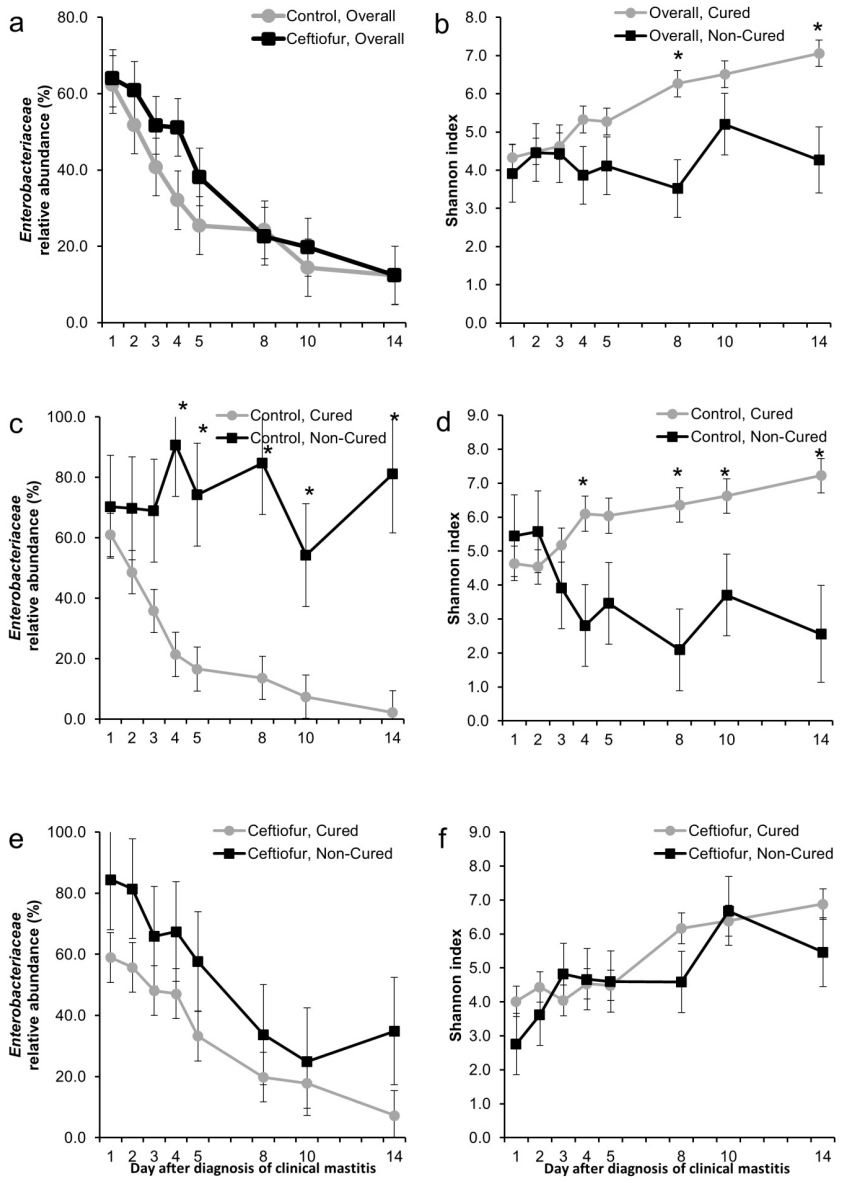


Figure 4.S7. Effect of intramammary treatment with ceftiofur hydrochloride (day 1) or cure on the relative abundance of *Enterobacteriaceae* and Shannon diversity index in cows with clinical mastitis associated with *Escherichia coli*. (a) Effect of intramammary treatment with ceftiofur hydrochloride (day 1) on the relative abundance of *Enterobacteriaceae* in cows with clinical mastitis associated with *E. coli*. Effect of eventual bacteriological cure on the relative abundance of *Enterobacteriaceae* in cows with clinical mastitis associated with *E. coli* on control cows (c) and treated cows (e). Effect of cure on the Shannon index in cows with clinical mastitis associated with *E. coli* (b), on control cows (d) and treated cows (f). Asterisks represent significant differences at $\alpha = 0.05$ between groups within the same study day.



CHAPTER 5

NORMAL MILK MICROBIOME IS REESTABLISHED FOLLOWING EXPERIMENTAL INFECTION WITH *ESCHERICHIA COLI* INDEPENDENT OF INTRAMAMMARY ANTIBIOTIC TREATMENT WITH A THIRD-GENERATION CEPHALOSPORIN IN BOVINES

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ABSTRACT

The use of antimicrobials in food animals and the emergence of antimicrobial resistance are global concerns. Ceftiofur is the only third-generation cephalosporin labeled for veterinary use in the USA, and it is the drug of choice in the majority of dairy farms for the treatment of mastitis. Here, we use next-generation sequencing to describe longitudinal changes that occur in the milk microbiome before, during, and after infection and treatment with ceftiofur. Twelve animals were intramammary challenged with Escherichia coli in one quarter and randomly allocated to receive intramammary treatment with ceftiofur (5d) or untreated controls. Serial samples were collected from −72 to 216 h relative to challenge from the challenged quarter, an ipsilateral quarter assigned to the same treatment group, and from a third quarter that did not undergo intervention. Infection with E. coli dramatically impacted microbial diversity. Ceftiofur significantly decreased LogCFUs but had no significant effect on the milk microbiome, rate of pathogen clearance, or somatic cell count. At the end of the study, the microbial profile of infected quarters was indistinguishable from pre-challenge samples in both treated and untreated animals. Intramammary infusion with ceftiofur did not alter the healthy milk (i.e., milk devoid of lots or serous appearance and collected from a mammary gland that shows no clinical signs of mastitis) microbiome. Our results indicate that the mammary gland harbors a resilient microbiome, capable of reestablishing itself after experimental infection with E. coli independent of antimicrobial treatment.

Keywords: Milk microbiome, Mastitis, E. coli, Ceftiofur, Dairy cattle, Antimicrobial treatment, Milk, Third-generation cephalosporin, Cephalosporins

INTRODUCTION

Mastitis is a prevalent, costly (Heikkila et al., 2012, Rollin et al., 2015) disease in dairy cows that is defined by increase in milk somatic cell count (**SCC**) as a result of inflammation in the mammary gland, leading to abnormal milk and varying degrees of clinical severity. This condition affects almost 25% of the 9.3 million dairy cows present in the United States every year (USDA, 2016), and negatively impacts animal welfare (Medrano-Galarza et al., 2012, Fogsgaard et al., 2015, Peters et al., 2015) and productivity (Grohn et al., 2004, Bar et al., 2008, Schukken et al., 2009). Recent studies have reported that approximately 80% of all antimicrobials used on American dairy farms are for the treatment or prevention of mastitis (Pol and Ruegg, 2007). Prevention measures, improved management, and sanitation have reduced the number of contagious mastitis cases and have led to a change in the etiology of the disease in the last decade (Bushnell, 1984, Jones, 2012, USDA, 2016), making opportunistic environmental pathogens, including coliforms, major contributors to clinical mastitis.

Antimicrobial resistance is a global concern and has led to increasing attention regarding the judicious use of antibiotics. Although conflicting evidence is available on whether human, companion, and/or livestock medicine is responsible for the emergence of antimicrobial resistance (Ma et al., 2016, Pal et al., 2016), the livestock industry has been recognized as one of the main causes (Pal et al., 2016, Pitta et al., 2016); perhaps due to the amount of antimicrobials used in this sector (Muziasari et al., 2016), affecting humans either through direct contact or through the food chain (Singer et al., 2003, Lhermie et al., 2016). The increasing demand for animal protein is believed to be accompanied by a significant growth in need for antimicrobial

use in food animal production, which is predicted to rise by approximately 67% between 2010 and 2030 (Lhermie et al., 2016). Responsible use of antimicrobials in food animals is paramount for maintenance of both animal and human health (FDA, 2017) and has been one of the policies proposed by the One Health Initiative (The One Health Initiative, van Helden et al., 2013, Dar et al., 2016). Ceftiofur is the only third generation cephalosporin labeled for veterinary use in the United States (USDA, 2016), is considered a critically important antimicrobial for human medicine (WHO, 2012), and is the drug of choice in the majority of dairy operations for intramammary treatment of mastitis (USDA, 2016). To this date, the effectiveness of the treatment of coliform mastitis has been discussed in an ambiguous manner (Schukken et al., 2011a, Suojala et al., 2013). Nevertheless, all information regarding the effectiveness of antimicrobial therapy reported so far is based on clinical cure and the ability to isolate and culture a specific pathogen from a mastitic milk sample in a laboratory setting. Culture independent studies resulted in significant proof for the existence of a resident microbiota in milk in humans (Hunt et al., 2011, Boix-Amoros et al., 2016), bovines (Bhatt et al., 2012, Oikonomou et al., 2012, Lima et al., 2017), and other species (Quigley et al., 2013). In light of constantly advancing molecular techniques, the use of next generation sequencing led to a paradigm shift in the understanding of the dynamics of health and disease (Oikonomou et al., 2014, Falentin et al., 2016) and offers an opportunity to provide evidence that will substantiate antibiotic stewardship, helping the optimization of preventative, diagnostic, and treatment protocols for bovine mastitis. Therefore detailed information on the effect of treatment of *Escherichia coli* mastitis with third generation cephalosporins is indispensable.

Our group has recently described the dynamics of milk microbiome upon antimicrobial treatment with ceftiofur in animals naturally infected with mastitis pathogens (Ganda et al., 2016). In that study, cows from a commercial dairy farm were enrolled upon diagnosis of clinical mastitis and randomly allocated to receive extended intramammary therapy with ceftiofur, or to receive no treatment. We observed that in cows with mastitis caused by *E. coli,* treatment with ceftiofur did not offer an advantage when compared to no treatment in terms of clinical cure, pathogen clearance rate, or bacterial load. Moreover, the milk microbiome from the affected quarters was indistinguishable from the adjacent healthy quarters within 14 days following the onset of the disease regardless or intramammary antimicrobial administration.

Herein we describe a challenge model using a known strain of mastitis-causing *E. coli* to characterize the microbiome before, during, and after intramammary infection in a controlled setting. We aim to investigate the changes that occur upon introduction of a major pathogen, and the ability of the healthy mammary microbiota to restore equilibrium with or without external aid of antimicrobials. Moreover, the role of antimicrobials in the normal milk microbiota has not yet been investigated in controlled longitudinal studies. We hypothesize that intramammary administration of a broad-spectrum antimicrobial might favor the overgrowth of specific organisms and incite a shift in the microbial profile of milk from normal quarters.

Therefore, the objectives of this study were to generate knowledge on the complex microbial ecology and treatment of mastitis, more specifically: a) describe the milk microbiome before, during, and after the infection of bovine mammary glands with a strain of *E. coli* previously isolated from a mastitis case; b) compare microbial populations between infected and

uninfected quarters, and c) evaluate the impact of a third generation cephalosporin on both healthy and mastitic milk.

MATERIALS AND METHODS

Ethics Approval

All experimental procedures were carried out at Cornell University, according to a research protocol that was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2013-0056).

Challenge Strain

The strain used in this study (*E. coli* ECC-Z, Cornell University) (Dogan et al., 2006) hereafter referred as C1, was isolated from a clinical case of bovine mastitis, and was proven effective in previous experimental challenges to result in mild to moderate cases of clinical mastitis (Quesnell et al., 2012, Sipka et al., 2013). Before experimental challenge, frozen stocks of the strain were activated in Luria-Bertani (**LB**) broth, incubated at 37°C for 12h and subsequently streaked on a McConkey plates for *E. coli* colony isolation. DNA extraction was performed in isolated colonies using a QIAamp DNA minikit (Qiagen Inc., Valencia, CA), and a fragment of the 16S rRNA gene was amplified using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-3'), followed by sequencing at the Cornell University Core Laboratories Center (Ithaca, NY) through Sanger sequencing for confirmation of the identity of the isolate.

Animal Selection and Housing

Twelve mature (second or greater lactation) Holstein cows were selected from the Cornell Veterinary Medicine Teaching Dairy (Ithaca, NY). Six animals, two at a time, were challenged during March of 2014, and six animals were challenged two at a time between March and April of 2015. Experimental challenge and sampling took place at the Large Animal Teaching and Research Unit (**LARTU**), Department of Animal Sciences, Cornell University (Ithaca, NY). Selected animals had no previous cases of clinical mastitis in the current lactation, were between 246 and 461 days in milk, and had an average somatic cell count of 95,000 cells/mL, ranging from 41,000 cells/mL to 191,000 cells/mL, measured one week prior to transportation to LARTU.

Animals were housed individually in stalls bedded with sawdust, and were fed *ad libitum* the same diet provided at the farm from which they were sourced, calculated to meet or exceed the requirements for lactating Holstein cows with a body weight of 650 kg producing 45kg of 3.5% fat corrected milk. The feed was transported daily from the Cornell Teaching Dairy. Animals were milked twice daily, at 08:00 and 20:00.

Sampling Procedures and Experimental Infection

Sampling scheme, treatment allocation, and experimental design are depicted in (**Figure 5.1a,b**). Milk samples were collected every 12 hours during the three days that preceded intramammary infection with *E. coli* strain C1, henceforth indicated as time 0, and every six hours from live challenge with *E. coli* until the 9th day after infection, with the last time point being at 216 hours post-challenge. Only one quarter was challenged: 100 colony forming units

(**CFU**) of *E. coli* C1 suspended in 1mL of phosphate buffered saline (**PBS**) solution was deposited immediately ventral of the Furstenberg's rosette via the teat canal.

Each animal had three quarters sampled at each time point: the challenged quarter, an ipsilateral unchallenged quarter that was included in the same antibiotic treatment group as the challenged one, and a third quarter which did not undergo any intervention (i.e. no challenge, no treatment) and was sampled in every time point as a within-animal control. Before milk sample collection, each cow had teats dipped in iodine (Bovadine sanitizing teat dip, DeLaval Manufacturing, Kansas City, MO), which was left in contact for at least 30 seconds. Teats were wiped with dry sterile gauze and final teat disinfection was performed with gauze soaked in 70% (v/v) ethanol immediately prior to collection of the milk sample. The three initial streams of milk were discarded; milk was collected into sterile tubes in three different aliquots and immediately placed on ice. The first aliquot was collected and kept frozen at -20°C until DNA extraction, the second aliquot was used for CFU counting, and a third aliquot was submitted for SCC determined through flow cytometry (Fossomatic FC, Eden Prairie, MN) at the Dairy One Cooperative Inc. (Ithaca, NY). Linear score (**LS**) was calculated based on SCC according to the equation $LS = [ln(SCC/10^5)/ln(2)] + 3$. Health status and temperature of each cow were assessed at each sampling time, and cows showing signs of systemic illness were provided with appropriate supportive therapy, which included intravenous administration of fluids and intramuscular administration of non-steroidal anti-inflammatory drugs.

Treatment Administration

At 48 hours after infection, animals were randomly allocated into either the treatment group, which received five consecutive intramammary infusions of ceftiofur hydrochloride comprised of 125 mg of ceftiofur equivalents (as ceftiofur hydrochloride; Spectramast LC®, Zoetis, Florham Park, NJ) at 24-hour intervals in both challenged and ipsilateral unchallenged quarters, or the untreated control group, for which no substance was introduced into the mammary glands. The timing of first treatment was chosen in an attempt to mimic the dynamics of culture-based treatment of coliform mastitis in a commercial dairy farm, and the first dose was administered immediately after sample collection of the 48-hour sample.

CFU Counting and Strain Typing

Milk samples collected at time points following intramammary infection were inoculated on MacConkey agar in 100 µL aliquots and incubated at 37°C overnight for bacterial identification. Total CFU/mL was calculated based on quantitative culture of serial milk dilutions in triplicates by averaging the number of colonies in the triplicate and multiplying the number by the dilution factor.

Strain typing was performed through random amplification of polymorphic DNA (**RAPD**) with primers designed specifically for RAPD typing of Gram negative bacteria (forward: 5'-AGTAAGTGACTGGGGTGAGCG-3', reverse: 5'TACATTCGAGGACCCCTAAGTG-3'), which have been shown to discriminate between

different strains of mastitis-causing *E. coli* (Dogan et al., 2006). PCR products were evaluated for banding pattern using gel electrophoresis in a 1.5% agarose gel at 60V for 1.5 h.

DNA Isolation and Purification

Genomic DNA extraction was performed using the PowerFood DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA), following the manufacturer's recommendations with an extra incubation at 65°C for 10 minutes prior to cell disruption to maximize DNA yields (Quigley et al., 2012). A six mL aliquot of milk was divided into fat, whey, and pellet through centrifugation. Whey was discarded and the fat layer and pellet were used as starting sample in DNA extraction, as described previously (Ganda et al., 2016). Concentration and purity of isolated DNA were evaluated based on optical density at 230, 260 and 280 nm wavelengths (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE).

16S rRNA Gene Amplification, MiSeq Sequencing, and Bioinformatic Analyses

Isolated genomic DNA was used as a template for amplification of the V4 hypervariable region of the bacterial 16S rRNA gene using the primers 515F and 806R, which had been optimized for the Illumina MiSeq platform (Illumina Inc., San Diego, CA) (Caporaso et al., 2012) as described previously (Foditsch et al., 2015).

Six runs of the Illumina MiSeq sequencer were needed for sequencing of all samples. In each run, 279 samples and a sequencing control were pooled in an equimolar library and sequenced using the MiSeq reagent kit V2 for 300 cycles. Bioinformatics was performed using quality-filtered indexed reads, which were concatenated into a single FASTA file and uploaded

in the open-source pipeline Quantitative Insights into Microbial Ecology (**QIIME**) version 1.9.1(Caporaso et al., 2010b), using computer resources of the Cornell Boyce Thompson Institute (Ithaca, NY). Sequences were handled following the default settings of the pipeline. Quality filtering was performed as described previously (Bokulich et al., 2013). Open-reference taxonomic assignment into operational taxonomic units (**OTUs**) with 97% identity was achieved using UCLUST (Edgar, 2010), RDP classifier (McDonald et al., 2012), PyNAST (Caporaso et al., 2010a), and the Greengenes (DeSantis et al., 2006) reference database. Rare OTUs with less than 5 sequences in each run, and samples with less than 1000 reads were excluded from the database. Within sample diversity (α-diversity) was assessed through Shannon diversity index calculated in a randomly selected subset of the OTU database obtained through the script single_rarefaction.py in QIIME at a rarefication level of 1,500 reads per sample. Between samples microbial diversity (β-diversity) was assessed through phylogenetic-based weighted UniFrac (Lozupone and Knight, 2005) distances, calculated in QIIME through the script beta_diversity.py and the distance matrix obtained was used for comparison between groups.

Statistical Analyses

The UNIVARIATE procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) was used to perform descriptive analyses. Non-normally distributed variables (i.e. SCC and CFU) were normalized through log transformation. Longitudinal changes in the microbial profile was assessed through description of the relative abundances of the 25 most abundant bacterial families using the tabulate function of JMP Pro 12 (SAS Institute Inc., Cary, NC), and relative abundances of all remaining families were combined into a single cluster, defined as 'Other'.

Variables of interest were evaluated between challenged, unchallenged, treated, and untreated quarters with repeated measures ANOVA using the GLIMMIX procedure of SAS. Significance of pairwise comparisons were adjusted through the Tukey-Kramer multiple comparison correction. Outcomes were the relative abundance of *Enterobacteriaceae*, Shannon diversity index, LogSCC, LS, and LogCFUs; explanatory variables were challenge (challenged versus control quarter), treatment (treated versus untreated quarter), time relative to experimental challenge, and their two- and three-way interactions.

To assess the effect of treatment, stratified analysis of covariance was performed in challenged and unchallenged quarters separately. To account for possible differences that occurred between intramammary infection and first treatment at 48h, the average of values observed between challenge and treatment (i.e. 0, 6, 12, 24, 36, 42, and 48 hours relative to challenge) was included as a covariate in these models. Variables of interest were the relative abundance of *Enterobacteriaceae*, Shannon diversity index, LogSCC, LS, and LogCFUs; explanatory variables were treatment (treated versus untreated quarter), time relative to experimental challenge, and their two-way interactions. Teat nested within cow was considered a random effect in all statistical models. The first-order ante-dependence covariance structure was selected because it resulted in the smallest Schwarz's Bayesian information criterion value. Differences with $P \leq 0.05$ were considered significant. Descriptive analyses of sequencing results are presented as average and standard deviation. All other results are presented as least square means followed by the respective standard error of the mean.

Three animals (animals E, G, and J – Additional file 1) experienced elevated SCC on the three days preceding intramammary infusion of bacteria and for that reason did not develop an infection following the challenge with *E. coli*. A fourth animal animal (animal L – Additional file 1) acquired a natural intramammary infection in one of the unchallenged quarters caused by an *E. coli* strain different from the experimental challenge strain. Data collected from these four animals was only used to compare challenged infected versus challenged, not infected quarters. All other analysis assessing the effect of experimental infection and treatment with ceftiofur did not include data from animals E, G, J, and L.

Data were handled as follows: samples without a SCC value due to clotted milk (i.e. clinical mastitis) received a value of 30,000,000 SCC; samples with a CFU value that indicated too numerous to count received a value of 60,000 CFU. The rationale for choosing these arbitrary values was to assign a number that was larger than the largest observation for that variable in the dataset (i.e. largest SCC observed was 27,255,000 and largest CFU observed was 58,000).

Multivariate analysis of milk microbiome was implemented in QIIME and R (R Core Team, Vienna, Austria). Analysis of Similarities (**ANOSIM**) was performed in non-rarefied data using the vegan (Jari Oksanen, 2016) package in R. Groups shown to be significantly different in ANOSIM underwent Analysis of Composition of Microbiomes (**ANCOM**) (Mandal et al., 2015) carried out in QIIME version 2.0.6 (Caporaso et al., 2010b), in an attempt to identify which OTUs were driving the main differences between groups. Microbiome changes over time in challenged and control quarters were visualized through Principal Coordinates Analysis (**PCoA**)

of weighted UniFrac distances calculated in QIIME and plotted using EMPeror (Vazquez-Baeza et al., 2013).

RESULTS

Health Characteristics

All cows exhibited elevated rectal temperature (Mean 39.1°C, StdDev 0.9°C) at 18h post-challenge (**Figure 5.1c**); one animal had recurrent fever and received support therapy as described above.

Abnormal milk was observed within 18 hours of infection (i.e. presence of flakes, clots, or serous milk) and lasted until 132 and 192 hours in control and ceftiofur groups, respectively. Mild clinical signs of mammary inflammation were observed, which included abnormal milk and/or redness and swelling of the challenged quarters (**Figure 5.1d**).

Bacterial Isolation and Strain Typing

The C1 strain of *E. coli* used for intramammary infection was isolated from milk samples collected post-challenge from every challenged quarter, except the three quarters that presented elevated SCC on the days preceding intramammary infusion of bacteria, which were excluded from further analysis. Confirmation of strain was performed through RAPD strain typing (**Figure 5.1e**).

Effect of Intramammary Infection with E. coli and Intramammary Antimicrobial Therapy on CFU Results

Intramammary infection with *E. coli* significantly increased LogCFUs ($P<0.0001$); intramammary treatment with ceftiofur significantly decreased LogCFUs in challenged, infected quarters ($P<0.0001$). Tukey-adjusted comparisons in each sampling time revealed that ceftiofur-treated quarters had significantly lower LogCFUs at 78, 84, 102, 108, 120, 126, 132, 144, and 150 hours relative to challenge (**Figure 5.1f**). Nevertheless, bacterial growth in samples collected from both control and treated quarters presented a decrease in CFU counts over time.

Effect of Intramammary Infection with E. coli on Somatic Cell Count Measured as Linear Scores

Intramammary infusion with 100 CFU of *E. coli* increased SCC as early as six hours post-challenge, peaking around 18 hours and remaining significantly higher when compared to unchallenged quarters until the end of the study period ($P<0.0001$) (**Figure 5.2b**). A non-significant increase in somatic cell count on unchallenged quarters was also observed between 12 and 48 hours relative to challenge (**Figure 5.2d**). Finally, intramammary treatment with ceftiofur hydrochloride did not significantly decrease linear score throughout treatment, with only two time points exhibiting different linear scores between treated and untreated groups (**Figure 5.2c**).

Sequencing Results

A total of 53,019,538 sequences passed quality control and were available for downstream analysis. The dataset analyzed, which only included infected animals, comprised 34,193,997 reads with a mean of 39,622 and standard deviation of 31,034 reads per sample.

Taxonomic Classification

On average, only 22.2% of all reads were not classified at the family level (StdDev 19.3%), whereas the number of unclassified reads at the genus level was on average 50.6% (StdDev 23.7%). The most abundant families in the dataset were *Ruminococcaceae* (Mean 13.5% StdDev 12.0%) *Enterobacteriaceae* (Mean 13.4% StdDev 24.6%), *Aerococcaceae* (Mean 5.6%, StdDev 7.7%), *Lachnospiraceae* (Mean 5.4%, StdDev 5.4%), *Corynebacteriaceae* (Mean 5.2%, StdDev 6.5%), *Planococcaceae* (Mean 5.2%, StdDev 7.9%), *Bacillaceae* (Mean 4.7%, StdDev 5.9%), *Clostridiaceae* (Mean 4.5%, StdDev 3.9%), *Bacteroidaceae* (Mean 4.2%, StdDev 3.6%), and *Staphylococcaceae* (Mean 3.6%, StdDev 6.5%). Detailed information on bacterial profile per study animal is provided in the supplementary information.

Pre-Challenge Microbial Profile

The microbial profile prior to intramammary infusion of E. coli (-72h to 0h) was diverse (Figure 3). No differences were observed between challenged, unchallenged, treated, and untreated groups in the pre-challenge microbiome. The most abundant families in pre-challenge samples were Ruminococcaceae (Mean 16.8%, StdDev 10.1%), Lachnospiraceae (Mean 7.0%, StdDev 5.1%), Aerococcaceae (Mean 6.8%, StdDev 8.2%), Enterobacteriaceae (Mean 6.3%,

StdDev 13.5%), Planococcaceae (Mean 5.7%, StdDev 9.5%), Bacteroidaceae (Mean 5.4%, StdDev 3.3%), Corynebacteriaceae (Mean 5.1%, StdDev 7.3%), Clostridiaceae (Mean 4.2%, StdDev 3.1%), Bacillaceae (Mean 3.5%, StdDev 3.7%), and Staphylococcaceae (Mean 2.8%, StdDev 4.9%).

Effect of Experimental Infection with E. coli and Intramammary Antimicrobial Therapy on Milk Microbiome

Intramammary infection with *E. coli* dramatically changed the milk microbial profile. Before challenge, quarters presented a very diverse profile, with the family *Ruminococcaceae* being the most abundant, averaging 13.3% and 14.3% in challenged untreated and challenged ceftiofur groups, respectively (**Figure 5.3c,d**). After experimental infection with *E. coli*, the milk microbiome had a significant increase in the family *Enterobacteriaceae* ($P<0.0001$) (**Figure 5.4b**), which represented over 30% of the relative abundance from 12 to 150 hours, peaking at 64.7% at 12 hours post-challenge in the untreated group (**Figure 5.3c**). Likewise, animals that eventually received intramammary treatment had *Enterobacteriaceae* as the predominant group, representing over 30% of the relative abundance from 12 to 60 hours, peaking at 77.9% at 18 hours (**Figure 5.3d**). Intramammary treatment with ceftiofur hydrochloride did not significantly improve the clearance rate of *Enterobacteriaceae*, nor significantly decreased the relative abundance of *Enterobacteriaceae* in any time point when compared to challenged untreated quarters (**Figure 5.4c**).

The microbial profile of unchallenged quarters was more diverse than what was observed in the post-infection challenged quarters (**Figure 5.3**). There was no remarkable change in the

relative abundances of the 25 most abundant families with intramammary treatment of unchallenged quarters with ceftiofur (**Figure 5.3b**).

Effect of Experimental Infection with E. coli and Intramammary Antimicrobial Therapy on Bacterial Diversity

Shannon diversity index was high and similar in all groups previous to experimental infection (**Figure 5.5a**). Diversity levels sharply decreased after experimental challenge with *E. coli* ($P<0.0001$), and the lowest diversity was observed between 30 and 78 hours post infection (**Figure 5.5a,b**). Administration of five doses of ceftiofur in 24-hour intervals starting at 48hours post-infection did not alter bacterial diversity in a consistent manner; Differences were observed in the diversity indexes before initiation of treatment regimen (at 36 and 48 hours). Nevertheless, Tukey-adjusted comparisons revealed that ceftiofur-treated animals had significantly different diversity indexes at 78, 102, and 180 hours relative to challenge (**Figure 5.5c**). By the end of the study period, treated and untreated quarters did not present significantly different diversity (**Figure 5.5c**).

Unchallenged quarters presented high and stable diversity indexes throughout the study (**Figure 5.5a,d**). No difference was observed in diversity levels in unchallenged quarters between control and ceftiofur infused quarters.

Effect of Pre-challenge Linear Scores on Intramammary Infection with E. coli

Animals that were challenged with 100 CFU of *E. coli* and were successfully infected had significantly lower linear scores in all time points preceding challenge when compared to

cows that were found not to be infected with the strain C1 (**Figure 5.6a**). Animals infected with the challenge strain presented a sharp rise in LS as soon as 18 hours after challenge, and sustained higher LS throughout the study period when compared to challenged and not infected quarters (**Figure 5.6a**). The microbial profile of not infected quarters only had an increase in the relative abundance of the family *Enterobacteriaceae* representing 19% of the microbial profile at the 12 hour time point post challenge (**Figure 5.6b**), whereas infected quarters had their milk microbiome dominated by the family *Enterobacteriaceae* which represented over 75% of the relative abundance at the 12 hour time point post challenge and accounted for over 30% of the microbial profile until 132 hours post challenge (**Figure 5.6c**).

Multivariate Analysis of Milk Microbiome and Effect of Intramammary Antimicrobial Therapy

The relative abundance of the family *Enterobacteriaceae* was the main driver of the variation on weighted UniFrac distances in the dataset comprised of all samples from infected cows (**Figure 5.7a**). Samples from challenged quarters were discretized into seven categories according to time relative to experimental challenge, as well as treatment group. A significant difference in the milk microbiome was detected between the seven categories in ANOSIM, which was corroborated by clustering observed in weighted UniFrac PCoA (**Figure 5.7b**). Analysis of composition of microbiomes identified ten OTUs that significantly differed between groups, four of which were assigned to the family *Enterobacteriaceae*, one was assigned to the family *Planococcaceae* and five OTUS were not taxonomically assigned at the family level.

Stratified ANOSIM revealed a significant difference when the microbiome pre-challenge (-72 to 0h) was compared to post-challenge pre-treatment (6h to 48h). Grouping was observed in weighted UniFrac PCoA, with the family *Enterobacteriaceae* being the main driver of variation in this comparison (**Figure 5.8a**). Seven OTUs were deemed significantly different between groups in ANCOM, two of which were assigned to the family *Enterobacteriaceae* and five of which were not taxonomically assigned at the family level.

The microbiome of samples from challenged treated and challenged untreated quarters were compared through ANOSIM before initiation of treatment (6h to 48h) and during treatment administration (54h to 150h). No differences were detected between treatment and control group in either ANOSIM or weighted UniFrac PCoA before initiation of treatment (**Figure 5.8b**) or during treatment administration (**Figure 5.8c**). When the microbiome of pre-challenge samples was compared to the of samples collected after treatment cessation (**156 to 216h**) through ANOSIM no significant differences were identified, which was corroborated through lack of grouping in weighted UniFrac PCoA (**Figure 5.8d**). In an attempt to identify if quarters that were not successfully infected after experimental infection had a different pre-challenge microbiome from quarters that were successfully infected, we performed ANOSIM in pre-challenge samples. No difference was observed on the milk microbiome prior to experimental infection between challenged infected and challenged uninfected quarters (ANOSIM: R= -0.17, P=0.99). In agreement with ANOSIM, PCoA of weighted UniFrac distances did not reveal any clustering pattern (data not shown). All samples from an individual animal were sequenced in order within a run; batch effects that could have arisen due to samples being sequenced in six

different runs were examined through weighted UniFrac PCoA, and no clustering due to sequencing run was observed (data not shown).

Figure 5.1. Experimental challenge timeline (a). Schematic of challenge and treatment in each quarter (b), effect of intramammary infection with *E. coli* and treatment with ceftiofur hydrochloride (48, 72, 96, 120, and 144 hours) on Temperature (c), California Mastitis Test (CMT) results (d). RAPD strain typing results (e) effect of intramammary infection with *E. coli* and treatment with ceftiofur hydrochloride (48, 72, 96, 120, and 144 hours) on Colony Forming Units (CFU) (f). Asterisks represent differences after Tukey-Kramer multiple comparison correction and α=0.05 between groups within the same time point.

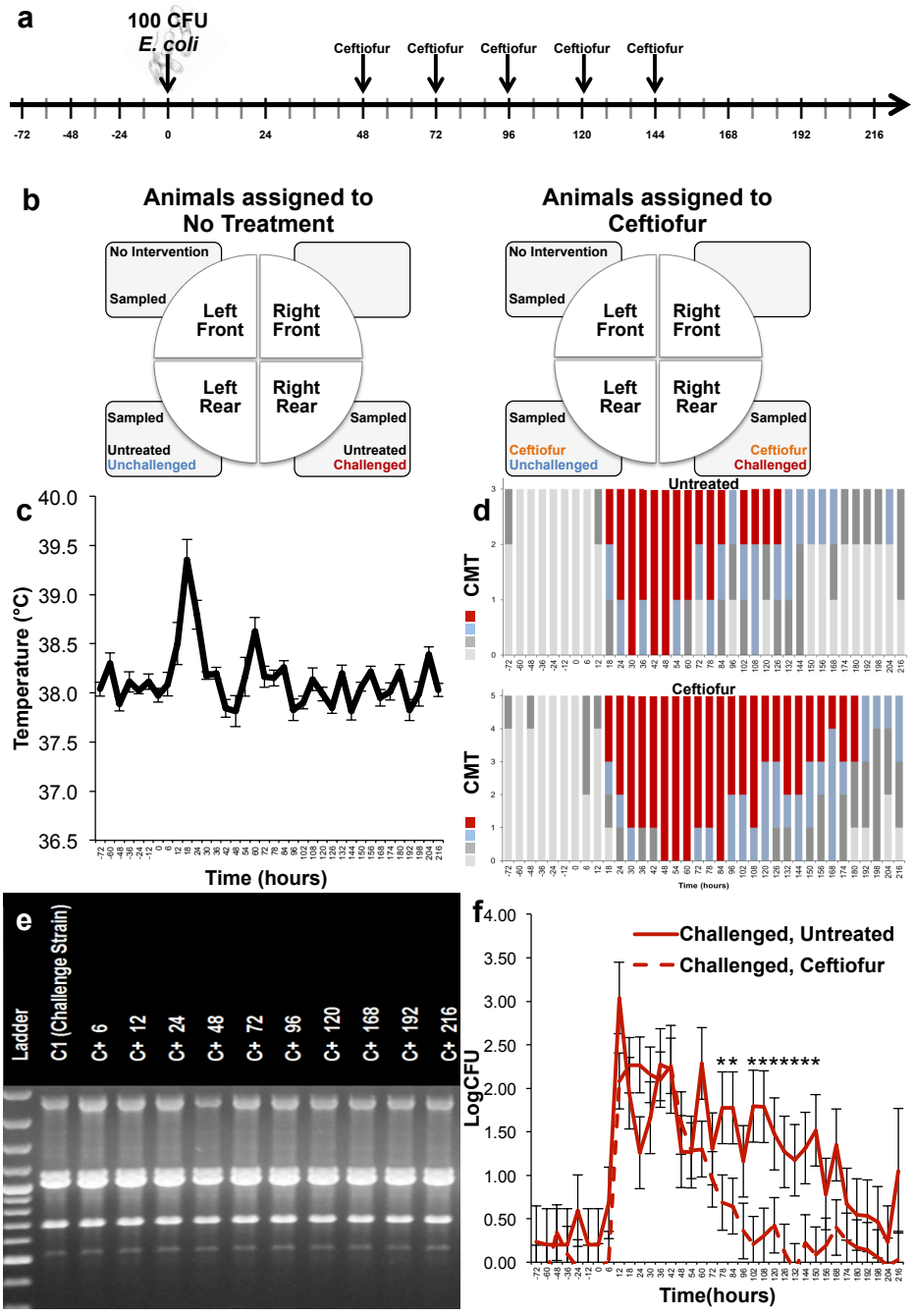


Figure 5.2. Effect of experimental infection with *Escherichia coli* (0 h) and intramammary treatment with ceftiofur hydrochloride (48, 72, 96, 120, and 144 h) on Linear Scores. Effect of challenge and treatment (a, $N = 24$ quarters), effect of intramammary challenge with *Escherichia coli* (b, $N = 24$), stratified analysis of covariance assessing the effect of intramammary treatment with ceftiofur hydrochloride in challenged (c, $N = 8$), and unchallenged quarters (d, $N = 16$). *Asterisks* represent differences after Tukey-Kramer multiple comparison correction and $\alpha = 0.05$ between groups within the same time point. *Black diamonds* represent experimental infection with 100 CFU of *E. coli*, and *grey circles* represent intramammary treatment with ceftiofur.

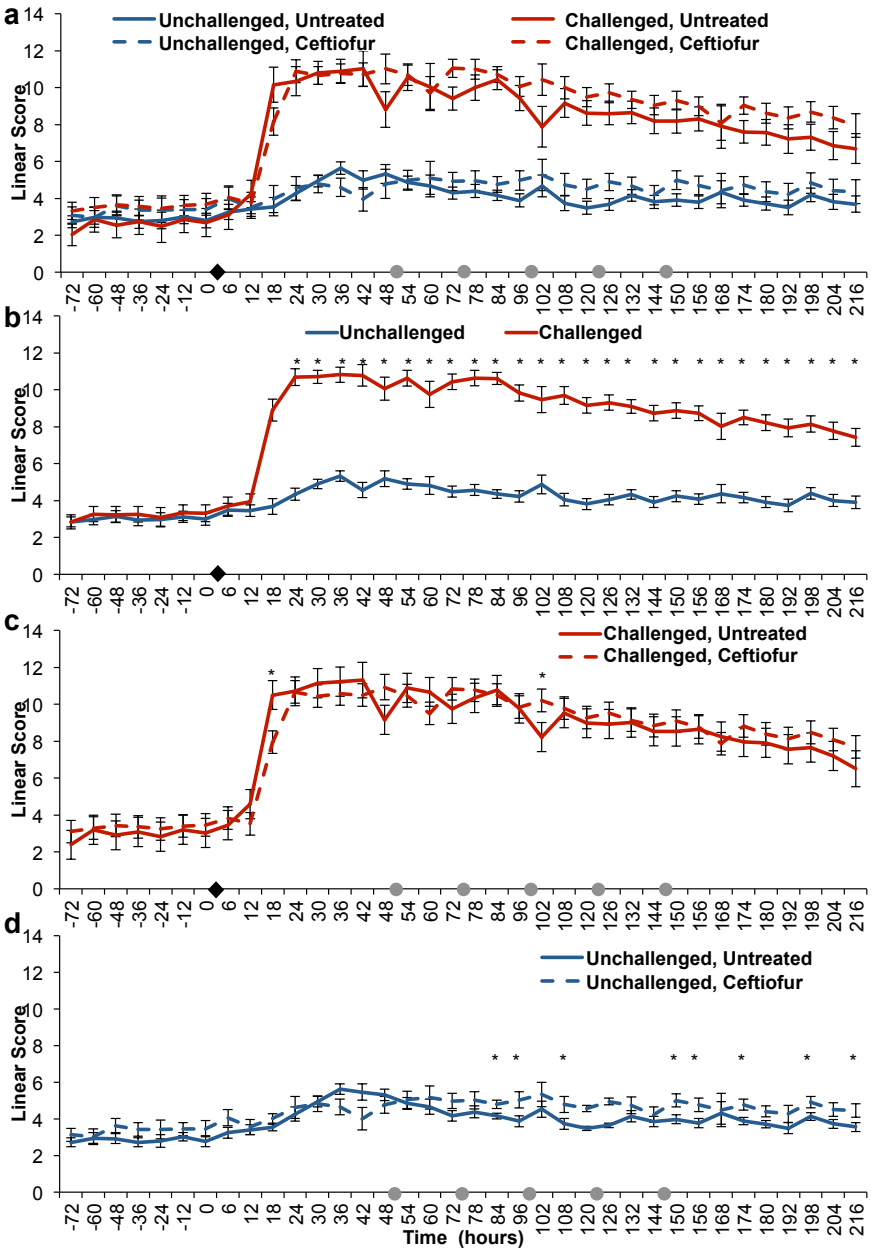


Figure 5.3. Effect of intramammary infection with *E. coli* and subsequent treatment with ceftiofur hydrochloride (48, 72, 96, 120, and 144 h) on relative abundance of the 25 most prevalent families in unchallenged untreated quarters (a, $N = 11$), in unchallenged ceftiofur-infused quarters (b, $N = 5$), in challenged untreated quarters (c, $N = 3$), and in challenged and ceftiofur-treated quarters (d, $N = 5$). *Black diamonds* represent experimental infection with 100 CFU of *E. coli*, and *grey circles* represent intramammary treatment with ceftiofur.

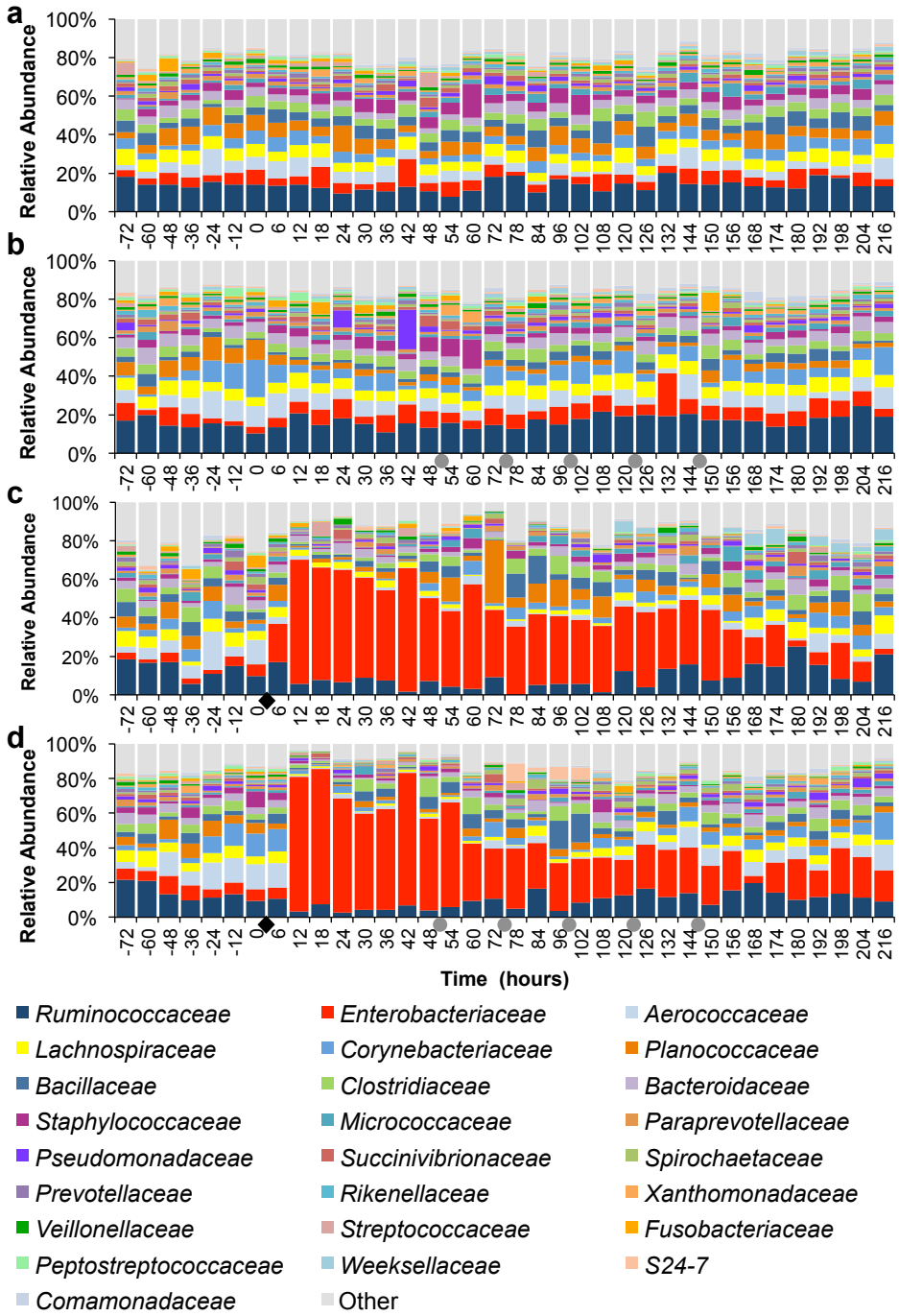


Figure 5.4. Effect of experimental infection with *Escherichia coli* (0 h) and intramammary treatment with ceftiofur hydrochloride (48, 72, 96, 120, and 144 h) on relative abundance of *Enterobacteriaceae*. Effect of challenge and treatment (a, $N = 24$ quarters), effect of intramammary challenge with *Escherichia coli* (b, $N = 24$) and stratified analysis of covariance assessing the effect of intramammary treatment with ceftiofur hydrochloride in challenged (c, $N = 8$) and unchallenged quarters (d, $N = 16$). *Asterisks* represent differences after Tukey-Kramer multiple comparison correction and $\alpha = 0.05$ between groups within the same time point. *Black diamonds* represent experimental infection with 100 CFU of *E. coli*, and *grey circles* represent intramammary treatment with ceftiofur.

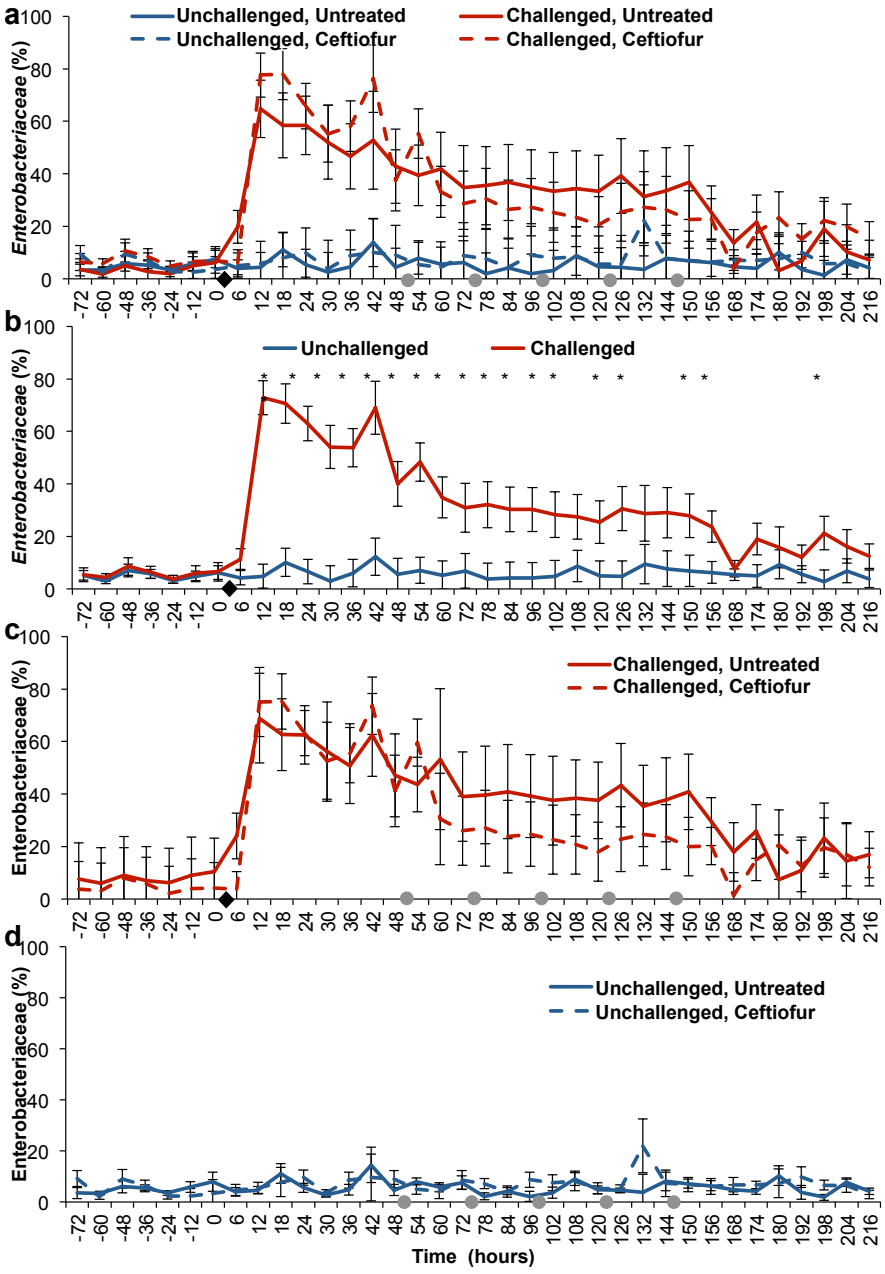


Figure 5.5. Effect of experimental infection with *Escherichia coli* (0 h) and intramammary treatment with ceftiofur hydrochloride (48, 72, 96, 120, and 144 h) on Shannon diversity index. Effect of challenge and treatment (a, $N = 24$ quarters), effect of intramammary challenge with *Escherichia coli* (b, $N = 24$) and stratified analysis assessing the effect of intramammary treatment with ceftiofur hydrochloride in challenged (c, $N = 8$) and unchallenged quarters (d, $N = 16$). Asterisks represent differences after Tukey-Kramer multiple comparison correction and $\alpha = 0.05$ between groups within the same time point. *Black diamonds* represent experimental infection with 100 CFU of *E. coli*, and *grey circles* represent intramammary treatment with ceftiofur.

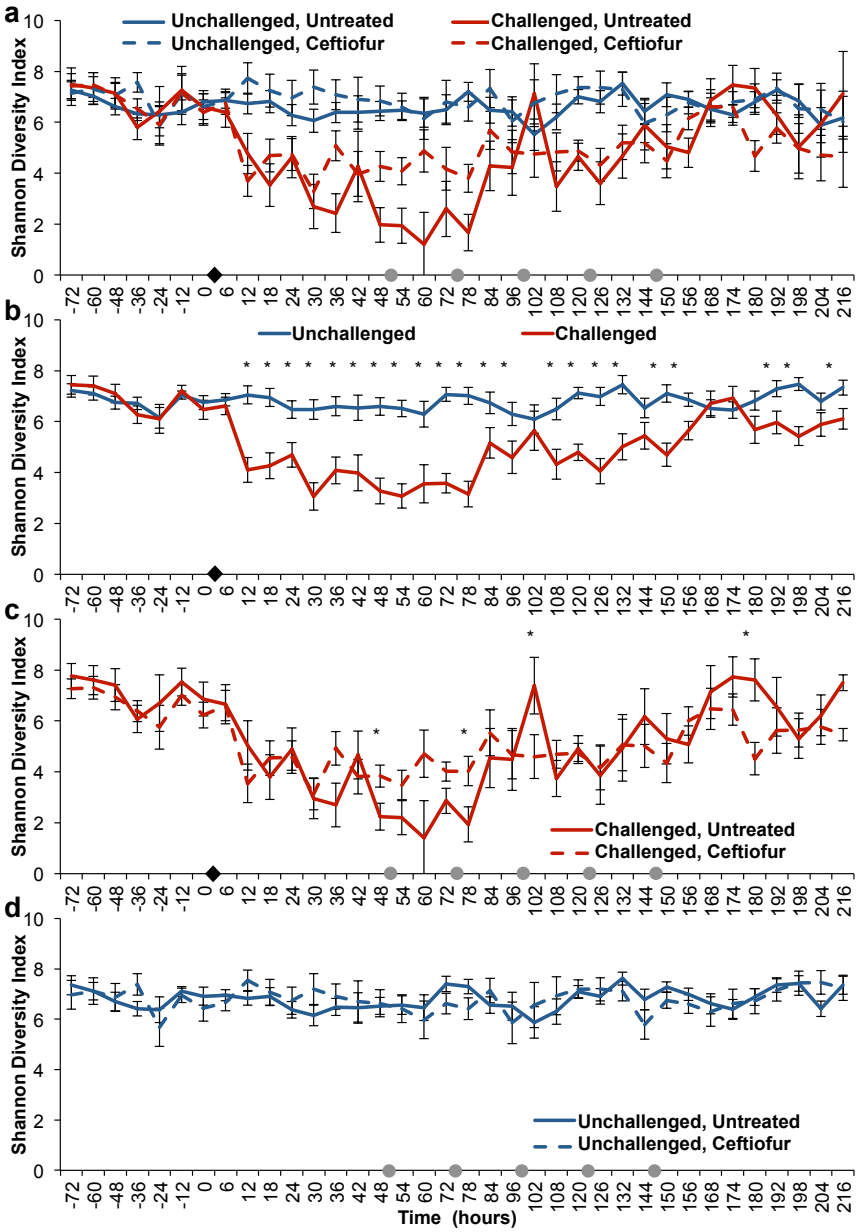


Figure 5.6. Effect of pre-challenge linear scores on intramammary infection success (a). Depiction of the relative abundance of the 25 most prevalent families in challenged uninfected quarters (b, $N = 3$), and challenged and infected quarters (c, $N = 8$). *Black diamonds* represent experimental infection with 100 CFU of *E. coli.*

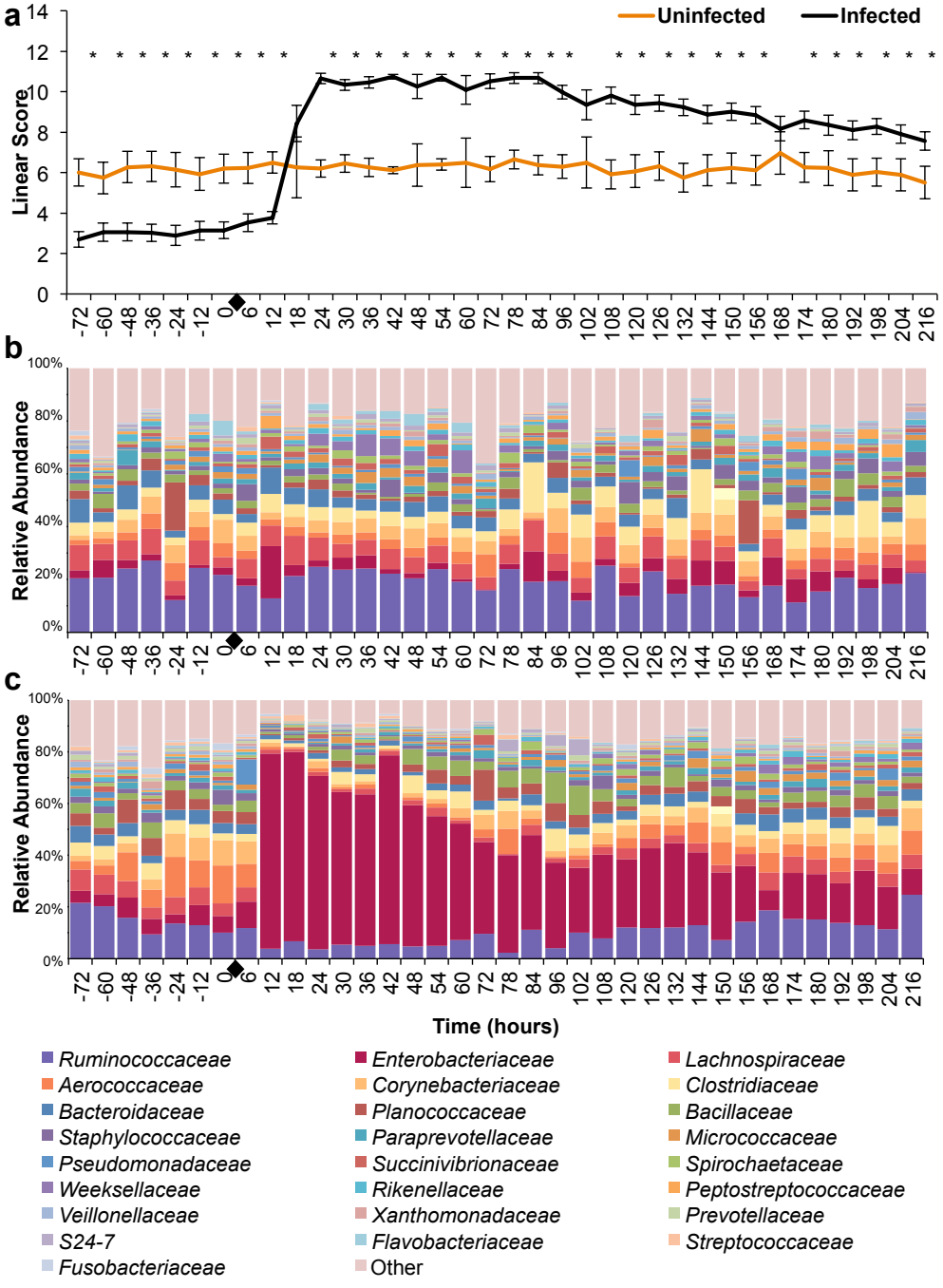


Figure 5.7. Biplot depicting weighted UniFrac distances of all samples and the coordinates of the five most abundant family-level taxa (*orange spheres*) in the context of relative abundance of *Enterobacteriaceae* (a). Analysis of similarity (ANOSIM) and principal coordinate analysis (PCoA) of weighted UniFrac distances comparing the effect of challenge, treatment and time in challenged quarters only (b). For comparison and plotting purposes, time points were discretized into seven categories.

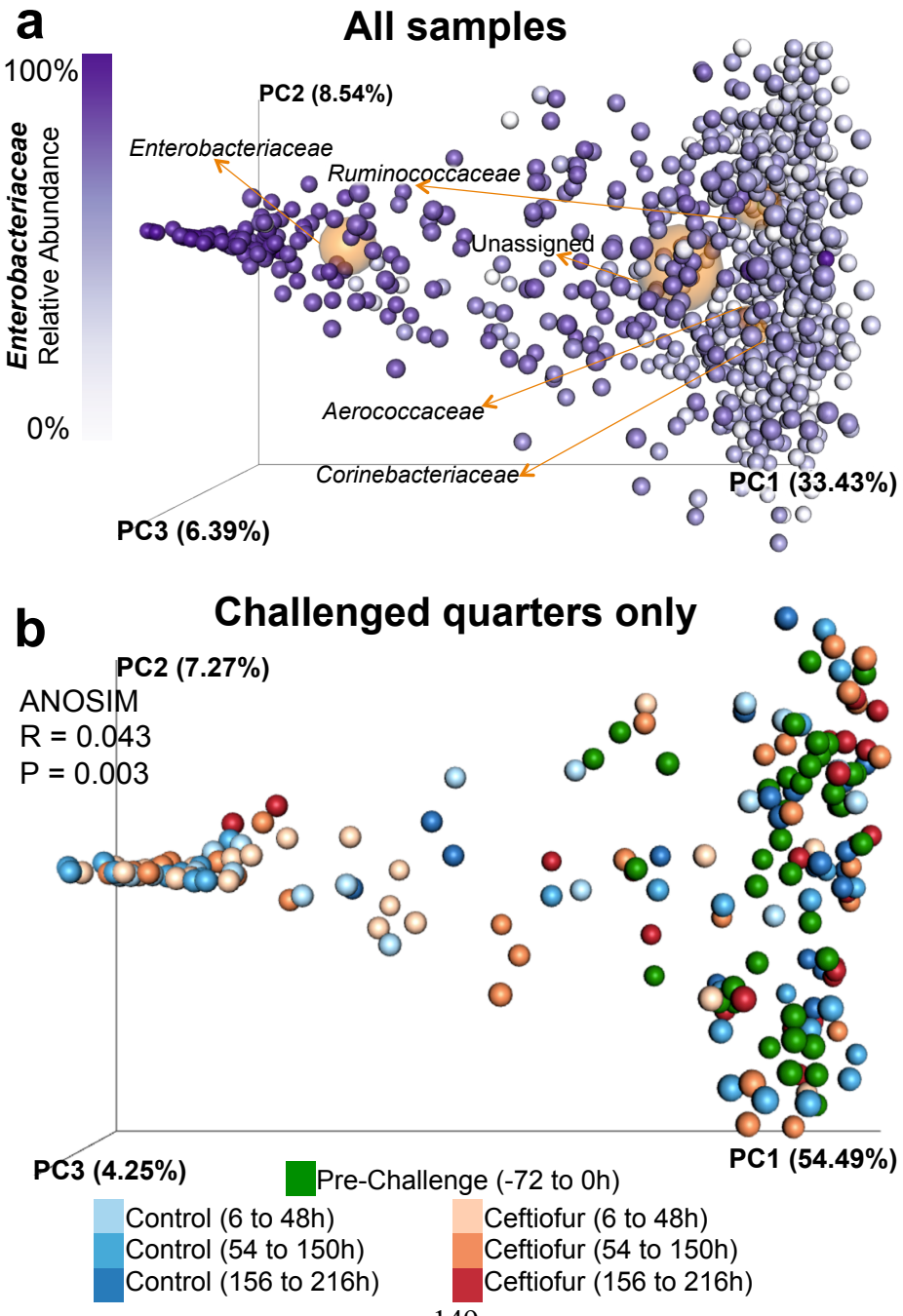
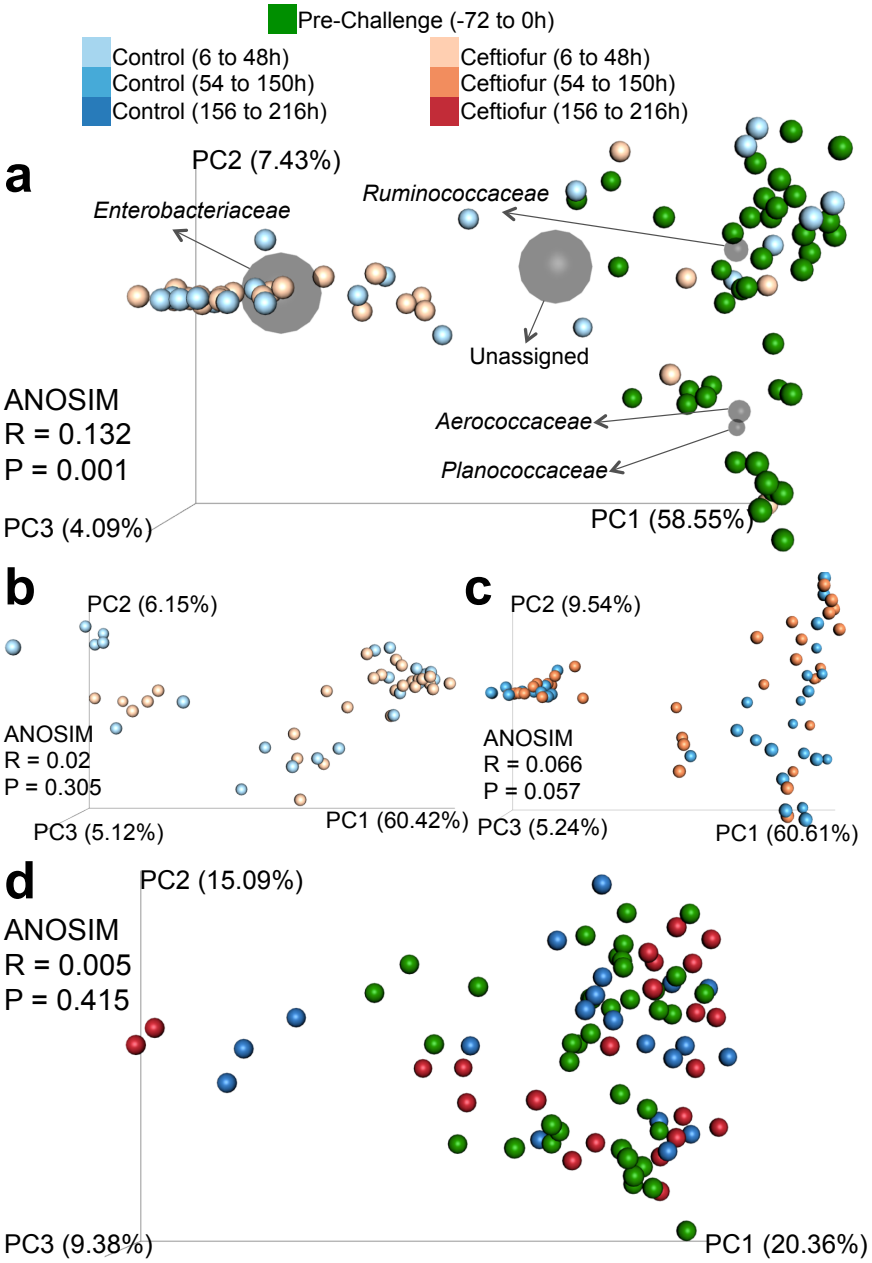


Figure 5.8. Multivariate analysis of milk microbiome. Effect of intramammary infection with *E.* coli on UniFrac distances and depiction of the five most abundant family-level taxa. Analysis of similarity (ANOSIM) and principal coordinate analysis (PCoA) of weighted UniFrac distances comparing pre-challenge (−72 to 0 h) and post-challenge (6 to 48 h) samples (a). Weighted UniFrac distances and ANOSIM comparing control and ceftiofur before initiation of treatment (b) and during treatment (c). ANOSIM comparing pre-challenge (−72 to 0 h) samples to control and ceftiofur samples after cessation of treatment (156 to 216 h) (d). For comparison and plotting purposes, time points were discretized into categories.



DISCUSSION

We have used an *in-vivo* experimental model of bovine mastitis and state-of-the-art technology to describe in detail the dynamic changes that the milk microbiome undergoes upon infection, treatment, and resolution of mastitis. To the best of our knowledge, this is the first study to investigate an experimental challenge of bovine mastitis using next generation sequencing, and the first investigation on the effects of third generation cephalosporins on the endogenous microbiota of healthy milk. Here we show that extended intramammary treatment with ceftiofur has no effect on the microbiome of milk from *E. coli* induced mastitis. Using multivariate analysis of weighted UniFrac distances and ANOSIM, we demonstrate that the milk microbiome returns to a similar state of that of unchallenged quarters 9 days after experimental intramammary infection with *E. coli*, regardless of receiving antimicrobial therapy. Our results show a significant decrease on the LogCFUs recovered from milk samples in challenged and treated quarters; however no beneficial effect of antimicrobial treatment was observed in somatic cell count, rate of decrease of *Enterobacteriaceae*, or microbial diversity in quarters challenged with *E. coli*.

We observed a dramatic decrease in microbial diversity following the experimental challenge with 100 CFU of *E. coli*. Other studies that investigated infections in both human (Fernandez et al., 2013) and bovine milk (Bhatt et al., 2012, Oikonomou et al., 2012, Oikonomou et al., 2014) have also reported that reduced microbial diversity was associated with mastitis. Interestingly, we observe here that diversity indexes of challenged quarters returned to indexes comparable to uninfected quarters by the end of the study period. This is similar to results observed by our group in a study that investigated the microbiome of animals naturally infected

with mastitis (Ganda et al., 2016). Treatment of challenged quarters with ceftiofur significantly decreased LogCFUs; however this effect could only be observed during treatment administration, with no significant differences in LogCFUs detected 12 hours after the last intramammary infusion (156h post-challenge). These findings are in agreement with our previous study (Ganda et al., 2016) that investigated the effect of ceftiofur in animals naturally infected with mastitis. In that study we were able to identify a significant reduction in bacterial load of treated animals as measured through number of 16S rRNA copies during treatment administration; on the other hand we failed to identify any differences in bacterial load after cessation of treatment. Despite the significant drop in LogCFUs observed in treated challenged quarters, we could not identify a treatment effect in the relative abundance of *Enterobacteriaceae.* Taking in consideration the significant reduction in live bacteria detected in the present study, it is possible that the relative abundance of bacteria remains unchanged but treatment with ceftiofur has an effect in impairing the viability of bacteria exposed to the antimicrobials.

Herein we were unable to recognize a treatment effect neither on the rate of change of *Enterobacteriaceae* nor in the overall microbial profile of challenged quarters. These findings are in agreement with our previous work (Ganda et al., 2016), in which no significant differences were observed in the rate of decrease of *Enterobacteriaceae* in animals naturally infected with *E. coli*. Moreover, we failed to identify a significant effect of treatment on the total milk microbiome as measured by ANOSIM, which is in line with results from our earlier investigation. Several studies have investigated the effect of ceftiofur in food animals (Chambers et al., 2015, Fleury et al., 2015, Baron et al., 2016); however these studies evaluated parenteral or

oral administration of ceftiofur in different microbiome niches, such as the gut. To the best of our knowledge, our group is the first to evaluate the effect of intramammary ceftiofur in bovine milk. The effect of chemotherapy in human milk microbiome and metabolome has been previously reported (Urbaniak et al., 2014). In bovine milk, modifications in the metabolome have also been described after the use of parenteral enrofloxacin (Junza et al., 2016). The evidence of ceftiofur-induced modifications in the microbiome of food animals and the indication of possible drug-induced changes in milk prompt further investigation to simultaneously characterize the effects of cephalosporins in the mammary gland microbiome, metabolome, and resistome. It is possible that the observed lack of change in the relative abundance could be accompanied by differences in the metabolic profile of bacteria exposed to antimicrobials. Using technologies such as shotgun metagenomics, metabolomics, and proteomics (Addis et al., 2016), we could detect differences not evident by 16S rRNA sequencing, and conclusions about the effects of cephalosporins in the diseased mammary gland may change.

In our study we observed a dramatic change on the milk microbial profile upon infection with *E. coli*. Nevertheless, the most remarkable result of this investigation was the lack of discrimination between the microbiome of pre-challenge samples and the microbiome of milk samples from the same quarters collected after disappearance of clinical signs. Multivariate analysis of milk microbiome identified a significant difference when pre challenged samples were compared to ones collected in the first two days after challenge (-72 to 0h versus 6 to 48h). However we failed to identify any differences between the microbiome of treated and untreated quarters during treatment administration (time points 54 to 150h). Interestingly, no difference could be observed in the overall microbiome assessed through ANOSIM between groups after

treatment cessation, indicating that the milk microbiome is capable of returning to the original microbial status. The restoration of the microbiota to a healthy milk profile is in line with the findings of our investigation in naturally infected animals, in which no differences on the microbiome of healthy quarters and cured quarters could be observed 14 days after diagnosis of mastitis. Conversely, work by Falentin et al., (2016) identified long-lasting effects in quarters with normal milk that had a history of mastitis and suggested that such effects could be due to clinical mastitis and the antimicrobials used for the treatment thereof. Nevertheless, work performed by that group consisted of a cross-sectional study, and several components related to study design, sample collection, and the pathogens associated with mastitis in those animals could contribute for differences in the findings between the two studies. Those authors identified high abundance of members of the family *Staphylococcaceae*, with some samples having as much as 30% of staphylococci reads assigned to *S. aureus*, which is known to have adapted to persist in the mammary environment, and attach to the cell lining (Schukken et al., 2011b). Regardless of *S. aureus* and *E. coli* both being considered major mastitis pathogens, the mammary environment is known to be a reservoir of *Staphylococcus* while *E. coli* is mainly considered an environmental mastitis causing bacteria (Schukken et al., 2011b).

Antimicrobial infusion in healthy mammary glands did not have a significant effect in the microbial profile. This was an interesting finding, given that alterations in the flora of healthy individuals exposed to antimicrobials favoring the blooming of pathogenic bacteria have been described (Theriot et al., 2014). Previous reports of mastitis outbreaks following 'blitz' therapy (Bradley and Green, 1997), which consists in intramammary treatment of all lactating animals in the herd for elimination of a contagious pathogen, led us to hypothesize that shifts in the

microbiome of healthy milk would occur in consequence to antimicrobial exposure, as it has been observed in other niches (Theriot et al., 2014, Chambers et al., 2015). Nevertheless, intriguing work by Zaura and colleagues (2015) have reported a surprising resilience of the salivary microbiome upon exposure to different antimicrobials. While authors of the latter study observed significant and long-lasting changes in the fecal microbiome, the microbiome of the saliva presented only short-term ecological consequences, representing two radically different responses in two niches of the same individuals. It is possible that the microbiome of the mammary gland present itself as stable and resilient, comparable to what was observed in the salivary microbiome. This hypothesis is corroborated by the highly diverse microbiome of healthy milk described in many studies (Cabrera-Rubio et al., 2012, Addis et al., 2016, Fitzstevens et al., 2016). One could also speculate that the healthy milk microbiome does not contain strains of bacteria that are resistant to ceftiofur and capable of taking advantage due to the lack of competition imposed by antimicrobial exposure. On the contrary of what is observed in the gut, the very low bacterial load in healthy milk reported in our former study might indicate that the milk microbial environment is not as competitive, and is less favorable to the overgrowth of bacteria and subsequent change in the microbial profile.

In this study, three out of the twelve challenged cows did not develop an infection following the challenge with *E. coli*. Several factors could account for this occurrence, most importantly the fact that these animals had significantly higher SCC in the time points prior to experimental infection. This finding is in agreement with Schukken (2011) and Burvenich (2003) which have reported that the success of any intramammary infection is dependent upon the stage of lactation and the initial amount of milk leukocytes. In addition, our data follows the reasoning

of Burvenich (2003), which stated that the severity and outcome of *E. coli* mastitis are cow-dependent, rather than entirely pathogen-dependent; however, it is important to highlight that in our study we have evaluated infection with a single characterized strain of *E. coli*. The dynamics of *E. coli* associated mastitis and its resolution are multifactorial, involving aspects of the animal's immune system, and features of the pathogen involved (Richards et al., 2015, Keane, 2016). Due to the anatomic structure of the udder, it is generally assumed that infection in one quarter should not affect the immune status of neighboring quarters. Recent studies have contested this hypothesis, providing evidence of interdependence between infected and healthy quarters (Merle et al., 2007, Blagitz et al., 2015). Jensen and colleagues (2013) have evaluated the transcriptional response of uninfected quarters in animals challenged with two major mastitis pathogens, and described that the response in non-affected quarters was greater in animals with *E. coli* associated mastitis. Although other immune parameters were not evaluated in the current study, the numerical increase in somatic cell count in unchallenged quarters after intramammary infection with *E. coli* observed here is in agreement with Jansen et al., (2013) and Blagitz et al., (2015), indicating that the immune response in the mammary gland is to some extent influenced by the status of adjacent quarters.

CONCLUSION

We have demonstrated here that the bovine mammary gland harbors a resilient microbiome, capable of reestablishing itself after dramatic changes due to an infectious event with an environmental pathogen. While all cows were inoculated with the same bacterial load, unique responses were observed in different animals. No differences were observed in the microbial profile of unchallenged mammary glands that were exposed to extended

intramammary antimicrobial therapy. The milk microbiome was shown to be diverse and stable, indicating that bacteria within the mammary gland are tightly regulated. Our results corroborate for judicious use of antimicrobials in the dairy industry, demonstrating that due to the resilience of the mammary gland microbiome, certain cases of mastitis are capable of resolving independently of the use of intramammary antimicrobials.

DECLARATIONS

Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no conflict of interest.

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AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: RCB, GO, YHS. Performed the experiments: EKG, NG, AS, BP. Analyzed the data: EKG. Wrote the paper: EKG. Revised the manuscript: YHS, GO, RCB. All authors reviewed the manuscript.

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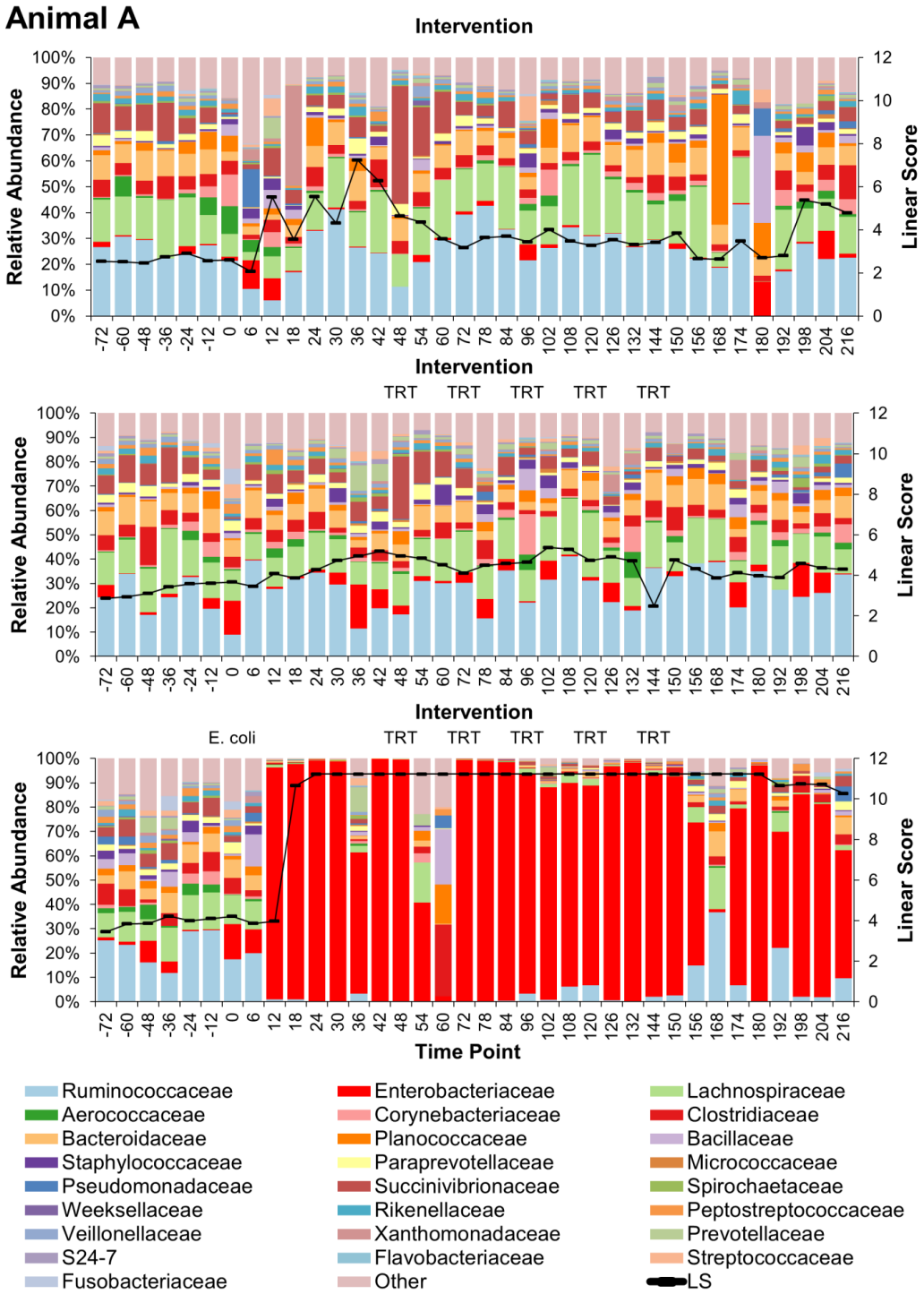
Figure 5.S1. Description of the microbiome from different quarters over time on animal A.



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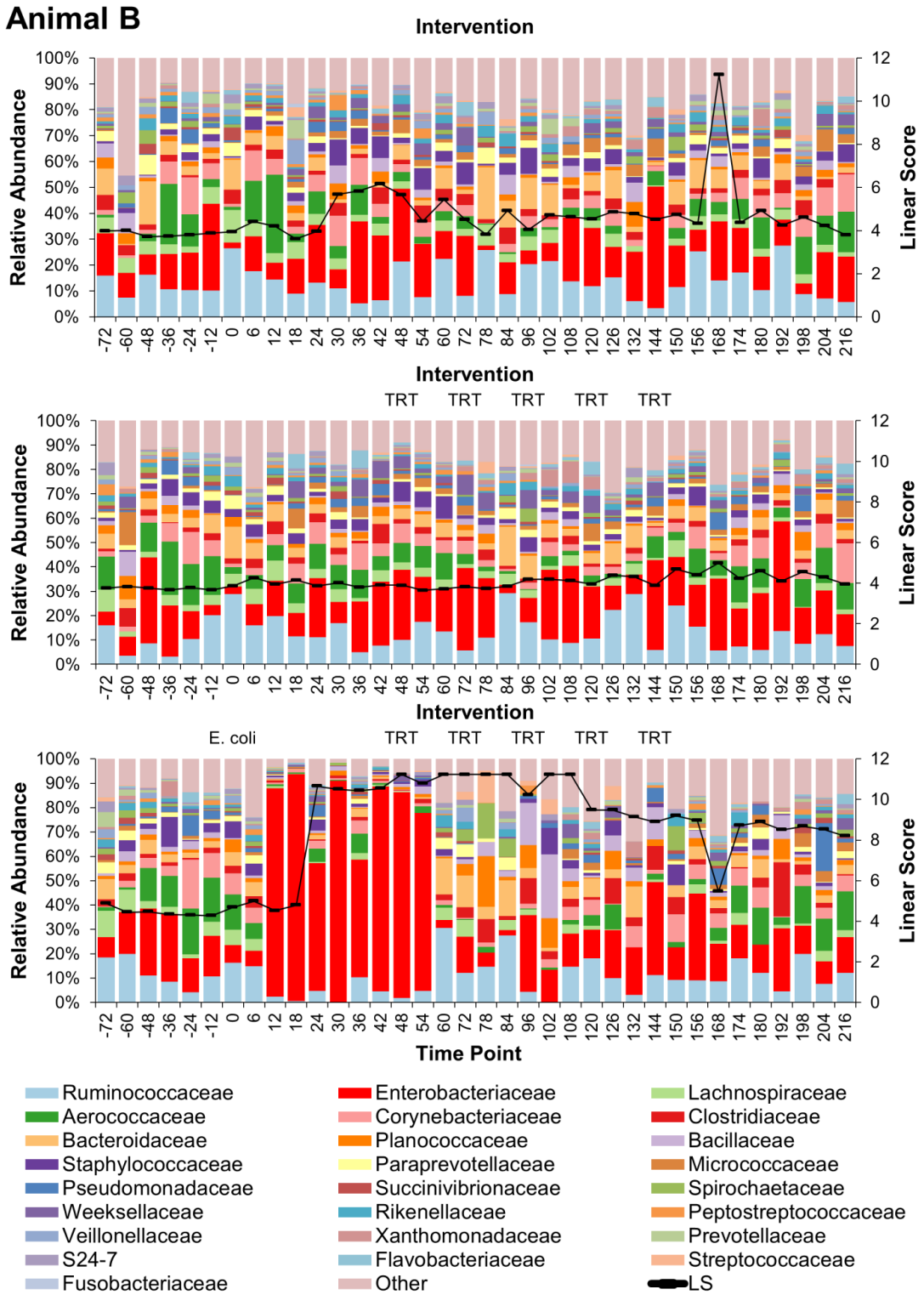

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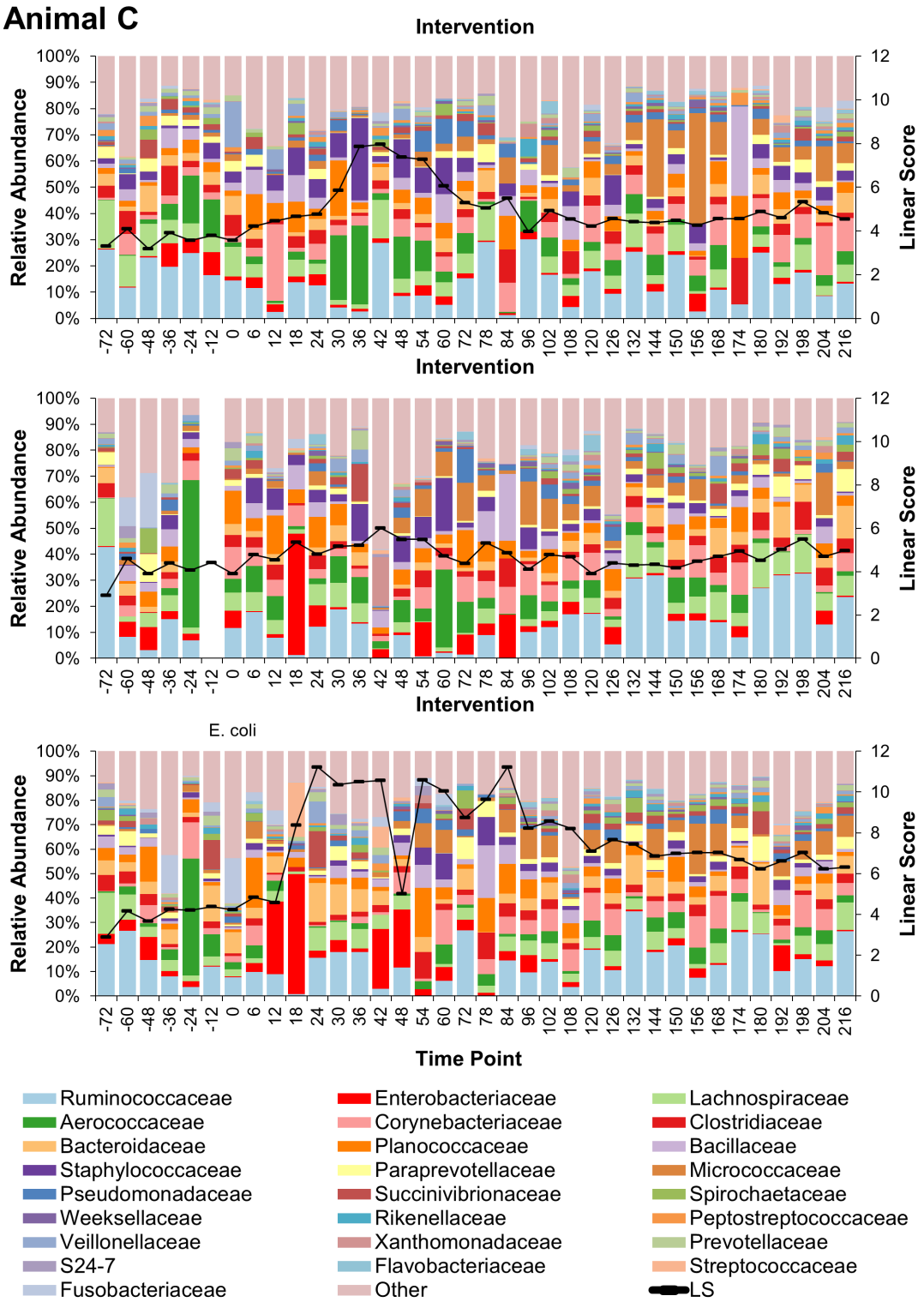

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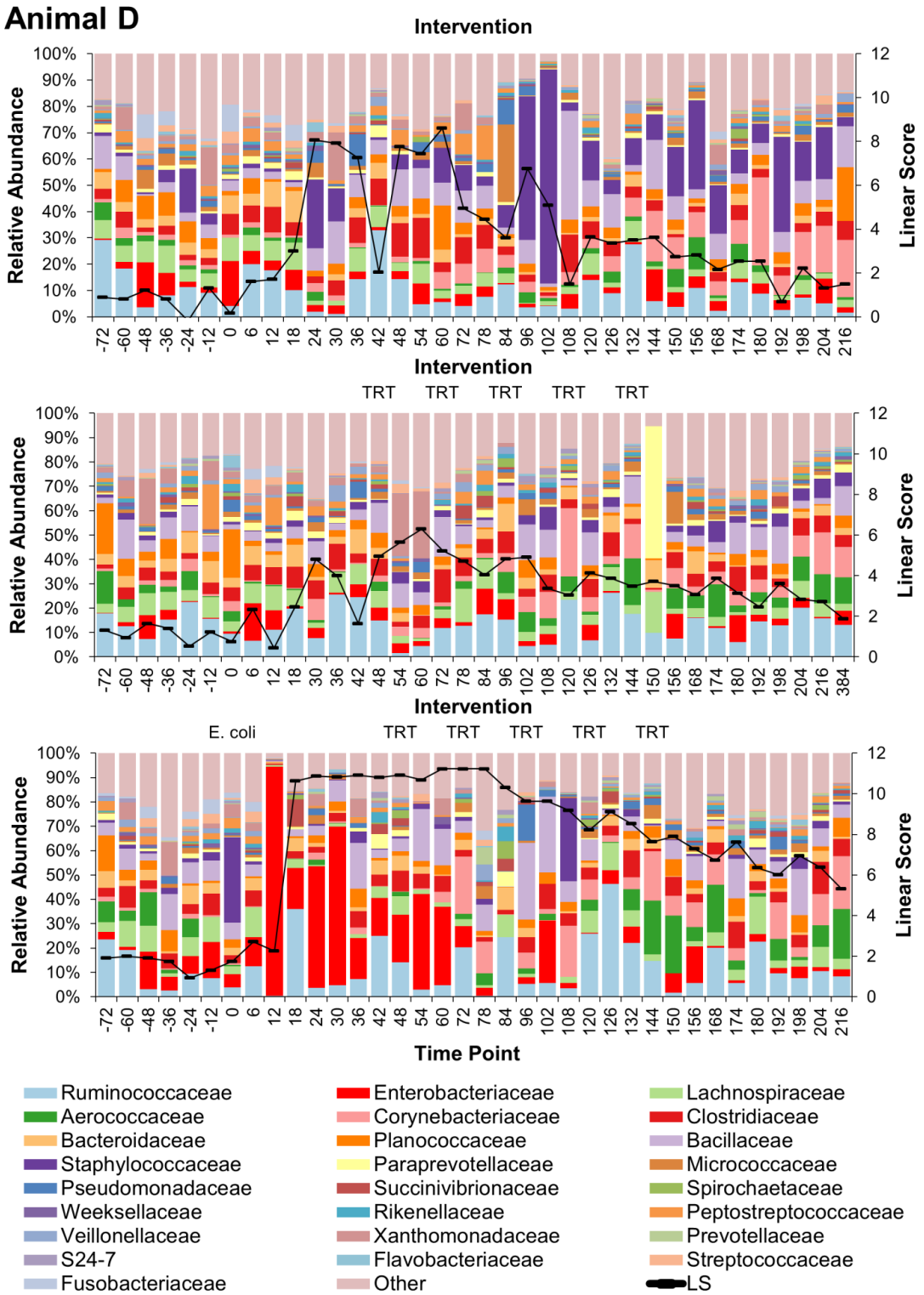

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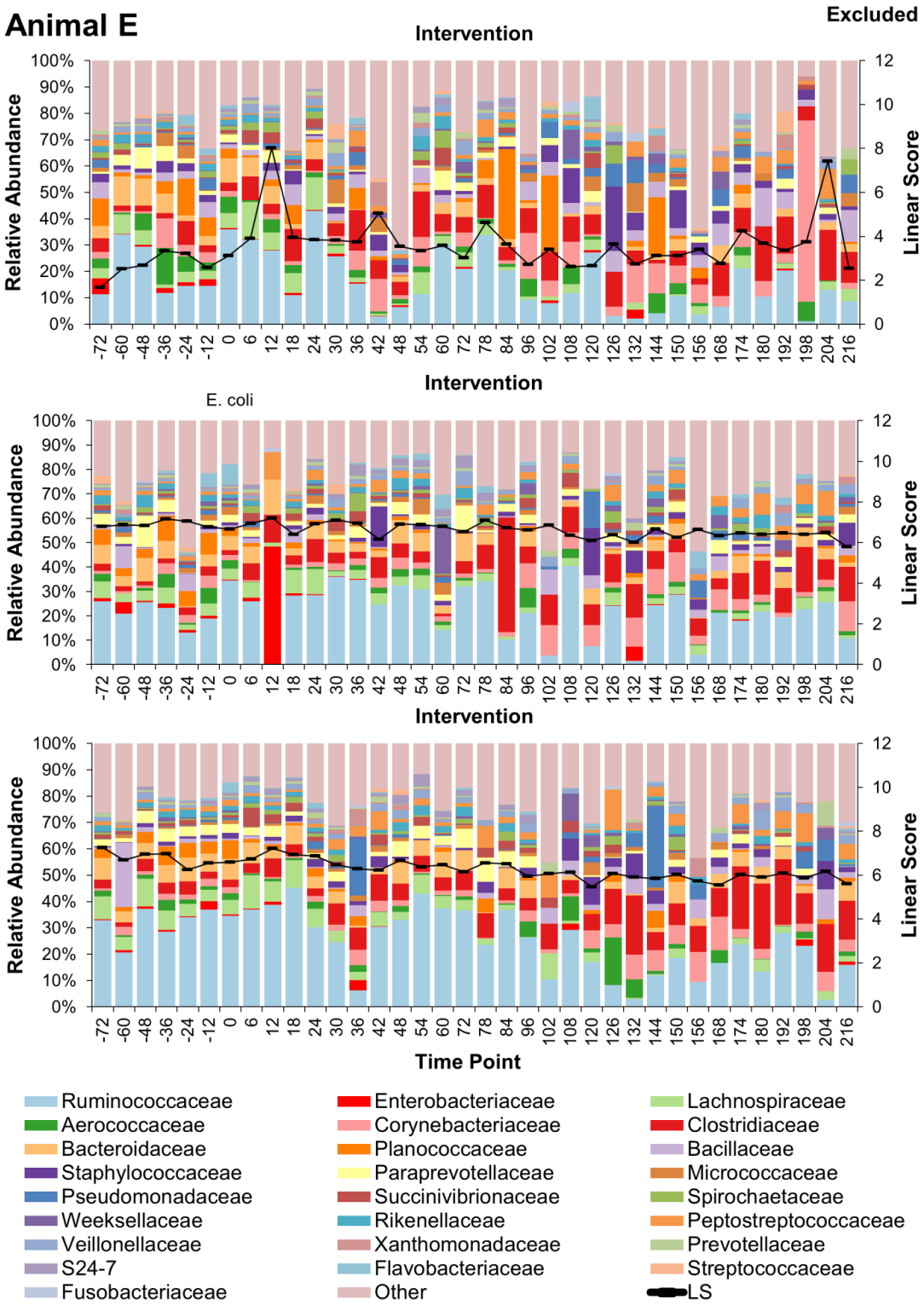

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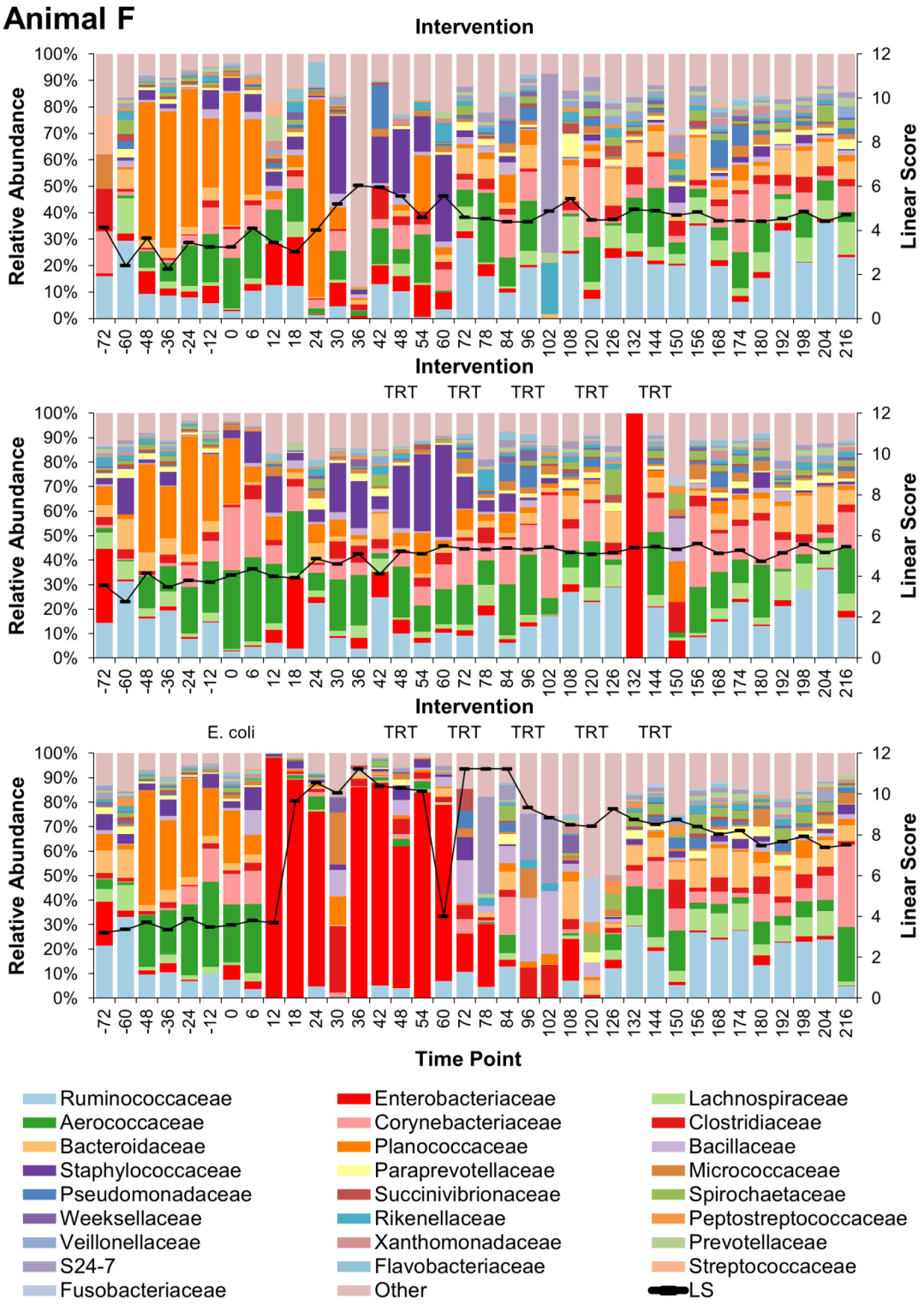

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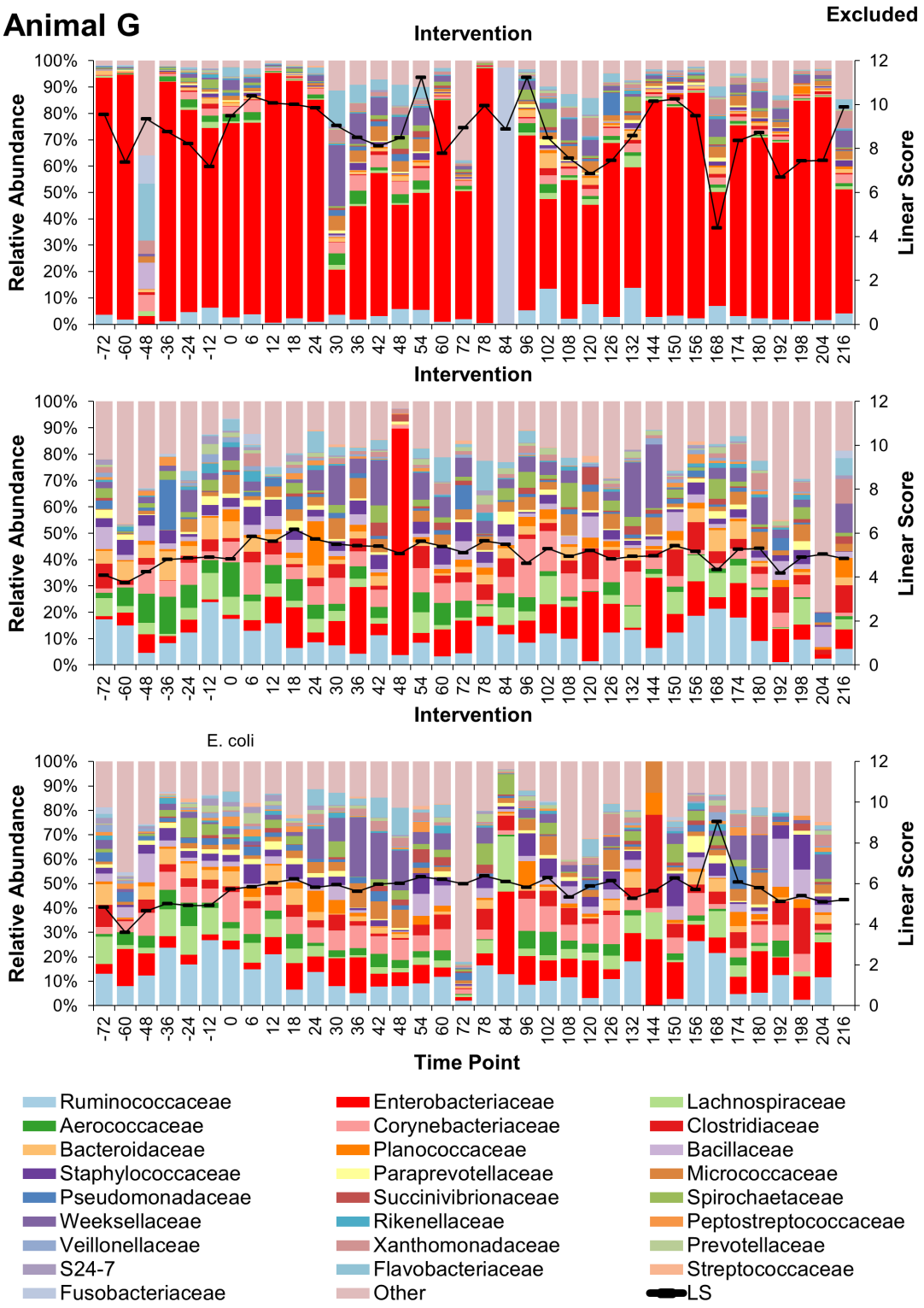

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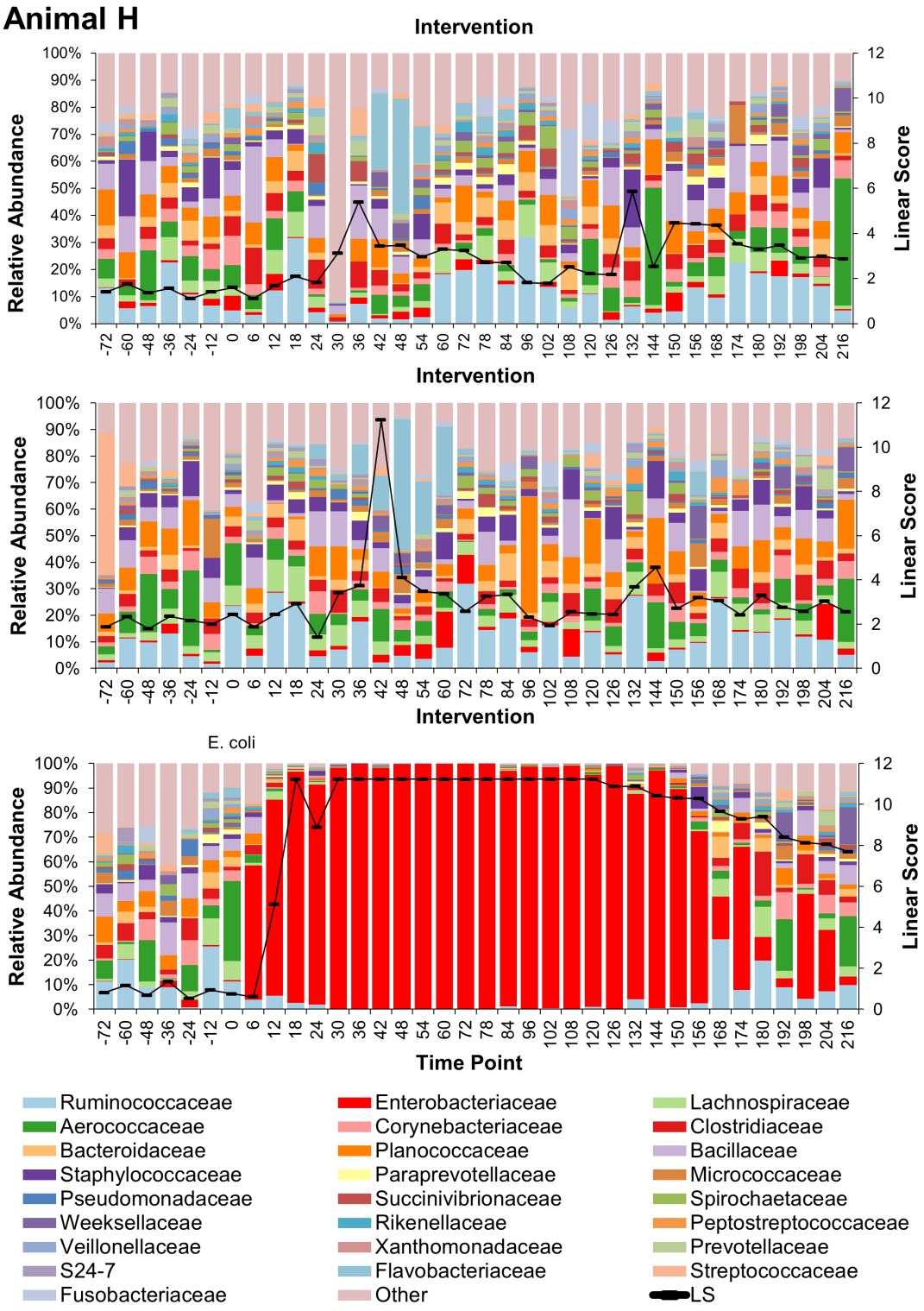

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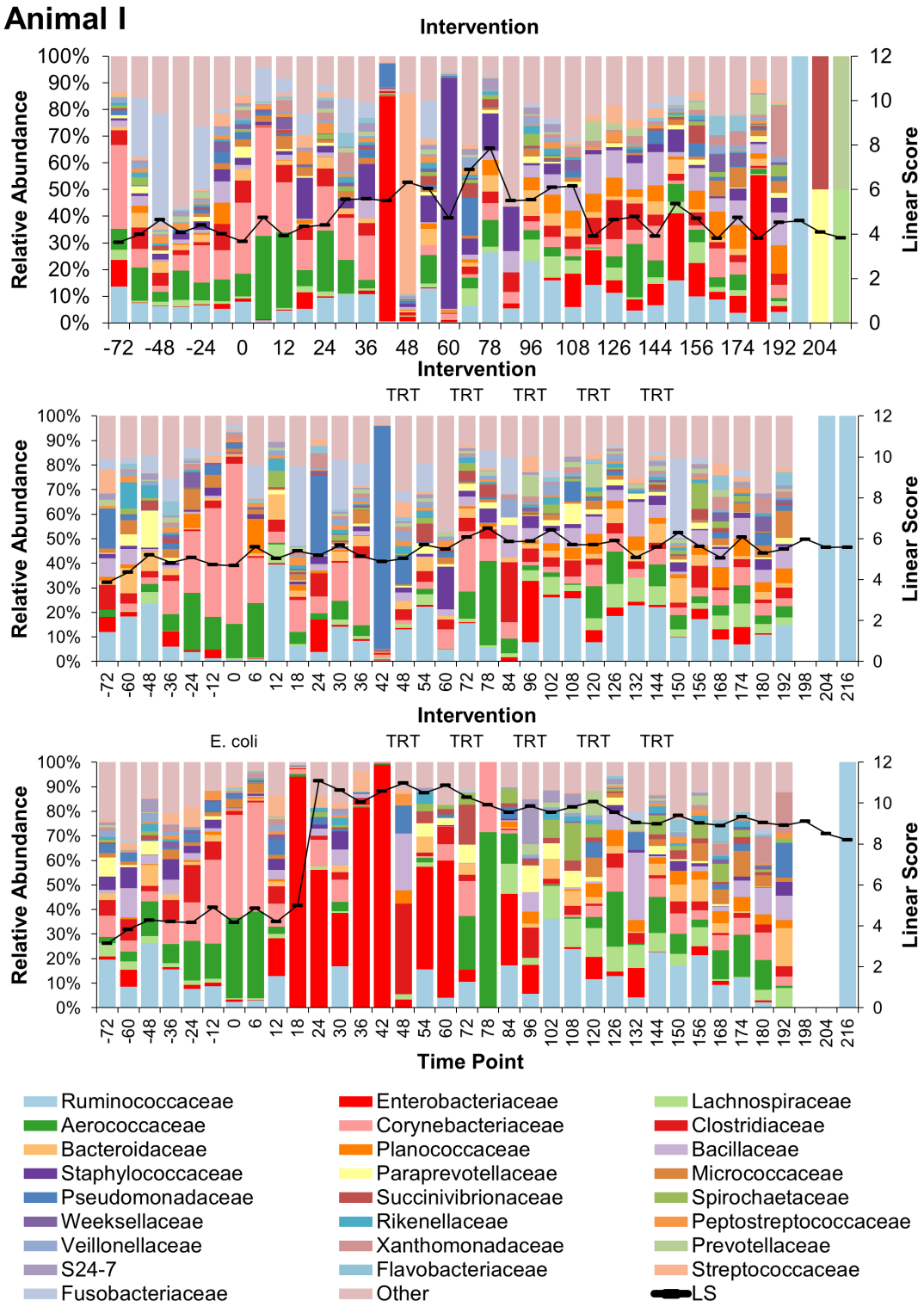

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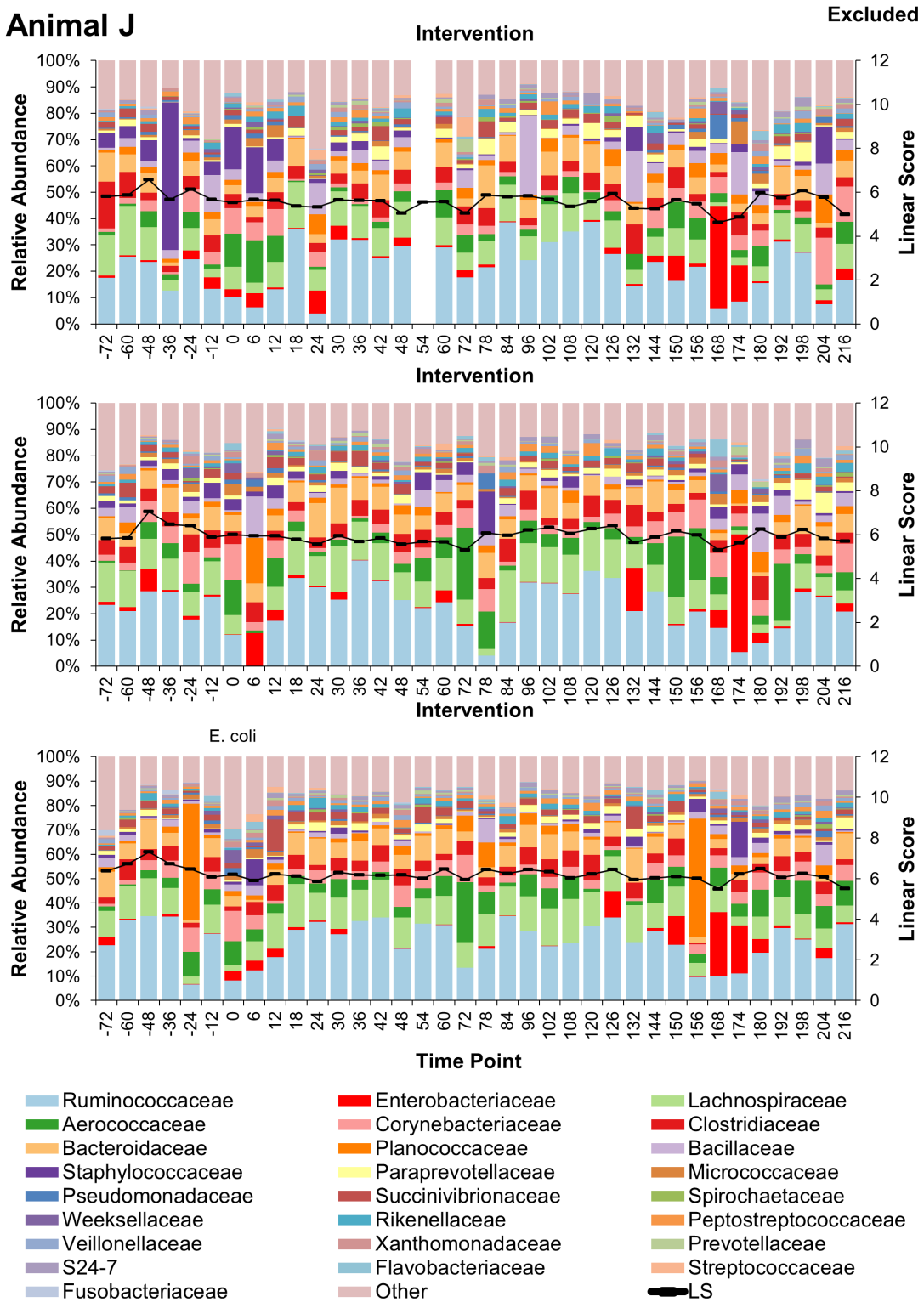


Figure 5.S11. Description of the microbiome from different quarters over time on animal K.

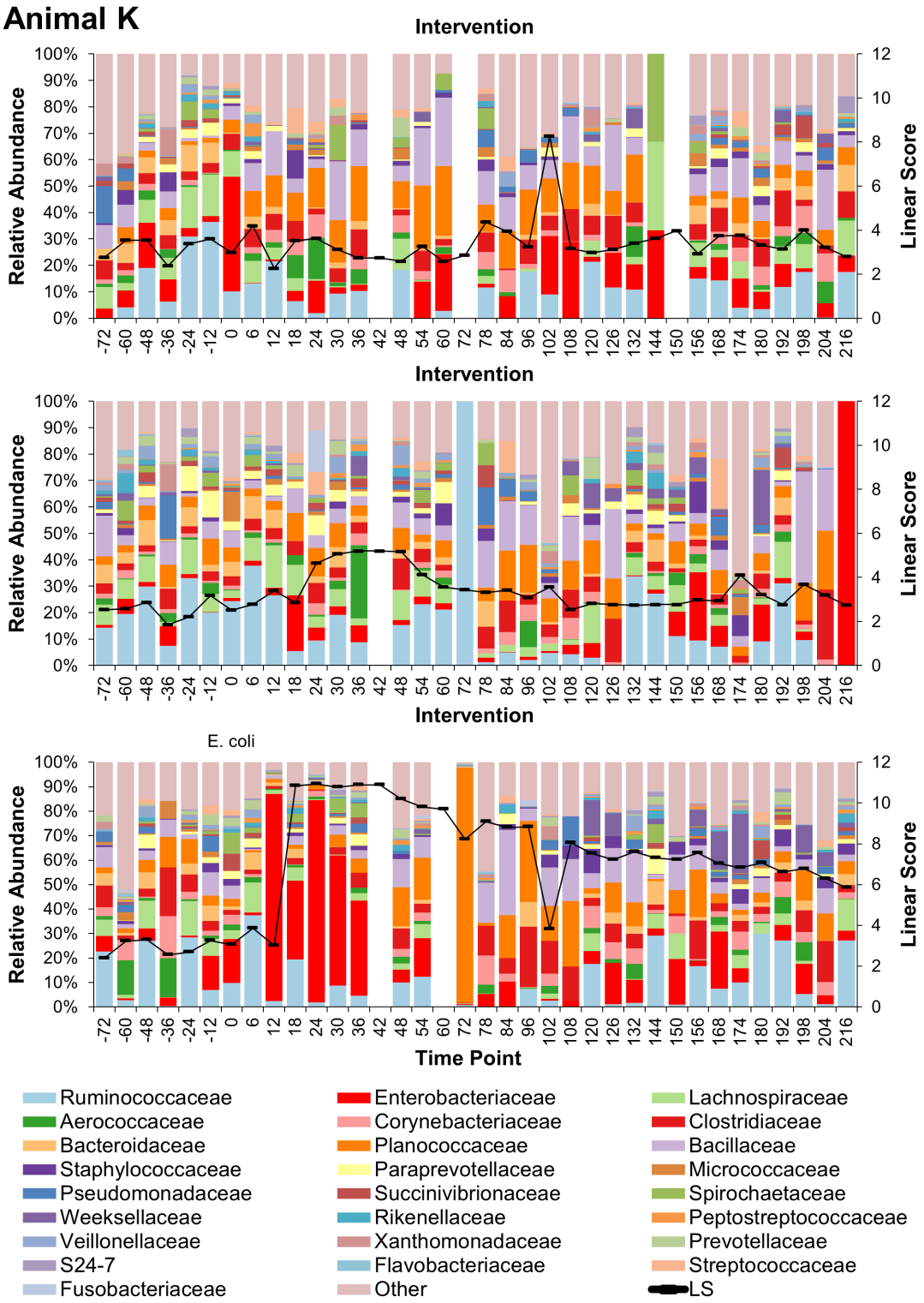
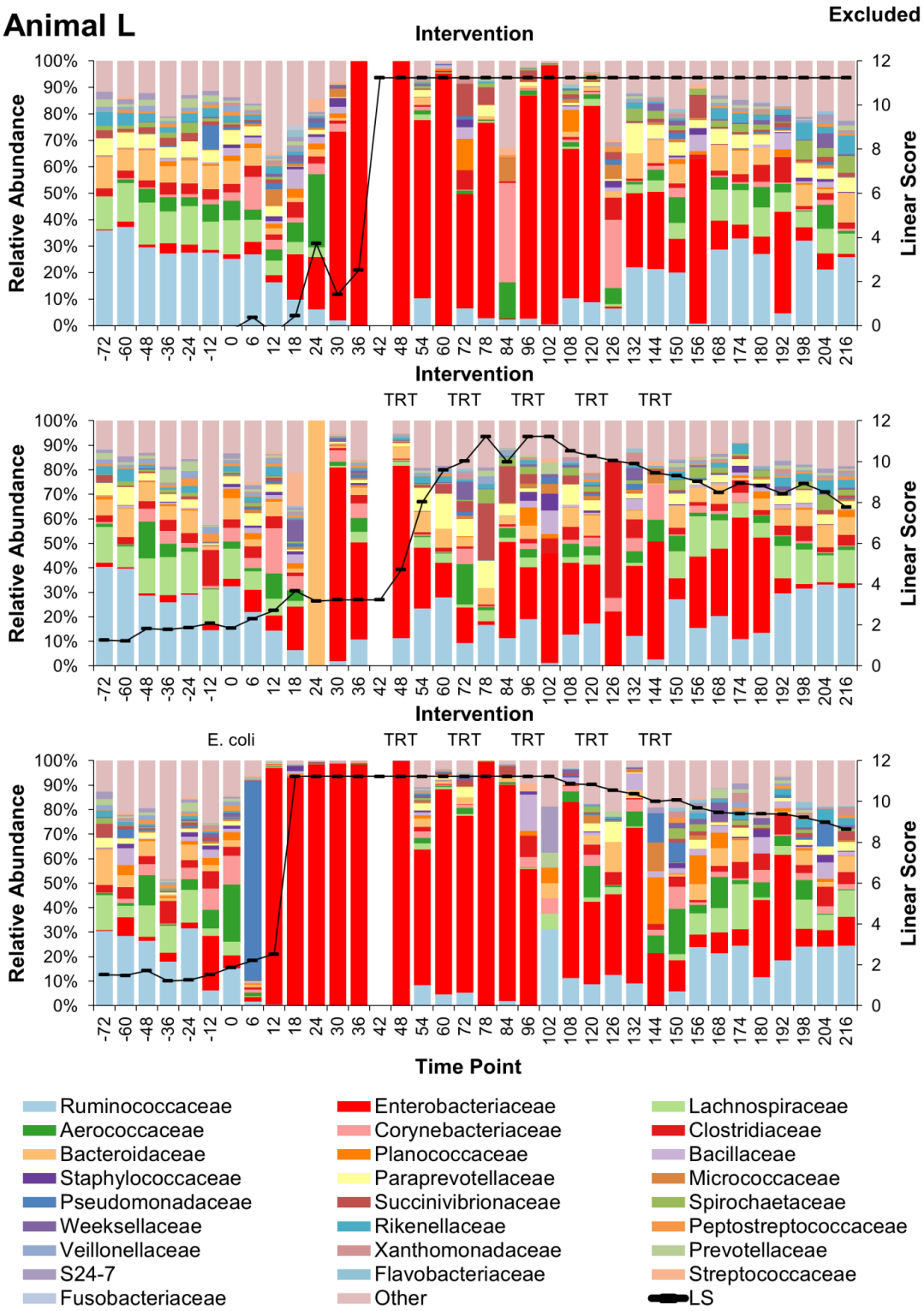


Figure 5.S12. Description of the microbiome from different quarters over time on animal L.



CHAPTER 6

CONCLUSION AND FUTURE DIRECTIONS

The overall objective of the research conducted here is to provide data that can facilitate effective management, treatment, and understanding of bovine mastitis. In chapter two, we conclude that the diagnostic system evaluated is suitable for use under field conditions, presenting overall sensitivity and specificity of 83% and 92% compared with results from standard laboratory diagnosis. Accumast® represents a unique approach for on-farm identification of bacteria present in milk, through the straightforward color-based interpretation. Through the use of this tool, diagnosis of mastitis pathogens can be performed by individuals with limited microbiology training, which facilitates selective antimicrobial therapy of mastitic cows.

While investigating the potential of injectable trace mineral supplementation to improve udder health, we identified that animals cured from subclinical mastitis had higher concentrations of phosphorus and selenium, irrespective of receiving or not supplementation. Furthermore, trace mineral supplementation tended to improve subclinical mastitis cure in cows with three or more lactations. Nevertheless, no overall effect was observed in reducing the incidence of clinical mastitis; however mineral supplementation significantly reduced the incidence of chronic clinical mastitis in primiparous animals. Further research is necessary to separate the effects of injectable supplementation of trace minerals and the mineral profile in cured animals. Supplementation with trace minerals did not affect somatic cell count, milk yield, or milk composition.

The findings from both chapters four and five indicate that the milk harbors a resilient microbiome, and that the antimicrobial treatment of mild and moderate cases of bovine mastitis caused by *Escherichia coli* or yielding negative results upon aerobic culture is not advantageous. The research methods employed during these studies contribute to a better understanding of the microbial dynamics of bovine mastitis. Intramammary infusion of *E. coli* severely affected microbial diversity as well as significantly increased levels of *Enterobacteriaceae*. Ceftiofur had a significant effect in decreasing bacterial load in both studies, however this effect could only be observed during treatment administration. Nevertheless, the most remarkable result was the lack of difference in microbial profile, pathogen load, pathogen clearance and diversity between treated and untreated quarters after treatment cessation. We learn from these studies that judicious use of antimicrobial can be achieved without long term changes in the milk microbiome and without compromising animal health.

Nevertheless, several avenues of research remain open in this topic. For instance, we identified through the significant reduction in LogCFU that intramammary treatment with ceftiofur impairs the viability of *E. coli* without affecting the relative abundance of *Enterobacteriaceae*. To date, no data is available regarding possible changes that can occur in the metabolic and transcriptional profile of bacteria present in milk exposed to antimicrobial agents. It is important to highlight that although a vast amount of information is provided in this dissertation we have focused in specific mastitis pathogens, and have only evaluated the effects of one antimicrobial on the milk microbiome. The etiology of bovine mastitis is complex, and several classes of antimicrobials are currently labeled for intramammary treatment of mastitis.

Based on the research carried out here we cannot predict what would be the changes in the microbial profile when other pathogens and other drugs come into play.

It will be interesting to assess how active are the microbes present in milk. Through sequencing the 16S rRNA we are able to identify which organisms are present in milk, but it is not possible to assess the viability of such organisms. Moreover, no conclusions can be drawn from the studies presented here in regards of increase in antimicrobial resistance.

In conclusion, further research using other 'omics' technologies such as metabolomics and proteomics will have the ability to detect differences and treatment effects that are not evident by microbiome profiling through sequencing of the 16S fraction of ribosomal RNA.