



## The *Lactococcus* genus as a potential emerging mastitis pathogen group: A report on an outbreak investigation

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### ABSTRACT

The bacterium *Lactococcus lactis* is widely used in food production and in medical applications, and is considered safe for human and animal use. However, studies have also linked *Lactococcus* bacteria to infection. For example, certain variants of *Lactococcus* species have been associated with bovine mastitis (e.g., *Lactococcus lactis* and *Lactococcus garvieae*). In this study, we investigated an outbreak of bovine mastitis thought to be associated with *Lactococcus* bacteria by using microbiological and molecular techniques. We used bacterial isolation, next-generation sequencing, DNA fingerprinting, and other methods to test our hypothesis that *Lactococcus* microbes were the primary pathogen causing the mastitis outbreak. Twenty-eight *Lactococcus* isolates were obtained from mastitic milk of 28 dairy cows. The isolates were identified as *L. lactis* (27 isolates) and *L. garvieae* (1 isolate). Phylogenetic analysis based on 16S rDNA gene sequence comparison indicated similarity among the *L. lactis* isolates as well as between the isolates and reference sequences. The DNA fingerprinting analysis based on random amplified polymorphic DNA results of the 27 *L. lactis* isolates identified different random amplified polymorphic DNA profiles, which suggests they originated from multiple sources. Microbiome analysis determined *Lactococcus* to be the dominant genus in the majority of the mastitic milk samples, whereas it was found in low relative abundance in healthy milk samples. The *Lactococcus* genus was detected in all environmental samples tested, and sampling of bulk tank milk corroborated that *Lactococcus* was not abundant in healthy milk from the same dairy herd. In summary, our findings suggest that *Lactococcus* bacteria are a potential etiological agent in the mastitis outbreak studied. Further studies should be conducted to understand the importance of *Lacto-*

*coccus*, especially *L. lactis*, as pathogenic microbes in veterinary medicine and food safety.

**Key words:** mastitis, *Lactococcus*, microbiome, next generation sequencing

### INTRODUCTION

Mastitis is an important disease in dairy cows causing reproduction problems (Hertl et al., 2010), culling (Gröhn et al., 2005), and economic losses due to reduced milk production, treatment expense, and discarded milk (Bar et al., 2008). Clinical mastitis is also a painful disease and is associated with behavioral changes (Medrano-Galarza et al., 2012).

For treatment of mastitis, identifying the microorganisms responsible should be considered (Royster and Wagner, 2015). Many microbial species have been identified as etiological agents, typically through bacterial culture (Oikonomou et al., 2012). *Lactococcus* species have been isolated from bovine mastitis, and their association with the disease has been discussed (Werner et al., 2014; Plumed-Ferrer et al., 2015a). Lactococci are gram-positive, nonmotile cocci, homofermentative, poorly  $\alpha$ -hemolytic, and exclusively produce L(+)-lactic acid (Casalta and Montel, 2008). They are members of the group lactic acid bacteria (**LAB**) and are routinely found on animal skin and plants (Casalta and Montel, 2008). Lactic acid bacteria are generally not considered harmful to humans (Mofredj et al., 2007) or animals (Klostermann et al., 2008; Espeche et al., 2012; Bouchard et al., 2015) and have been used for the prevention and treatment of human (Mofredj et al., 2007) and animal diseases (Klostermann et al., 2008; Espeche et al., 2012; Bouchard et al., 2015). Lactic acid bacteria have been reported to produce proteins, chemical mediators, and other molecules that stimulate local immune responses (Mofredj et al., 2007).

Interestingly, in dairy cows, the potential of LAB for treatment or prevention (or both) of mastitis has been considered (Klostermann et al., 2008; Espeche et al., 2012; Bouchard et al., 2015). Klostermann et al. (2008) evaluated the use of a live culture suspension of

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*Lactococcus lactis* DPC3147 to treat naturally infected mastitic animals. Trials were conducted for subclinical and acute clinical mastitis, which demonstrated that treatment with *L. lactis* DPC3147 culture potentially had a similar level of efficacy as common antibiotics (Klostermann et al., 2008).

*Lactococcus lactis*, in particular, is of considerable economic importance (Cavanagh et al., 2015) and is widely used as a starter culture in dairy fermentation (Casalta and Montel, 2008). *Lactococcus lactis* is known to produce bacteriocin (Klostermann et al., 2008) and is frequently used as a probiotic (Furtado et al., 2014). Moreover, *L. lactis* is included on the Qualified Presumption of Safety list of the European Food Safety Authority (Plumed-Ferrer et al., 2013) and is accepted as generally recognized as safe (Casalta and Montel, 2008). However, some species of *Lactococcus* have been reported to be the cause of human (Davies et al., 2009; Hadjisymeou et al., 2013; Inoue et al., 2014) and animal infections (Plumed-Ferrer et al., 2013, 2015a; Khoo et al., 2014). Nevertheless, it is unclear if these cases represent the emergence of novel pathogenic strains or were detected due to the availability of improved identification methods (Plumed-Ferrer et al., 2015b).

Werner et al. (2014) confirmed, by using a DNA sequencing approach, that the majority of isolates from bovine mastitis milk samples, which were phenotypically identified as *Streptococcus* spp., were in fact *L. lactis*. *Lactococcus* species are closely related to streptococci and *Streptococcus*-like genera such as *Enterococcus* and *Aerococcus*. Therefore, the role of *Lactococcus* spp. as an etiological agent of mastitis may have been underreported throughout the years (Werner et al., 2014). Considering this, *Lactococcus* species isolated from bovine intramammary infections are now being characterized both genotypically and phenotypically (Plumed-Ferrer et al., 2013, 2015a; Werner et al., 2014). However, the mechanism of pathogenicity is not yet fully understood (Plumed-Ferrer et al., 2015b), and few studies have been conducted on lactococci as potential bovine mastitis pathogens.

Therefore, the aim of the present study was to use current microbiological and molecular techniques to investigate an outbreak of mastitis that was thought to be associated with *Lactococcus* infection. Specifically, we hypothesized that a member(s) of the *Lactococcus* genus was the primary pathogen causing the mastitis outbreak. To explore our hypothesis, we used next-generation sequencing of the 16S rRNA gene, random amplified polymorphic DNA-PCR (RAPD-PCR), and phylogenetic techniques.

## MATERIALS AND METHODS

### Animal Care Statement

All experimental procedures in this study conformed to 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), which regulate 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 Cornell University (protocol number: 2013–0056).

### Farm and Management

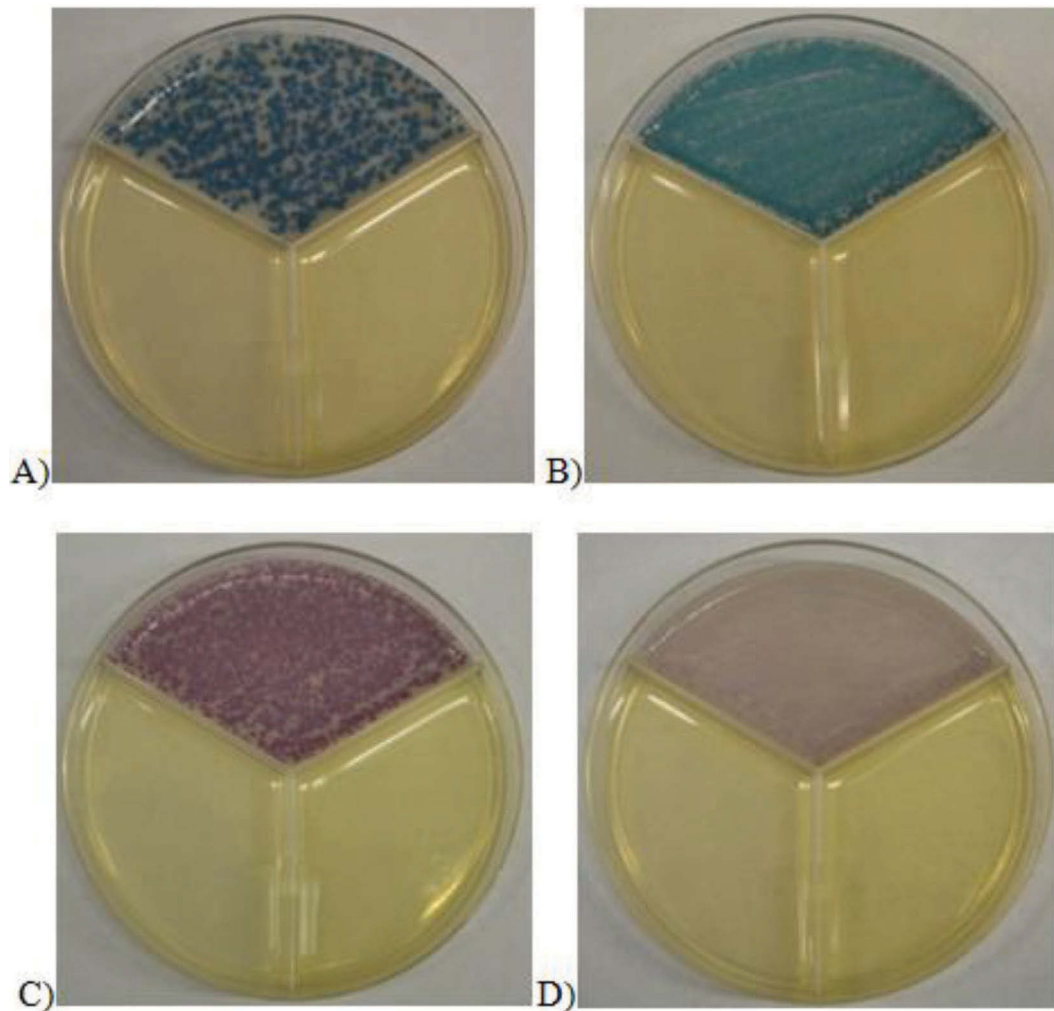
The study was conducted on a single commercial dairy farm located in upstate New York. During the experimental period, from July until October 2015, the farm milked approximately 1,200 cows. Primiparous and multiparous cows were housed separately in freestall barns bedded with sand. Cows were fed a TMR 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). Cows were milked thrice daily in a double-20 milking parlor.

The target length of the dry period was 55 d. Cows were dried off by abrupt interruption of milking and dry cow therapy was equally performed for all quarters of all cows and consisted of intramammary infusion with ceftiofur hydrochloride (Spectramast DC, Zoetis, Madison, NJ) followed by the administration of an internal teat sealant (Orbeseal, Zoetis). Before the outbreak of *Lactococcus* spp., the common mastitis pathogens encountered based on aerobic mastitic milk culture were represented by approximately 33% gram-negative microbes, mainly *Escherichia coli* and *Klebsiella*; 33% gram-positive microbes, mainly *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus* spp., and *Staphylococcus* spp.; and 33% were culture negative from a total of approximately 500 mastitic milk samples evaluated during a period of 1 yr prior to the *Lactococcus* outbreak. The historical clinical cure rates following intramammary antibiotic therapy of clinical mastitis were approximately 70%. The bulk tank SCC ranged from 150,000 to 250,000. The herd is a closed herd with no other major concomitant disease problems. The incidence of displaced abomasum, ketosis, metritis, and retained placenta were 2.5, 5, 12, and 6%, respectively.

### Enrollment Criteria, On-Farm Culture, and Sample Collection

Clinical mastitis was identified by the presence of abnormal milk, such as a watery appearance, flakes, and clots; the identification was performed thrice daily in the milking parlor by farm employees. On a daily basis, milk samples from all clinical mastitis cases (from mild to severe cases of mastitis) were cultured using the AccuMast-Mastitis Culture System (FERA Animal Health, Dryden, NY). The AccuMast system consists of a single Petri dish with 3 selective chromogenic media used to identify and differentiate specific mastitis pathogens (Ganda et al., 2016). Ganda et al. (2016) validated the on-farm use of AccuMast by comparing its results with the results of a referral laboratory and also to 16S rRNA gene sequencing. Unfortunately, no *Lactococcus*

mastitis cases were identified in that study. The present study was motivated by the fact that the farm manager of the study farm noticed the appearance of a unique coloration pattern for an increasing number of cows cultured using the AccuMast system (Figure 1). Plates were incubated aerobically at 37°C for a period of 16 to 20 h when interpretation of test results was performed by a trained farm employee, and affected cows that had culture results consistent with suspected infection by a *Lactococcus* species (Figure 1D) were eligible to enter the study. Before intramammary antibiotic treatment was started and within 24 h of the initial diagnosis, a fresh milk sample was aseptically collected from cows that were considered *Lactococcus* positive by a trained farm employee, a total of 28 samples from 28 different cows. Briefly, the teat ends were cleaned, disinfected, and the first streams were discarded before a sample of



**Figure 1.** Growth characteristics of different bacteria on the second section of the AccuMast-Mastitis Culture System (FERA Animal Health, Dryden, NY). Growth of (A) *Streptococcus uberis* (ATCC 700407); (B) *Streptococcus agalactiae* (ATCC 27956); (C) *Enterococcus faecalis* (ATCC 29212); and (D) *Lactococcus lactis* isolated from mastitic milk in this study. Color version available online.

approximately 8 mL of milk was collected in a sterile vial from each clinically diseased quarter. Additionally, quarter milk samples from 30 clinically healthy cows were collected to serve as a baseline comparison for the microbiome studies. All samples were kept refrigerated, (for approximately 2 h) while being transported to our laboratories at Cornell University for further processing.

To investigate the potential environmental sources of *Lactococcus* spp., we collected random sand bedding samples from different cow pens: 5 samples from the superficial layer and 5 samples from the deep layer for microbiome investigation. A bulk tank milk sample was also collected for microbiome investigation, transported on ice to the laboratory, and then stored at  $-20^{\circ}\text{C}$  until further processing. All samples were collected as soon as the outbreak was identified by farm employees.

### **Bacterial Isolation and Bacterial Identification by the Sanger Sequencing Method**

Mastitic milk samples considered *Lactococcus* spp. positive were aseptically inoculated onto CHROMagar StrepB (CHROMagar, Paris, France) using sterile swabs, and incubated under aerobic conditions at  $37^{\circ}\text{C}$  for up to 24 h. Single, light pink colonies from each sample were selected and streaked onto the same chromogenic culture medium and incubated under the same conditions. Good bacterial growth was observed after the first inoculation; thus, this step was repeated 3 times to generate a pure culture from each mastitic milk sample. Next, the bacterial colonies were inoculated into Bacto Brain Heart Infusion broth (Becton, Dickinson and Company, Sparks, MD) and incubated overnight at  $37^{\circ}\text{C}$ ; these cultures were used to prepare stocks (bacterial cultures stored in 50% glycerol at  $-20^{\circ}\text{C}$  for further use) and to extract DNA.

The DNA was extracted from each bacterial isolate using InstaGene Matrix (Bio-Rad Laboratories Inc., Hercules, CA) following the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). The 16S ribosomal DNA gene was amplified using 10 pmol of each fd1forward and rP2 reverse primers (Weisburg et al., 1991), Econo-Taq Plus Green 1 $\times$  Master Mix (Lucigen, Middleton, WI), 280 to 350 ng of template DNA, and ultrapure distilled water to bring the final reaction volume to 100  $\mu\text{L}$ . The conditions for amplification were  $94^{\circ}\text{C}$  for 5 min,  $57^{\circ}\text{C}$  for 2 min, and  $72^{\circ}\text{C}$  for 2 min followed by 29 cycles of  $94^{\circ}\text{C}$  for 2 min,  $57^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 2 min, with a final extension of  $72^{\circ}\text{C}$  for 10 min (Wood et al., 1998). Agarose gel electrophoresis (1.2% wt/vol)

was used to verify the presence of PCR products. The DNA was visualized with 0.5  $\mu\text{g/mL}$  ethidium bromide.

The PCR products were purified using a Gel/PCR Fragments Extraction Kit (IBI Scientific, Peosta, IA) in accordance with the manufacturer's recommendations. The purified samples were submitted to the Cornell University Life Science Core Laboratories Center for Sanger sequencing using 8 pmol of primer fd1 and 300 ng of PCR products. FASTA sequences were compared against sequences stored in GenBank using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; Benson et al., 2009; Sayers et al., 2009).

### **Phylogenetic Analysis**

Using Geneious software version 9.0.4 (Biomatters, Auckland, New Zealand), the 16S rDNA sequences (Sanger sequencing), amplified from all individual bacterial isolates, were aligned with each other and with relevant reference sequences obtained from the Ribosomal Database Project (Cole et al., 2014): *Lactococcus garvieae* (JCM8735; AB012306), *L. lactis* (MRS1; AJ488173), *L. lactis* ssp. *cremoris* (CF4; AB181302), *L. lactis* ssp. *lactis* (MR26, AF493057), *Lactococcus piscium* (fish isolate HR1A-68.; X53905), *Lactococcus raffinolactis* (NCDO617; X54261), *Streptococcus agalactiae* [(T); JCM 5671; AB023574], *Streptococcus dysgalactiae* (ATCC 35666; AJ319643), and *S. uberis* [(T); JCM 5709; AB023573]. Sequences were aligned using the Clustal W method (Larkin et al., 2007) and the final alignment was corrected manually. Evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and the phylogenetic tree was constructed by applying the neighbor-joining method (Saitou and Nei, 1987).

### **RAPD-PCR**

The RAPD-PCR was performed as previously described by Akopyanz et al. (1992) using primer 1254. The reactions were performed using 10 pmol of 1254 primer, 1 $\times$  EconoTaq Plus Green Master Mix (Lucigen), 280 to 350 ng of template DNA, and ultrapure distilled water was used to bring the final reaction volume to 50  $\mu\text{L}$ . The cycling program was 4 cycles of  $94^{\circ}\text{C}$  for 5 min,  $36^{\circ}\text{C}$  for 5 min, and  $72^{\circ}\text{C}$  for 5 min, followed by 30 cycles of  $94^{\circ}\text{C}$  for 1 min,  $36^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 2 min, and then a final extension at  $72^{\circ}\text{C}$  for 10 min (Akopyanz et al., 1992). The products of the reaction were visualized by electrophoresis using a 2% (wt/vol) agarose gel with 0.5  $\mu\text{g/mL}$  ethidium bromide. The profiles were compared visually and clustered according to suggested similarity.



### Metagenomic DNA Extraction and Next-Generation Sequencing of the Bacterial 16S rRNA gene

Samples of mastitic milk, milk from healthy cows, bulk tank milk, and sand bedding were analyzed by metagenomic sequencing of the 16S rRNA gene using the Illumina MiSeq platform (Illumina Inc., San Diego, CA). The DNA was extracted from all samples by using a PowerSoil DNA Isolation Kit (MO BIO Laboratory Inc., Carlsbad, CA) following the manufacturer's instructions. The 515f-806rB region of the 16S rRNA gene was amplified according to a previous study (Caporaso et al., 2010). Barcoded primers were used as previously described by Lima et al. (2015).

Amplification of the V4 hypervariable region of the 16S rRNA gene was completed using 10 pmol of each primer, EconoTaq Plus Green 1× Master Mix (Lucigen), 10 to 50 ng of template DNA, and ultrapure water to bring the final reaction volume to 25 µL. All reactions were set up in triplicate, and the PCR cycles were 94°C for 3 min, 35 cycles of 94°C for 45 s, 50°C for 1 min, 72°C for 90 s, and 72°C for 10 min. Replicates were pooled, visualized by electrophoresis through 1.2% (wt/vol) agarose gels stained with 0.5 mg/mL ethidium bromide and purified using a Gel/PCR Fragments Extraction Kit (IBI Scientific). The amplicons were quantified using a Qubit 2.0 Fluorometer (Invitrogen by Life Technologies, Carlsbad, CA), standardized to the same concentration and pooled for sequencing on the Illumina MiSeq platform. A final equimolar library was sequenced using the MiSeq Reagent Nano Kit V2 (300 cycles, Illumina Inc.).

### Analysis of Results

The results from Sanger sequencing, RAPD-PCR, and MiSeq Reporter Metagenomics Workflow for relative abundance and taxonomy, with a focus on genera, were descriptive. To illustrate the relative abundances of the 30 most abundant bacterial genera in the different samples evaluated, we generated a heatmap using JMP software (version 11, SAS Institute Inc., Cary, NC). Analysis of variance, implemented in JMP, was used to compare the relative abundances of *Lactococcus* between milk from mastitic and healthy cows.

## RESULTS

Initial screening using the AccuMast-Mastitis Culture System identified 28 mastitic milk samples with colonies characteristic of *Lactococcus* spp. The typical colonies of *Lactococcus* are shown in Figure 1D. The samples were inoculated onto CHROMagar to identify and confirm the presence of a typical lactococcal colony.

In all samples, typical colonies were obtained and the isolates were identified by Sanger sequencing of the 16S rDNA gene as *L. lactis* (27 isolates) and *L. garvieae* (1 isolate).

Alignment of each of the 16S rDNA gene sequences of the 27 *L. lactis* isolates and an *L. lactis* reference (MRS1; AJ488173) strain revealed a pairwise identity of 99.8%. The single *L. garvieae* isolate had a pairwise identity of 98.9% with the *L. garvieae* reference sequence (JCM8735; AB012306). High similarity among the isolates was evident from the phylogenetic tree generated using the 16S rDNA gene sequences (Figure 2). Furthermore, we observed higher similarity between the isolates and the *L. lactis* ssp. *cremoris* (CF4; AB181302) compared with the *L. lactis* ssp. *lactis* (MR26, AF493057).

We DNA fingerprinted the 27 *L. lactis* strains using the RAPD technique, which all showed a good RAPD banding pattern using primer 1254. Visual analysis suggested different RAPD profiles (Figure 3). Whereas several distinct RAPD profiles were observed, some isolates had a similar pattern (Figure 3).

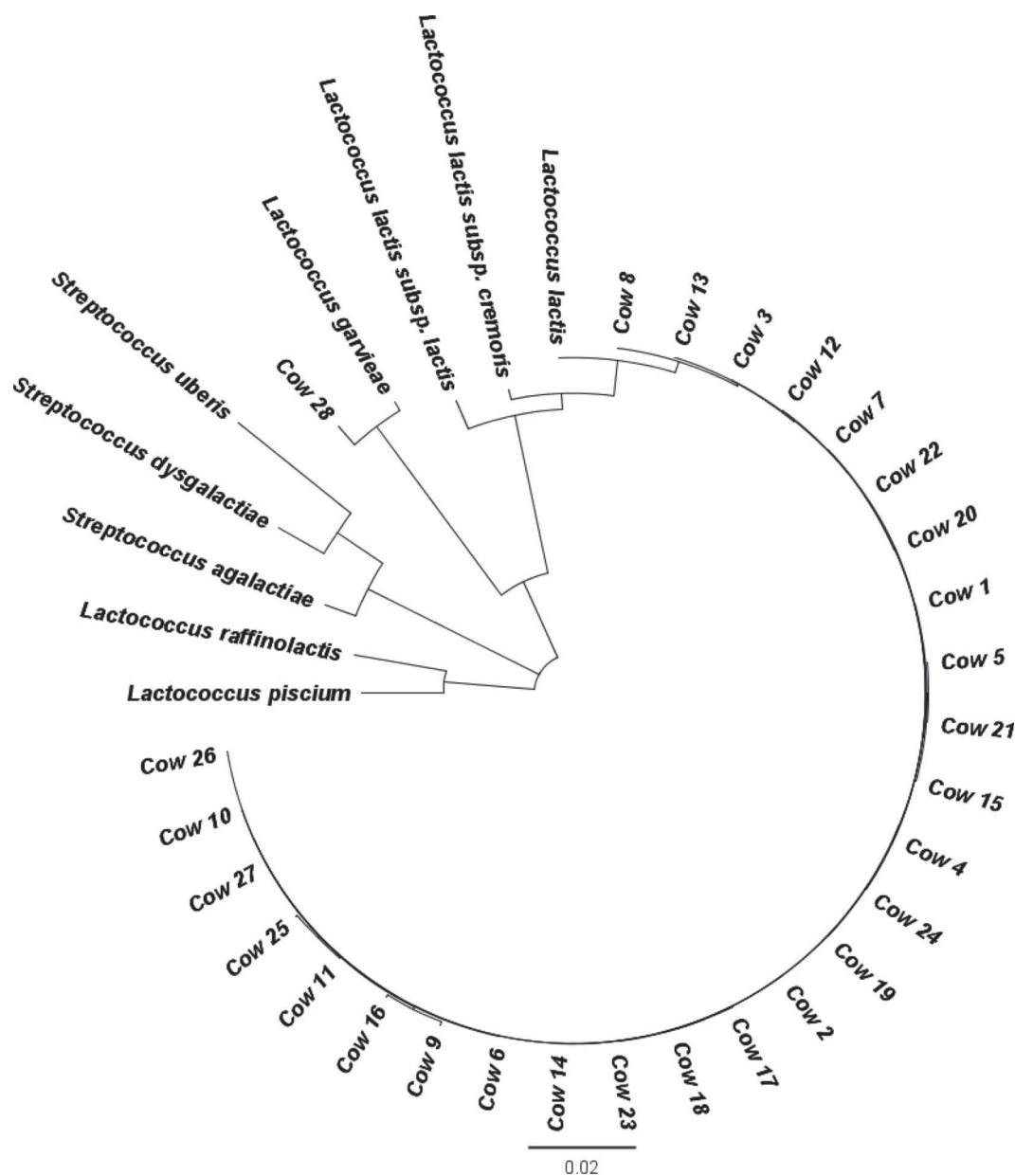
Quality-filtered reads for the 16S rRNA gene sequences were demultiplexed, yielding 4,899,000 sequences in total with a median length of 301 bases per read and an average coverage of 71,000 (SEM = 2,150) sequences per sample. We found 634 genera in mastitic milk samples and the 17 most abundant are shown in Figure 4. *Lactococcus* was detected in all samples, with 1.95 to 96.6% relative abundance, being the dominant genus in the majority of samples. A higher relative abundance of the *Lactococcus* genus was evident in the microbiome of mastitic milk samples compared with the microbiome of healthy milk samples (Figure 5). The average relative abundance of the genus *Lactococcus* for healthy milk was below 0.1%, which was 20 times lower than the relative abundance of *Lactococcus* in the mastitic milk. In sand bedding and bulk tank milk samples, 690 genera were identified, and the 30 most abundant are shown in Figure 6. *Lactococcus* was present in all samples. Interestingly, *Bergeyella* and *Acinetobacter* were the most abundant in the sand bedding samples, and *Chryseobacterium* and *Enhydrobacter* in the bulk tank sample. Accordingly, as in the healthy milk samples, we observed a low relative abundance of the *Lactococcus* genus in the bulk tank milk sample, specifically 0.5% (Figure 6).

## DISCUSSION

Our investigation of an outbreak of bovine mastitis on a dairy farm showed that species within the *Lactococcus* genus are a potential cause of the disease. It is possible that *Lactococcus* strains already existed in

the study herd before the investigated outbreak given the possibility that the referral laboratory misclassified the cases as *Streptococcus* spp. (Werner et al., 2014). However, the outbreak may have been the consequence of the introduction of new *Lactococcus* strains or changes in the environment that selectively favored the pre-existing *Lactococcus* strains. *Lactococcus lactis* was the dominant species detected and *L. garvieae* was the only other *Lactococcus* species identified. Phylogenetic tree analysis, using the 16S rRNA gene, confirmed that

all isolates were clustered with reference *L. lactis* sequences and only one isolate clustered with a known *L. garvieae* sequence. Microbiome analysis determined the *Lactococcus* genus to be dominant in most mastitic samples, with a high relative abundance, and *Lactococcus* genus was also identified in all investigated environmental samples. Conversely, *Lactococcus* bacteria were found at only low relative abundance in healthy milk and in bulk tank milk. These data support our hypothesis that bacteria of the genus *Lactococcus* were



**Figure 2.** Phylogenetic tree based on the sequences of 28 isolates from mastitic milk samples (where each cow is represented by an isolate) and the reference sequences used: *Lactococcus garvieae* (JCM8735; AB012306), *Lactococcus lactis* (MRS1; AJ488173), *L. lactis* ssp. *cremoris* (CF4; AB181302), *L. lactis* ssp. *lactis* (MR26, AF493057), *Lactococcus piscium* (fish isolate HR1A-68.; X53905), *Lactococcus raffinolactis* (NCDO617; X54261), *Streptococcus agalactiae* [(T); JCM 5671; AB023574], *Streptococcus dysgalactiae* (ATCC 35666; AJ319643), and *Streptococcus uberis* [(T); JCM 5709; AB023573].

the primary pathogen causing the mastitis outbreak. We used molecular techniques (e.g., next-generation sequencing) with increased accuracy and reliability to support our conclusions.

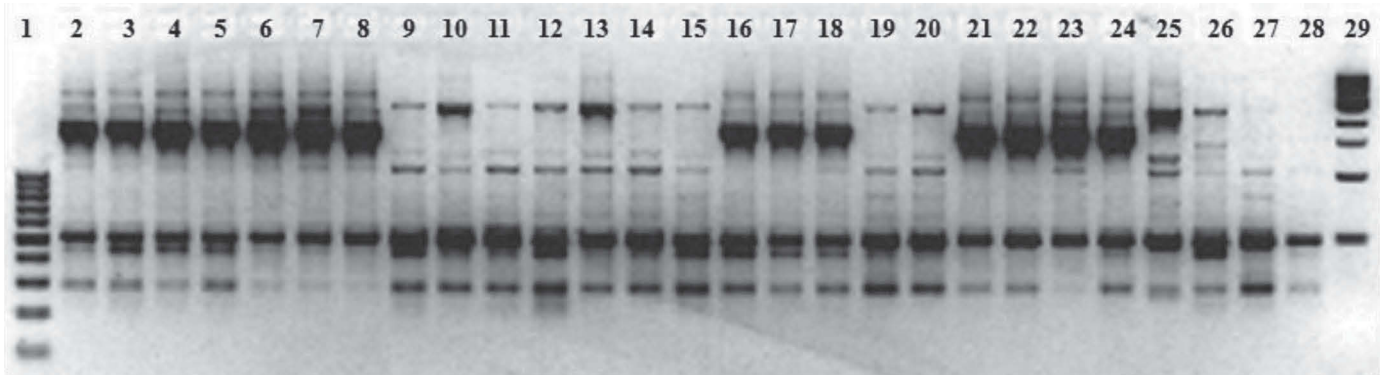
Methods based on PCR have greater accuracy than traditional methods for the identification of species (Kuang et al., 2009; Plumed-Ferrer et al., 2013). Therefore, we used Sanger sequencing, which identified *L. lactis* and *L. garvieae* in mastitic milk samples, consistent with other recent studies (Plumed-Ferrer et al., 2013, 2015a; Werner et al., 2014). The questionable reliability of traditional methods for identifying common and emerging mastitis pathogens is a concern. Phenotypic and biochemical methods for identification of streptococci and streptococci-like bacteria are thought to be inaccurate and inconsistent (Fortin et al., 2003; Werner et al., 2014). For example, Werner et al. (2014) found that traditional methods overestimated the abundance of *S. uberis* and failed to identify *L. lactis* ssp. *lactis*. Similarly, Plumed-Ferrer et al. (2013) concluded that phenotypic tests have likely underestimated the incidence of *Lactococcus* in clinical samples throughout the years. Furthermore, in another study, the API 20 Strep System misidentified *Lactococcus* isolates as *Enterococcus faecium* and *Enterococcus* spp.; their true identities were subsequently determined by 16S rRNA gene sequencing to be *L. lactis* ssp. *lactis* and *L. garvieae* (Wyder et al., 2011).

Phylogenetic analysis using the 16S rRNA gene revealed that the majority of the *Lactococcus* isolates were closely clustered with the *L. lactis* ssp. *cremoris* strain (CF4; AB181302). In humans, infections caused by *Lactococcus*, specifically *L. lactis* ssp. *cremoris*, are generally rare (Hadjisymeou et al., 2013); however, during the past 50 yr numerous case reports of infection have been published (Davies et al., 2009). Many of these cases were associated with unpasteurized dairy

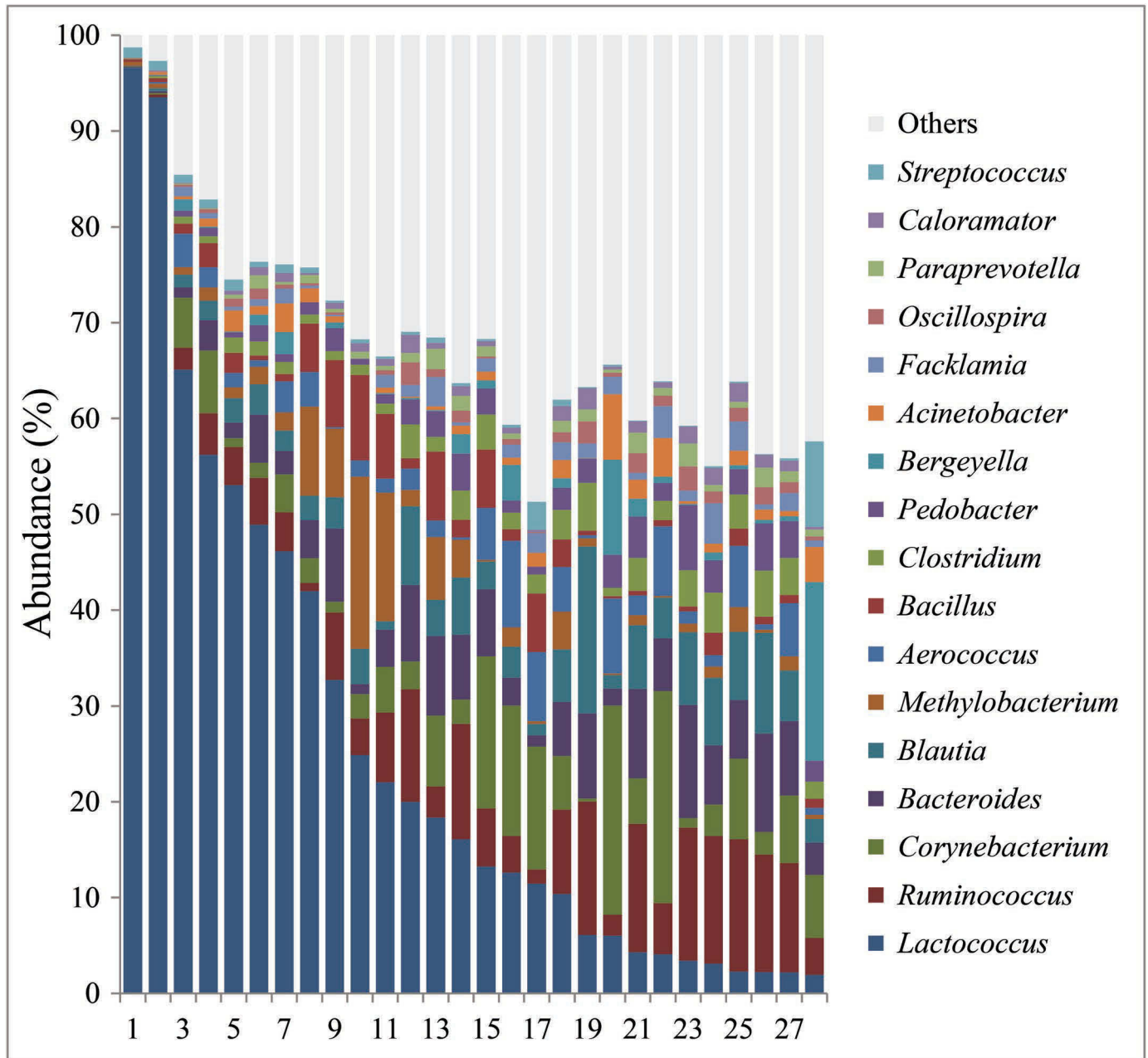
products or immunodeficiency, and in one case the bacterium was isolated from a dairy product (Hadjisymeou et al., 2013; Inoue et al., 2014). *Lactococcus* bacteria remain viable in transit through the gastrointestinal tract, which is believed to be the mode of infection in humans (Hadjisymeou et al., 2013). The pathogenicity of *L. lactis* ssp. *cremoris* is unknown and its route of infection is not well understood (Inoue et al., 2014). Both *L. lactis* ssp. *cremoris* and *L. garvieae* have been isolated from bovine mastitis (Plumed-Ferrer et al., 2013, 2015b). *Lactococcus garvieae* is a known important pathogen in aquaculture, and some human cases of *L. garvieae* infection had a history of patient contact with raw fish (Navas et al., 2013).

All of the isolates we identified as *L. lactis* from different animals were DNA-fingerprinted by RAPD-PCR. Several different RAPD profiles were present and a few patterns seemed to occur in multiple cows, which could indicate point source infection, contagious transmission, or lack of discriminatory power, as previously explored for other mastitis pathogens (Daly et al., 1999; Munoz et al., 2007). Herein, we should consider the possibility that different cows were being infected by a common source, which could explain the fact that some *Lactococcus* isolates had similar RAPD-PCR agarose gel profiles. Only a few studies have used RAPD-PCR for DNA fingerprinting of *Lactococcus* isolates (Tailliez et al., 1998; Mangin et al., 1999; Plumed-Ferrer et al., 2015a), and further studies are still needed to validate this technique for differentiation of *Lactococcus* isolates. Additional studies are warranted to elucidate the mode of transmission of *Lactococcus* and also to evaluate the short and long-term effect of *Lactococcus* mastitis on cow health, milk production, quality, and composition.

We assessed the microbial diversity in mastitic milk, sand bedding, and bulk tank milk. The 16S rRNA gene sequencing of microbes in mastitic milk identi-



**Figure 3.** Random amplified polymorphic DNA-PCR analysis using primer 1254 to fingerprint 27 *Lactococcus lactis* strains isolated from mastitic milk. Lane 1 = 100-bp DNA ladder (Omega Bio-Tek, Norcross, GA), lanes 2 to 28 = isolates from cows 3, 17, 20, 4, 21, 5, 2, 23, 9, 7, 25, 15, 19, 14, 22, 24, 1, 8, 12, 18, 16, 6, 10, 26, 13, 27, and 11, respectively; and lane 29 = 1-kb ladder (New England BioLabs Inc., Ipswich, MA).



**Figure 4.** Mean relative abundance of the 17 most prevalent bacterial genera identified in mastitic milk samples (others = sum of all other genera). Color version available online.

fied *Lactococcus* in all samples; in fact, it was the most abundant genus in the majority of samples (Figure 4). Whereas previous studies using 16S rRNA gene sequencing did not identify this genus as being among the most abundant in milk samples (Oikonomou et al., 2012, 2014), other studies using culture-independent methods showed that *Lactococcus* was widely distributed in raw milk, pasteurized milk (Quigley et al., 2013), and raw milk from bovine mastitis (Kuang et al., 2009). Therefore, we compared the relative abundance

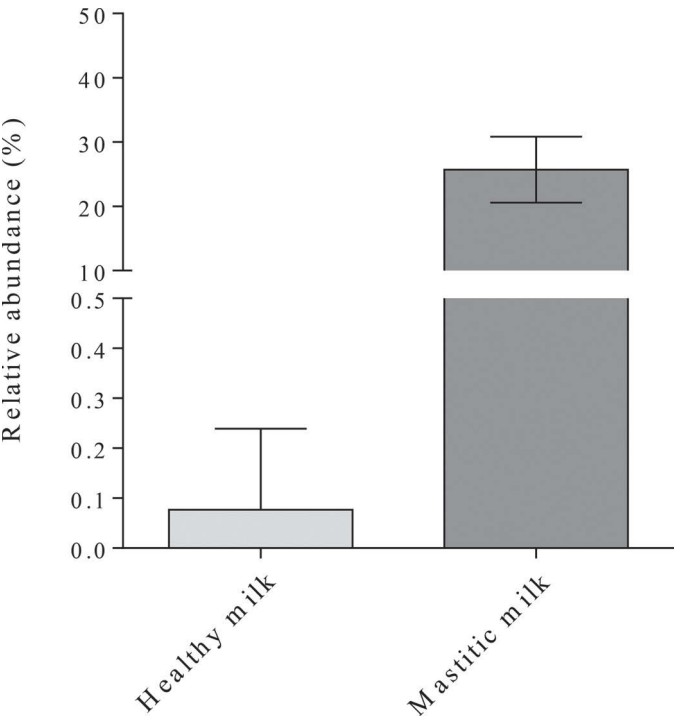
of the *Lactococcus* genus in mastitic and nonmastitic milk samples (Figure 5). The results showed a higher relative abundance of *Lactococcus* in mastitic milk, suggesting that species within this genus are potential etiologic agents of disease.

Microbial diversity analysis of sand bedding and bulk tank milk identified *Lactococcus* in all samples; it was present among the 30 most prevalent bacterial genera (Figure 6). In bulk tank milk, *Lactococcus* was not highly abundant, which confirms that *Lactococcus* was

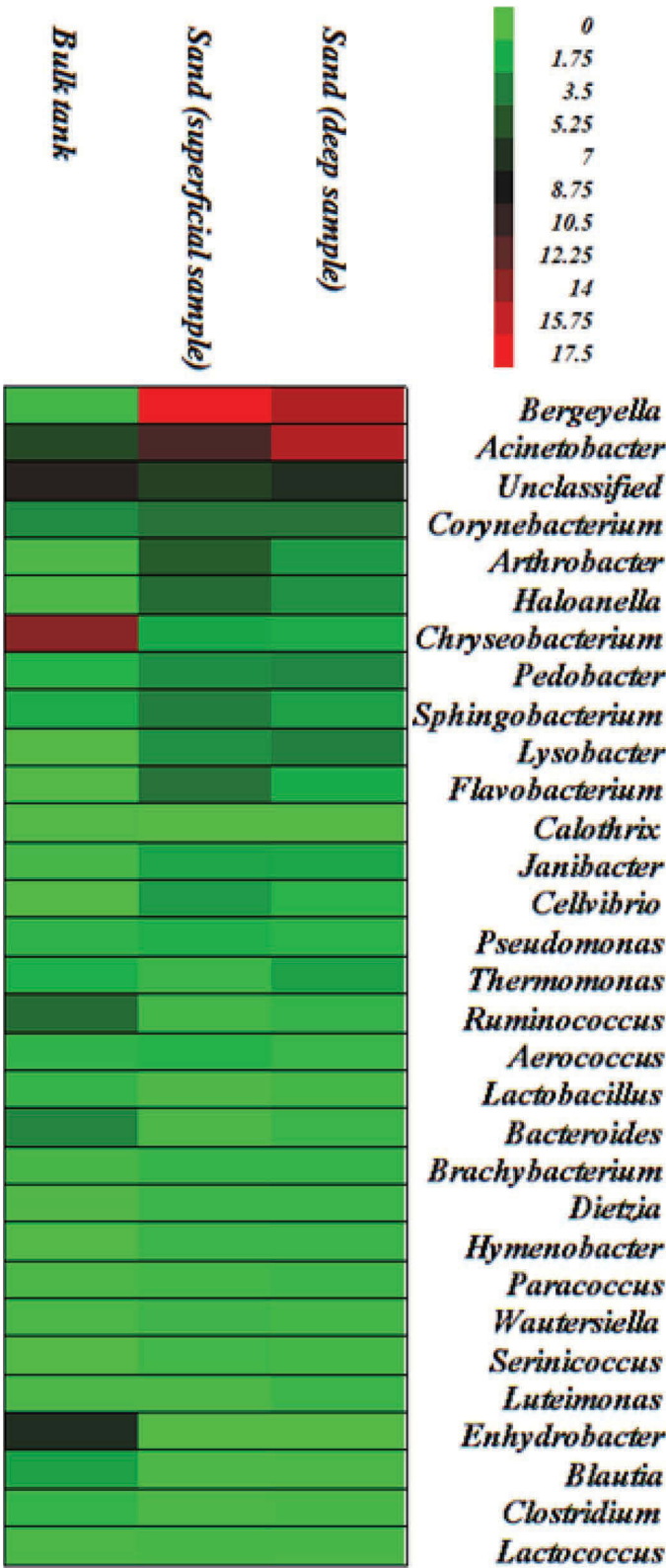


not common in milk of healthy cows in the study herd. In addition, *Chryseobacterium* and *Enhydrobacter* were the most prevalent genera in bulk tank milk. *Chryseobacterium* spp. are psychrotrophic bacteria frequently detected in dairy products (Delbès et al., 2007; Munsch-Alatossava et al., 2012). *Enhydrobacter* is a rare genus with only a single species, *Enhydrobacter aerosaccus* (Kawamura et al., 2012), and has been identified in dairy products at a low level (Oki et al., 2014). It was not expected to be abundant within the milk samples; however, little information has been published regarding this genus. Further studies on *Enhydrobacter* are necessary to assess its importance in the dairy industry.

In sand bedding samples, *Lactococcus* was detected in all samples, which could indicate that sand bedding was a potential reservoir for *Lactococcus*. However, it is also possible that the sand bedding evaluated herein was not the primary source of contamination but was simply contaminated by milk leakage from infected cows while they were lying down; samples analyzed in the present study were collected directly from the free-stalls. Nevertheless, the identification of *Lactococcus* in the sand bedding indicates that this microbe could be transmitted from cow to cow via the contaminated sand or the sand bedding could be a direct primary source of contamination.



**Figure 5.** Mean of relative abundance of *Lactococcus* in healthy milk (n = 30) versus mastitic milk (n = 28). Error bars represent the standard deviation of the mean relative abundance.



**Figure 6.** Mean relative abundance of the 30 most prevalent bacterial genera identified in the sand bedding and bulk tank milk samples. The scale represents the relative abundance of each bacterial genus. Color version available online.

The *Bergeyella* and *Acinetobacter* genera were highly abundant in sand bedding samples. Whereas little is known about *Bergeyella* (Han et al., 2006), *Acinetobacter* is found in natural ecosystems and in the spoilage flora of foods (Kämpfer, 2014), and is a known opportunistic human pathogen (Touchon et al., 2014). The exact environmental reservoirs of *Acinetobacter* spp. are unknown (Touchon et al., 2014). The high abundance of *Bergeyella*, *Acinetobacter*, *Chryseobacterium*, and *Enhydrobacter* found in this study urges further research to determine their potential importance to the dairy industry.

## CONCLUSIONS

Based on the results of microbiological and molecular biology analyses, we suggest that *Lactococcus* is a potential etiologic agent of mastitis in the outbreak studied. We identified *L. lactis* and *L. garvieae* in milk of affected cows, and the *L. lactis* isolates showed different RAPD patterns suggesting they originated from multiples reservoirs. The microbiome was assessed for several different samples. *Lactococcus* bacteria were highly abundant in most mastitic milk samples, but showed a low relative abundance in healthy milk samples. Furthermore, *Lactococcus* was detected in all environmental samples analyzed. We suggest that species within the *Lactococcus* genus are potential etiological agents of bovine mastitis. Further studies should be conducted to understand the pathogenic significance of the genus and *L. lactis* in particular in veterinary medicine and food safety.

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