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Molecular characterization of bacterial isolates and microbiome: study of mastitic milk, bulk tank milk, and cheese processing plants

Marjory Xavier Rodrigues

Thesis presented to obtain the degree of Doctor in Science. Area: Food Science and Technology

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This work is dedicated to my mom,

who always is with me,

never leaves me.

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“Take the first step in faith.
You don’t have to see the whole staircase,
just take the first step.”

Martin Luther King

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RESUMO

Caracterização molecular de isolados bacterianos e microbioma: estudo de leite de vacas com mastite, leite de tanque e de planta de processamento de queijo

O presente estudo apresentou como objetivo avaliar isolados bacterianos e microbioma de lácteos. Os objetivos específicos foram: caracterizar *Staphylococcus* spp. isolados de leite de vacas com mastite, avaliar a presença de *Lactococcus* em leite de vacas com mastite como um potencial agente causador de mastite, avaliar a associação entre microbioma de leite de tanque e parâmetros da qualidade de leite, e caracterizar *Staphylococcus* spp. isolados de linhas de processamento de queijo Minas frescal. A detecção de genes codificadores de fatores de virulência (enterotoxinas (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selj*, *selk*, *sell*, *selm, seln*, *selo, selp, selq*, *ser*, *ses*, *set*, *selu*, *selv* e *selx*), hemolisinas (*hla*, *hlb*, *hld*, *hlg* e *hlg-v*), toxinas exfoliativas (*eta*, *etb* e *etd*), leucocidina de Panton-Valentine (*pvl*), toxina da síndrome do choque tóxico (*tst*)), genes codificadores de resistência a antibióticos (resistência a tetraciclina (*tet*K, *tet*L e *tet*M), eritromicina (*erm*A, *erm*B e *erm*C), meticilina (*mec*A e *mec*C) e tobramicina (*ant(4')-Ia*)), tipagem molecular (*spa*, SCC*mec* e *agr* types), e fenotipagem quanto à resistência a antibióticos foram realizadas em estafilococos isolados de leite de vacas com mastite e de amostras de planta de processamento de queijo. *Staphylococcus aureus* foi identificado na maioria dos isolados de ambas as origens. Diversos genes de fatores de virulência foram detectados, com destaque para a distribuição de genes codificadores de enterotoxinas estafilocócicas (85,0%-85,7% dos isolados foram positivos para um ou mais genes codificadores de enterotoxinas), sendo o gene relacionado com a toxina H o mais frequente. *Staphylococcus aureus* meticilina resistente foram identificados em isolados de leite de vacas com mastite (4.1%) e em processamento de queijo (6.0%); o perfil genotípico e fenotípico destes isolados foram descritos. t605 foi o mais freqüente na população de *S. aureus* estudada. Em leite de vacas com mastite, *Lactococcus* foi sugerido como o agente causador de um surto de mastite numa fazenda leiteira. Usando sequenciamento de nova geração, a abundância de *Lactococcus* foi observada no microbioma das amostras. O isolamento e sequenciamento de DNA confirmaram a presença de *Lactococcus lactis* e *Lactococcus garvieae*. O microbioma de amostras ambientais e de leite de tanque da fazenda mostrou o gênero *Lactococcus* entre os mais comuns, sugerindo outras fontes deste gênero. Contemplando parâmetros da qualidade de leite, o microbioma de leite de tanque de várias fazendas leiteiras foi relacionado com contagem de células somáticas e contagem bacteriana. O *core microbiome* foi descrito e muitos gêneros bacterianos de importância foram identificados. Dentre as análises realizadas associando microbioma com parâmetros da qualidade de leite, foi destacada a identificação de *Streptococcus* em amostras classificadas com alta contagem de células somáticas e alta contagem bacteriana. Diversos táxons bacterianos com abundância relativa significativamente maior em amostras classificadas com alta e baixa contagem de células somáticas e contagem bacteriana foram mostrados. Reação em cadeia da polimerase em tempo real também foi realizada e associada com diversidade bacteriana, táxons bacterianos e contagem bacteriana. Estes levantamentos confirmam a necessidade de controlar e prevenir a contaminação bacteriana na indústria de lácteos, do rebanho leiteiro até os consumidores.

Palavras-chave: Qualidade de leite; Mastite; *Staphylococcus*; Fatores de virulência; Resistência a antibióticos; Tipagem molecular; *Lactococcus*; *DNA fingerprintin*g; Análise filogenética; Comunidade bacteriana; Sequenciamento de nova geração

ABSTRACT

Molecular characterization of bacterial isolates and microbiome: study of mastitic milk, bulk tank milk and cheese processing plant

The present study aimed to evaluate bacterial isolates and the microbiome of dairies. The specific aims were: to characterize *Staphylococcus* spp. isolated from mastitic milk, to evaluate the presence of *Lactococcus* in mastitic milk as a potential causative agent of mastitis, to evaluate the association between microbiome and milk quality parameters, and to characterize *Staphylococcus* spp. isolated from production lines of *Minas Frescal* cheese. The detection of genes encoding virulence factors (enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selj*, *selk*, *sell*, *selm, seln*, *selo, selp, selq*, *ser*, *ses*, *set*, *selu*, *selv*, and *selx*), hemolysins (*hla*, *hlb*, *hld*, *hlg*, and *hlg-v*), exfoliative toxins (*eta*, *etb*, and *etd*), Panton-Valentine leukocidin (*pvl*), and toxic shock syndrome toxin (*tst*)), genes encoding antibiotic resistance (resistance to tetracycline (*tet*K, *tet*L, and *tet*M), erythromycin (*erm*A, *erm*B, and *erm*C), methicillin (*mec*A and *mec*C), and tobramycin (*ant(4')-Ia*)), molecular typing (*spa*, SCC*mec*, and *agr* types), and phenotyping regarding antibiotic resistance were performed in staphylococci isolates from mastitic milk, and from cheese processing plant samples. *Staphylococcus aureus* was identified in the majority of isolates from both origins. Several virulence factor genes were detected. The distribution of genes encoding staphylococcal enterotoxins (85.0% - 85.7% of isolates were positive for one or more enterotoxin gene) was highlighted and the gene related to H toxin was the most prevalent. *Methicillin-resistant Staphylococcus aureus* were identified in isolates from mastitic milk (4.1%) and cheese processing (6.0%); the genotyping and phenotyping of these isolates were described. t605 had the highest frequency in the *S. aureus* population studied. In mastitic milk, *Lactococcus* was suggested as the causative agent of an outbreak of mastitis in a dairy farm. Using next generation sequencing, the abundance of *Lactococcus* was observed in microbiome samples. Bacterial isolation and DNA sequencing confirmed the presence of *Lactococcus lactis* and *Lactococcus garvieae*. The microbiome of environmental samples and bulk tank milk from the dairy farm showed the *Lactococcus* genus among the most common bacterial taxa, suggesting other sources of this genus. Regarding milk quality parameters, the microbiome of bulk tank milk from several dairy farms was associated with somatic cell count and bacterial count. The core microbiome was described and many genera of importance were identified. Among the associations performed between microbiome and milk quality parameters, the identification of *Streptococcus* in samples classified with high somatic cell count and high bacterial count was highlighted. Several bacterial taxa with relative abundance significantly higher in samples classified as high and low cell count and bacterial count were shown. Real-time polymerase chain reaction was also performed associated with bacterial diversity, bacterial taxa, and bacterial count. These findings highlight the need to control and prevent bacterial contamination in the dairy industry, from herd to consumers.

Keywords: Milk quality; Mastitis; *Staphylococcus*; Virulence factors; Antibiotic resistance; Molecular typing; *Lactococcus*; DNA fingerprinting; Phylogenetic analysis; Bacterial community; Next generation sequencing

1 INTRODUCTION

Raw milk is considered a sterile secretion; however, microbial contamination can occur during milk handling, storage, and processing activities (De SILVA; KANUGALA; WEERAKKODY, 2016). High-quality milk is independent of scale of production and thus animals must be provided with good feed, milked in a clean environment, in well-ventilated parlors, and their overall health maintained (CERVA, 2011).

Regarding dairy cow health, mastitis is a huge concern. It is the most prevalent disease associated with production in dairy cows worldwide and affects milk yield and composition (SEEGERS; FOURICHON; BEAUDEAU, 2003). It is a complex disease and multi-etiological (FOOD AND AGRICULTURE ORGANIZATION, 2014), with high clinical and economic significance (SHAHEEN; TANTARY; NABI, 2016). This disease is easily recognized in the clinical form by the visible presence of abnormal characteristics in milk. The subclinical form is frequent; however, no abnormal characteristics are detected visually in milk (ROYSTER; WAGNER, 2015). Both clinical and subclinical mastitis generally occurs as a result of bacterial intramammary infection due to contagious (transmitting cow to cow) and environmental pathogens (present in the cows' environment) (ROYSTER; WAGNER, 2015). In herds the contagious agents are the most common (ROYSTER; WAGNER, 2015) and *Staphylococcus aureus* has been identified as one of the most important (BARDIAU et al., 2014). Studies have reported a high prevalence of this pathogen (OTE et al., 2011), in addition to the presence of others, such as *Staphylococcus hyicus*, *Staphylococcus chromogene*s, *Staphylococcus epidermidis*, and *Staphylococcus intermedius* (LANGE et al., 2015).

S. aureus is among the most important human pathogens with the ability to cause a wide range of infections, e.g. skin, soft tissue, and bone infections (WORLD HEALTH ORGANIZATION, 2014), and food poisoning (JORGENSEN et al., 2005). *S. aureus* carries virulence factors (JARRAUD et al., 2002) and antibiotic resistance (MOON et al., 2007), having an ability to produce different extracellular toxins causing several types of diseases and symptoms (BALABAN, RASOOLY, 2000). The virulence factors described and characterized in *S. aureus* include staphylococcal enterotoxins, Panton-Valentine leukocidin, toxic shock syndrome toxin, hemolysins, and exfoliative toxins (OTE et al., 2011). Furthermore, some coagulase-negative

staphylococci (CNS) strains have mechanisms of virulence, which was first described in *S. aureus* (PODKOWIC et al., 2013).

Additionally, the overuse of antibiotics in animal and human treatments enabled the emergence of *Staphylococcus* antibiotic resistance. Methicillin-resistant *S. aureus* (MRSA) remains a relevant threat to public health worldwide, they demonstrate resistance to a wide range of antibiotics and are easily transmitted (CHATTERJEE; OTTO, 2013). High levels of MRSA increases patients risk and the requirement for a second-line of more toxic drugs, thus increasing costs, side-effects and may promote resistance in staphylococci and/or other bacteria (WORLD HEALTH ORGANIZATION, 2014). Considering this, there are increasing efforts to understand the epidemiology and the genetic variability of *S. aureus* populations (CHATTERJEE, OTTO, 2013; KOREEN et al., 2004). Some methods are used to detect microevolution, e.g. Multi Locus Sequence Type (MLST) and staphylococcal protein A gene (*spa*) typing, while others are used to describe genetic changes such as gene deletions and duplications, e.g. Pulsed-Field Gel Electrophoresis (PFGE) and staphylococcal chromosome cassette *mec* (SCC*mec*) (CHATTERJEE, OTTO, 2013). Another typing method used is based on identifying the system of regulation of virulence factors in *S. aureus*, the *accessory gene regulation* (*agr*) typing. The *agr* locus encodes a two component signaling pathway, where the activating ligand is an agr-encoded auto inducing peptide (AIP) and polymorphisms in this peptide and its receptor divides strains into four major groups (JARRAUD et al., 2002). Within these groups, some members produce peptides to activate *agr* in other members, while peptides produced by a different group are usually inhibitory (JARRAUD et al., 2002). The association between *agr* groups and disease, profile of toxin genes, and characteristics genetics of strains have been reported (JARRAUD et al., 2002). Therefore, knowledge on *S. aureus* and others staphylococci is expanding and the actions of control and prevention can become more effective against these bacteria.

Conversely, emerging mastitis pathogens are also a huge concern and are still beyond the control of the dairy industry. For example, species within *Lactococcus* genus, widely used in the dairy industry (CASALTA; MONTEL, 2008) are now being considered as potential mastitis pathogens (PLUMED-FERRER et al., 2013; PLUMED-FERRER et al., 2015; WERNER et al., 2014). *Lactococcus* are very close to environmental streptococci and streptococci-like bacterial groups that include classical mastitis pathogens (WERNER et al., 2014). Many phenotypic and

biochemical methods typically used for identification of species can be inaccurate and unreliable particularly for some closely related species (WERNER et al., 2014). As a result, the presence of *Lactococcus* spp. as pathogens may have been underreported and consequently there is lack of information about their clinical importance in bovine mastitis (WERNER et al., 2014). Moreover, advancements in available technologies, predominately DNA sequencing, will greatly support the identification and characterization of bacteria. A recent study using DNA sequencing, described that 70% of isolates identified as streptococci by conventional microbiological assays were in fact lactococci, the majority of which were *L. lactis* (WERNER et al., 2014). Thus, applying new methods to identify bacteria has yielded increased reports on *Lactococcus* spp. as a cause of animal and human infections (PLUMED-FERRER et al., 2013).

Lactococcus lactis and *Lactococcus garvieae* species have been reported in bovine intramammary infections (MEHMETI et al., 2015; PLUMED-FERRER et al., 2013) and in human infections (AZOUZI et al., 2015; HADJISYMEOU; LOIZOU; KOTHARI, 2013; NAVAS; HALL; EL BEJJANI, 2013). However, *L. lactis* species is used in dairy product fermentation and is recognized as “Generally Recognized as Safety” (CASALTA; MONTEL, 2008), and is used in treating bovine mastitis (KLOSTERMANN et al., 2008). *L. garvieae* has also been used in the fermentation process a lower scale (CASALTA; MONTEL, 2008). Recently, Reguera-Brito et al. (2016) compared *L. garvieae* isolates from clinical human infection with isolates from food samples. They found genetic relatedness between human and food isolates and suggested that meat and dairy products may be important sources of human *L. garvieae* infection. Mehmeti et al. (2015) evaluated *L. garvieae* isolated from cow raw milk and found a high frequency, antibiotic resistance and high genetic diversity.

In summary, it is evident the importance of mastitis pathogens for microbiological quality of raw milk and dairy products. However, the microbiological quality is not exclusively associated with mastitis pathogens, since bacterial contamination can arise from several sources. The development and application of DNA-based technologies to identify large numbers of microorganisms in raw food material can contribute to improvements in food production and quality (GALIMBERTI et al., 2015). For example, next generation sequencing generates a large amount of DNA sequence data, which has helped metagenomic studies; these data provide information enabling identification of the microbial diversity and of non-culturable

causative agents of diseases (BERGHOLZ et al., 2014). In raw milk, a description of natural microbial community is limited, since previous studies focused on specific microorganisms, such as pathogenic and spoilage bacteria (FRICKER et al., 2011). Next generation sequencing has now been used to assess the raw milk microbiome (OIKONOMOU et al., 2014; QUIGLEY et al., 2013); however, raw milk microbiome assessed by independent-culture methods are only described and/or associated with disease, e.g. association of milk microbiome with bovine mastitis (OIKONOMOU et al., 2014). Association of microbiome with quality parameters was not found thus far. Parameters, such as somatic cell count (SCC) and bacterial count (by Standard Plate Count, SPC) are useful to indicate and facilitate the monitoring of herd health and milk quality (JAYARAO et al., 2004), and also are used by dairy industry to pay for very high quality (NIGHTINGALE et al., 2008).

Considering this, the present thesis aimed to evaluate bacterial isolates and the microbiome of dairies. Six chapters were written, in the present one an introduction on subjects explored and the importance of the study is provided. In the second chapter, staphylococci isolates from mastitic milk, obtained from dairy cows with subclinical mastitis, were identified at the species level, and characterized regarding virulence factor genes, antibiotic resistance genes, antibiotic resistance phenotyping, and molecular typing, such as *agr*, *spa*, and SCC*mec* typing; furthermore, several multiplex Polymerase Chain Reaction (PCR) were optimized. Next, an outbreak of mastitis is shown, in which *Lactococcus* spp. was indicated as a potential causative agent of the disease. Microbiome of mastitic and healthy milk was described and compared, and identification of species, phylogenetic analysis, and DNA fingerprinting of the bacterial isolates were performed. The bulk tank milk microbiome was assessed using high-throughput sequencing of the16SrRNA gene associated with milk quality parameters, SCC and SPC, and were discussed in the fourth chapter. The microbiome of bulk tank milk was described as well as the core microbiome across samples from nineteen dairy farms enrolled in the study. Bacterial taxa was associated with quartiles of SCC and SPC, and associated with samples classified with high and low SCC and SPC using response screening analysis, the findings were reported and, to the best of our knowledge, this study is pioneering on this focus. The fifth chapter follows a similar structure cited for the second chapter; however, the staphylococci isolates were obtained from production lines of *Minas* cheese processing plant, including e.g. isolates from raw milk, table, cheese mold,

handler, and cheese. Finally, the last chapter brings the concluding remarks of all chapters presented.

References

AZOUZI, F.; CHAHED, C.; MARZOUK, M.; FERJANI, A.; HANNECHI, N.; FEKIH, M.; SALEM, Y.B.; BOUKADIDA, J. Chorioamnionitis due to *Lactococcus lactis cremoris*: a case report. **Case Reports in Women's Health**, Amsterdam, v. 7, p. 1-2, July 2015.

BALABAN, N.; RASOOLY, A. Staphylococcal enterotoxins. **International Journal of Food Microbiology**, Amsterdam, v. 61, n. 1, p. 1-10, Oct. 2000.

BARDIAU, M.; DETILLEUX, J.; FARNIR, F.; MAINIL, J.G.; OTE, I. Associations between properties linked with persistence in a collection of *Staphylococcus aureus* isolates from bovine mastitis. **Veterinary Microbiology**, Amsterdam, v. 169, n. 1-2, p. 74-79, Feb. 2014.

BERGHOLZ, T.M.; MORENO SWITT, A.I.; WIEDMANN, M. Omics approaches in food safety: fulfilling the promise? **Trends in Microbiology**, Cambridge, v. 22, n. 5, p. 275-281, Mar. 2014.

CASALTA, E.; MONTEL, M. C. Safety assessment of dairy microorganisms: the *Lactococcus* genus. **International Journal of Food Microbiology**, Amsterdam, v. 126, n. 3, p.271-273, Sept. 2008.

CERVA, C. Milking and handling of raw milk: effect of storage and transport on milk quality. WHITE, C.H. **Encyclopedia of dairy sciences**. Cambridge: Cambridge Academic Press, 2011. p. 642-648.

CHATTERJEE, S.S.; OTTO, M. Improved understanding of factors driving methicillin-resistant *Staphylococcus aureus* epidemic waves. **Clinical Epidemiology**, Auckland, v. 5, p. 205-217, July 2013.

De SILVA, S.A.S.D.; KANUGALA, K.A.N.P.; WEERAKKODY, N.S. Microbiological quality of raw milk and effect on quality by implementing good management practices. **Procedia Food Science**, London, v. 6, p. 92-96, 2016.

FOOD AND AGRICULTURE ORGANIZATION. **Impact of mastitis in small scale dairy production systems**. 2014. Disponível em: <http://www.fao.org/3/a-i3377e.pdf>. Acesso em: 05 maio 2016.

FRICKER, M.; SKÅNSENG, B.; RUDI, K.; STESSL, B.; EHLING-SCHULZ, M. Shift from farm to dairy tank milk microbiota revealed by a polyphasic approach is independent from geographical origin. **International Journal of Food Microbiology**, Amsterdam, v. 145, suppl. 1, S24-S30, 2011.

GALIMBERTI, A.; BRUNO, A.; MEZZASALMA, V.; MATTIA, F.; BRUNI, I.; LABRA, M. Emerging DNA-based technologies to characterize food ecosystems. **Food Research International**, Barking, v. 69, p.424-433, Mar. 2015.

HADJISYMEOU, S., LOIZOU, P.; KOTHARI, P. *Lactococcus lactis cremoris* infection: not rare anymore? **BMJ Case Report**, London, p.1-3, May 2013.

JARRAUD, S.; MOUGEL, C.; THIOULOUSE, J.; LINA, G.; MEUGNIER, H.; FOREY, F.; NESME, X.; ETINNE, J.; VANDENESCH, F. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. **Infection and Immunity**, Bethesda, v. 70, n. 2, p. 631-641, Feb. 2002.

JAYARAO, B.M.; PILLAI,S.R.; SAWANT, A.A.; WOLFGANG, D.R.; HEGDE, N.V. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. **Journal of Dairy Science**, Champaign, v. 87, n. 10, p. 3561-3573, Oct. 2004.

JORGENSEN, H.J.; MATHISEN, T.; LOVSETH, A.; OMOE, K.; QVALE, K.S.; LONCAREVIC, S. An outbreak of *Staphylococcus* food poisoning cause by enterotoxin H in mashed potato made with raw milk. **FEMS Microbiology Letters**, Amsterdam, v. 252, n. 2, p. 267-272, Nov. 2005.

KLOSTERMANN, K., CRISPIE, F.; FLYNN, J.; ROSS, R. P.; HILL, C.; MEANEY, W. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. **Journal of Dairy Research**, Cambridge, v. 75, n. 3, p. 365-373, Aug. 2008.

KOREEN, L.; RAMASWAMY, S.V.; GRAVISS, E. A.; NAIDICH, S.; MUSSER, J.M.; KREISWIRTH, B.N. *spa* Typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. **Journal of Clinical Microbiology**, Washington, v. 42, n. 2, p. 792-799, Feb. 2004.

LANGE, C.C.; BRITO, M.A.V.P.; REIS, D.R.L.; MACHADO, A.A.; GUIMARÃES, A.S.; AZEVEDO, A.L.S.; SALLES, E.B.; ALVIM, M.C.T.; SILVA, S.F.; MEURER, I.R. Species-level identification of staphylococci isolated from bovine mastitis in Brazil using partial 16S rRNA sequencing. **Veterinary Microbiology**, Amsterdam, v. 176, n. 3/4, p. 382-388, Apr. 2015.

MEHMETI, I.; SKENDER, M.; DIEP, D.B.; NES, I.F. High frequency of the potential pathogen *Lactococcus garvieae* in raw milk from Kosovo. **Food Control**, Guildford, v. 53, p.189-194, July 2015.

MOON, J.S.; LEE, A.R.; KANG, H.M.; LEE, E.S.; KIM, M.N.; PAIK, Y.H.; PARK, Y.H.; JOO, Y.S.; KOO, H.C. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. **Journal of Dairy Science**, Champaign, v. 90, n. 3, p. 1176-1185, Mar. 2007.

NAVAS, M.E.; HALL, G.; EL BEJJANI, D. A case of endocarditis caused by *Lactococcus garvieae* and suggested methods for identification. **Journal of Clinical Microbiology**, Washington, v. 51, n. 6, p. 1990-1992, June 2013.

NIGHTINGALE, C., DHUYVETTER, K.; MITCHELL, R.; SCHUKKEN, Y. Influence of variable milk quality premiums on observed milk quality. **Journal of Dairy Science**, Champaign, v. 91, n. 3, p. 1236-1244, Mar. 2008.

OIKONOMOU, G.; BICALHO, M.L.; MEIRA, E.; ROSSI, R.E.; FODITSCH, C.; MACHADO, V.S.; TEIXEIRA, A.G.; SANTISTEBAN, C.; SCHUKKEN, Y.H.; BICALHO, R.C. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. **PLoS One**, San Francisco, v. 9, n. 1, p. 1-11, Jan. 2014.

OTE, I.; TAMINIAU, B.; DUPREZ, J.N.; DIZIER, I.; MAINIL, J.G. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. **Veterinary Microbiology**, Amsterdam, v. 153, n. 3/4, p. 285-292, Dec. 2011.

PLUMED-FERRER, C.; UUSIKYLÄ, K.; KORHONEN, J.; von WRIGHT, A. Characterization of *Lactococcus lactis i*solates from bovine mastitis. **Veterinary Microbiology**, Amsterdam, v. 167, n. 3/4, p. 592-599, Dec. 2013.

PLUMED-FERRER, C., BARBERIO, A.; FRANKLIN-GUILD, R.; WERNER, B.; MCDONOUGH, P.; BENNETT, J.; GIOIA, G.; ROTA, N.; WELCOME, F.; NYDAM D. V.; MORONI. P. Antimicrobial susceptibilities and random amplified polymorphic DNA-PCR fingerprint characterization of *Lactococcus lactis* ssp. *lactis* and *Lactococcus garvieae* isolated from bovine intramammary infections. **Journal of Dairy Science**, Champaign, v. 98, n. 9, p. 6216-6225, Sept. 2015.

PODKOWIK, M.; PARK, J.Y.; SEO, K.S.; BYSTROŃ, J.; BANIA, J. Enterotoxigenic potential of coagulase-negative staphylococci. **International Journal of Food Microbiology**, Amsterdam, v. 163, n. 1, p. 34-40, Apr. 2013.

QUIGLEY, L.; McCARTHY, R.; O'SULLIVAN, O.; BERESFORD, T.P.; FITZGERALD, G.F.; ROSS, R.P.; STANTON, C.; COTTER,P.D. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. **Journal of Dairy Science**, Champaign, v. 96, n. 8, p. 4928-4937, Aug. 2013.

REGUERA-BRITO, M.; GALÁN-SANCHÉZ, F.; BLANCO, M.M.; RODRÍGUEZ-IGLESIAS, M.; DOMINGUEZ, L.; FERNÁNDEZ-GARAYZÁBAL, J.F.; GIBELLO, A. Genetic analysis of human clinical isolates of *Lactococcus garvieae*: relatedness with isolates from foods. **Infection, Genetics and Evolution**, Amsterdam, v. 37, p. 185-191, Jan. 2016.

ROYSTER, E.; WAGNER, S. Treatment of mastitis in cattle. **Veterinary Clinics of North America: Food Animal Practice**, Philadelphia, v. 31, n. 1, p. 17-46, Mar. 2015.

SEEGERS, H.; FOURICHON, C.; BEAUDEAU, F. Production effects related to mastitis and mastitis economics in dairy cattle herds. **Veterinary Research**, Le Ulis, v. 34, n. 5, p. 475-491, Sept. 2003.

SHAHEEN, M.; TANTARY, H.A.; NABI, S.U.A treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention and control strategy. **Advances in Dairy Research**, Las Vegas, v. 4, n. 1, p. 1-10, Jan. 2016.

WERNER, B., MORONI,P.; GIOIA, G.; LAVÍN-ALCONERO, L.; YOUSAF, A.; CHARTER, M.E.; CARTER, B.M.; BENNETT, J.; NYDAM, D.V.; WELCOME, F.; SCHUKKEN,Y.H. Short communication: genotypic and phenotypic identification of environmental streptococci and association of *Lactococcus lactis* ssp. *lactis* with intramammary infections among different dairy farms. **Journal of Dairy Science**, Champaign, v. 97, n. 11, p. 6964-6969, Nov. 2014.

WORLD HEALTH ORGANIZATION. **Antimicrobial resistance**: global report on surveillance. 2014.Disponível em: <http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1>. Acesso em: 05 maio 2016.

2 ANTIBIOTIC RESISTANCE AND MOLECULAR CHARACTERIZATION OF *Staphylococcus* spp. FROM MASTITIC MILK

Abstract

The aims were to identify and to characterize *Staphylococcus* spp. isolated from mastitic milk, and to optimize multiplex Polymerase Chain Reactions (PCR). Staphylococci previously isolated from milk of dairy cows with subclinical mastitis were analyzed. PCR was applied to amplify *nuc, sodA, spa, agr* locus, virulence factors, and antibiotic resistance genes; DNA sequencing also was performed as well as antibiograms. In a group of forty-nine staphylococci, *S. aureus* (85.7% of the isolates) was a prevalent species, followed by *S. hyicus*, *S. xylosus*, *S. chromogenes*. Virulence factors genes were identified in the most isolates by multiplex PCR, successfully optimized in this study. Regarding enterotoxin genes, *seh* and *sel*x were highlighted. All hemolysin genes were detected, the profile clustering all genes represent 28.6% of isolates. The antibiotic resistance was evaluated; the majority of isolates (69.4 %) were resistant to penicillin. Among the genes encoding antibiotic resistance was identified *mec*A, two Methicillin-Resistant *S. aureus* found were typed as *spa* type 605, *agr* type II, and one identified as SCC*mec* type IVa. The *spa* types t605, t521, t267, and t9121, and the *agr* types I and II were observed. The findings emphasize the importance of prevention of *Staphylococcus* infection in dairy cows.

Keywords: Antibiotic; Staphylococci; Toxins; Virulence; Genes

2.1 Introduction

Bovine mastitis affects the dairy industry worldwide and it is associated with reduced milk quality, and milk production (SILVA et al., 2013). Coagulase-positive staphylococci (CPS) are widely studied as a common cause of clinical and subclinical mastitis (OTE et al., 2011; RAJIC-SAVIC et al., 2015), and the most important causative agent in this bacterial group is *Staphylococcus aureus* (OTE et al., 2011). In addition, the relevance of coagulase-negative staphylococci (CNS) as a cause of mastitis in dairy cows has also been shown (SILVA et al., 2014). CNS research has predominantly focused on humans, and the enterotoxigenic potential of CNS has not been extensively explored, although it has been suggested that CNS from bovine intramammary infection (IMI) could be a potential source of staphylococcal superantigens (SAgs) (PARK et al., 2011).

SAgs, e.g. staphylococcal enterotoxin (SE) and toxic shock syndrome toxin-1 (TSST-1), were first identified in *S. aureus* (PARK et al., 2011) and have been well characterized. The emetic activity of SEs has been demonstrated (HU; NAKANE, 2014); thus, there is a potential to cause foodborne disease (JORGENSEN et al., 2005). *S. aureus* may carry genes for production of other toxins such as Panton-

Valentine leukocidin, toxic shock syndrome toxin and exfoliative toxins (JARRAUD et al., 2002).

The importance of virulence factors in *Staphylococcus* genus and the genetic variability within the *S. aureus* population have been highlighted in medicine, and could potentially help in treatments (OTE et al., 2011). However, antibiotic resistance is a concern since studies have demonstrated the emergence of resistant isolates from bovine mastitis (SILVA et al., 2014; MOON et al., 2007).Thus, the aims of the present study were to identify, to characterize *Staphylococcus* spp. isolated from mastitic milk, and to optimize several multiplex Polymerase Chain Reactions (PCR) in order to simultaneously identify the presence of different virulence factor genes.

2.2 Materials and methods

2.2.1 Origin and collection of isolates

The collection of bacterial isolates belonging to Hygiene and Dairy Laboratory, University of São Paulo, was used. From this collection, isolates from mastitic milk previously identified as *Staphylococcus* spp. were selected. In total, forty-nine isolates were selected from three different dairy farms located in São Paulo State, Brazil.

The isolates were obtained in a previous study performed by Hygiene and Dairy Laboratory's group, in which dairy cows were diagnosed with subclinical mastitis after screening using California Mastitis Test. For the bacterial culturing, standard microbiological methods included colony morphology on Baird Parker Agar (BPA, Difco BD®, Nova Jersey, EUA) with egg yolk tellurite supplement (Laborclin®, Pinhais, Brazil), Gram staining, catalase, and coagulase test were completed to identify staphylococci, and all isolates were stored at -20 °C.

2.2.2 DNA extraction, polymerase chain reaction, and molecular typing

Each isolate was inoculated into Brain Heart Infusion (BHI, Oxoid™, Hampshire, UK) broth and incubated at 37 °C for 24 hours. Aliquots of each culture were centrifuged and the supernatant was discarded. The pellet was used to extract DNA using "AxyPrep™ Blood Genomic DNA Miniprep kit" (Axygen Scientific Inc., Union City, USA), according to manufacturer's instructions. Agarose gel electrophoresis was completed to verify the extraction, and the genomic DNA was stored at −20 °C.

Coagulase-positive and coagulase-negative staphylococci previously identified by coagulase test were confirmed by detecting the *coa* gene through PCR. The PCR amplification of *coa* gene described by Aarestrup, Dangler and Sordillo (1995) was modified by using 0.75 mM of $MgCl_2$ in each reaction, and the PCR cycles used were as follows: 95 °C for 5 min; 30 cycles at 95°C for 30s, 55 °C for 2 min, and 72°C for 4 min; and finally at 72 °C for 10 min. When confirmed as coagulase-positive, multiplex PCR was performed to identify *S. aureus*, *S. intermedius* and *S. hyicus* according to Sasaki et al. (2010). Other strains were identified by amplifying the *sod*A gene, and through DNA sequencing using Sanger method (SILVA et al., 2014).

The SEs (SEA-SEE, SEG-SElJ, SElK-SElQ, SER-SET, SElU, SElV, SElX), hemolysins (alpha, beta, delta, gamma component A, B and C and gamma-variant hemolysin), Panton-Valentine leukocidin (PVL), exfoliative toxins (ETA, ETB and ETD), and toxic shock syndrome toxin (TSST-1) genes were assessed by PCR. Primers used in this study are shown in Table 1.

Table 1 - Sequences used to amplify virulence factors

(to be continued)

Gene	Oligonucleotide sequence (5´→3´)	bp	Reference
sea	TTGGAAACGGTTAAAACGAA GAACCTTCCCATCAAAAACA	120	Johnson et al. (1991)
seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	478	Johnson et al. (1991)
sec	GACATAAAAGCTAGGAATTT AAATCGGATTAACATTATCC	257	Johnson et al. (1991)
sed	CTAGTTTGGTAATATCTCCT TAATGCTATATCTTATAGGG	317	Johnson et al. (1991)
see	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209	Mehrotra, Wang and Johnson (2000)

Table 1 - Sequences used to amplify virulence factors

(continuation)

Gene	Oligonucleotide sequence (5´→3´)	bp	Reference
seg	AAGTAGACATTTTTGGCGTTCC AGAACCATCAAACTCGTATAGC	287	Omoe et al.(2002)
seh	GTCTATATGGAGGTACAACACT GACCTTTACTTATTTCGCTGTC	213	Omoe et al. (2002)
sei	GGTGATATTGGTGTAGGTAAC ATCCATATTCTTTGCCTTTACCAG	454	Omoe et al.(2002)
selj	CATCAGAACTGTTGTTCCGCTAG CTGAATTTTACCATCAAAGGTAC	142	Nashev et al. (2004)
selk	TAGGTGTCTCTAATAATGCCA TAGATATTCGTTAGTAGCTG	293	Omoe et al. (2005)
sell	CACCAGAATCACACCGCTTA CTGTTTGATGCTTGCCATTG	240	Cremonesi et al.(2005)
selm	ATCATATCGCAACCGCTGAT TTCAGTTTCGACAGTTTTGTTGTC	626	Ote et al. (2011)
seln	ATGAGATTGTTCTACATAGCTGCAAT AACTCTGCTCCCACTGAAC	680	Ote et al. (2011)
selo	AAATGATTCTTTATGCTCCG AAAGCACATTGTCATGGTGA	300	Ote et al. (2011)
selp	TGATTTATTAGTAGACCTTGG ATAACCAACCGAATCACCAG	396	Ote et al. (2011)
selq	AATCTCTGGGTCAATGGTAAGC TTGTATTCGTTTTGTAGGTATTTTCG	122	Omoe et al. (2005)
ser	GGATAAAGCGGTAATAGCAG GTATTCCAAACACATCTAAC	166	Omoe et al. (2005)
ses	CCCCGGATCCGATGAATCTAGACCTAAAATAG CCCCGTCGACTTATTGGGAATAAAC	794	Ono et al. (2008)
set	CCCCGGATCCGATTCTCGTGAAGGTTTAAAAG CCCCGTCGACCTATTTTTCCATATATATATC	671	Ono et al. (2008)
selu	ATGGAGTTGTTGGAATGAAGT TTTTTGGTTAAATGAACTTCTACA	796	Fischer et al. (2009)

Table 1 - Sequences used to amplify virulence factors

(conclusion)

Gene	Oligonucleotide sequence (5´→3´)	bp	Reference
selv	GCAGGATCCGATGTCGGAGTTTTGAATCTTAGG TAACTGCAGTTAGTTACTATCTACATATGATATTTC GACATC	720	Thomas et al. (2009)
selx	AGCAGACGCGTCAACACAAA ACTTGTTCAATGTCATTAACACTTTTCAC	612	Wilson et al. (2011)
hla	CTGATTACTATCCAAGAAATTCGATTG CTTTCCAGCCTACTTTTTTATCAGT	209	Jarraud et al. (2002)
hlb	GTGCACTTACTGACAATAGTGC GTTGATGAGTAGCTACCTTCAGT	309	Jarraud et al. (2002)
hld	AAGAATTTTTATCTTAATTAAGGAAGGAGTG TTAGTGAATTTGTTCACTGTGTCGA	111	Jarraud et al. (2002)
hlg	GTCAYAGAGTCCATAATGCATTTAA CACCAAATGTATAGCCTAAAGTG	535	Jarraud et al. (2002)
hlg-v	GACATAGAGTCCATAATGCATTYGT ATAGTCATTAGGATTAGGTTTCACAAAG	390	Jarraud et al. (2002)
eta	ACTGTAGGAGCTAGTGCATTTGT TGGATACTTTTGTCTATCTTTTTCATCAAC	190	Jarraud et al. (2002)
etb	CAGATAAAGAGCTTTATACACACATTAC AGTGAACTTATCTTTCTATTGAAAAACACTC	612	Jarraud et al. (2002)
etd	AACTATCATGTATCAAGG CAGAATTTCCCGACTCAG	376	Yamaguchi et al. (2002)
tst	TTCACTATTTGTAAAAGTGTCAGACCCACT TACTAATGAATTTTTTTATCGTAAGCCCTT	180	Jarraud et al. (2002)
pvl	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAAGTGTATTGGATAGCAAAAGC	443	Lina et al. (1999)

Single PCR was initially performed for genes and positive (extracted DNA from strains belonging to Hygiene and Dairy Laboratory collection, University of São Paulo) and negative controls were incorporate into each run. Next, primers were combined in the same reaction when possible depending on amplification

characteristics, e.g. annealing temperature, number of PCR cycles and concentration of $MgCl_2$ (Table 2). The multiplex reactions were as follows: 1X PCR Buffer, 1U GoTaq® Hot Start Polymerase (Promega Corporation, Madison, USA), $MgCl_2$ (Promega Corporation, Madison, USA) concentration was variable (Table 2), 10 ρmol of each primer (synthesis by Sigma-Aldrich®, São Paulo, Brazil), 200 µM deoxynucleotides (Promega Corporation, Madison, USA), template DNA (approximately 40 ng) and ultrapure water to bring the final reaction volume to 25 µL. Genes that were not incorporate into multiplex PCR, were amplified by uniplex PCR using 2.5 mM of $MgCl_2$ and thermally cycled at 94 °C for 5 min, 30 cycles at 94°C for 2 min, 48 °C for 1 min, and 72°C for 1 min, and then once at 72 °C for 10 min.

Table 2 - Conditions of the multiplex PCR optimized in this study

(to be continued)

Set	Genes	Conc. $MgCl_2$(mM)	Condition of PCR*	
A	*sea* + *sec*	2,0	94 °C – 2 min 54 °C – 1 min 72 °C – 1 min	30 cycles
B	*seb* + *selk*	2,0	94 °C – 2 min 55 °C – 1 min 72 °C – 1 min	30 cycles
C	*sed* + *seh*	2,0	94 °C – 2 min 55 °C – 1 min 72 °C – 1 min	30 cycles
D	*see* + *selq*	2,0	94 °C – 2 min 54 °C – 1 min 72 °C – 1 min	30 cycles
E	*seg* + *selu*	2,0	94 °C – 2 min 54 °C – 1 min 72 °C – 1 min	30 cycles
F	*sei* + *selm* + *selo*	1,5	94 °C – 2 min 54 °C – 1 min 72 °C – 1 min	30 cycles

Table 2 - Conditions of the multiplex PCR optimized in this study

(conclusion)

Set	Genes	Conc. $MgCl_2$(mM)	Condition of PCR*	
G	*selj* + *sell*	3,0	94 °C – 2 min 64 °C – 2 min 72 °C – 1 min	35 cycles
H	*seln* + *selp* + *ser*	3,0	94 °C – 30 sec 58 °C – 30 sec 72 °C – 1 min	35 cycles
I	*pvl* + *tst*	3,0	94 °C – 30 sec 55 °C – 30sec 72 °C – 1 min	30 cycles
J	*hla* + *hlb* + *hld*	2,0	94 °C – 30 sec 63 °C – 30 sec 72 °C – 1 min	30 cycles
K	*hlg* + *hlg-v*	2,0	94 °C – 30 min 48 °C – 30 sec 72 °C – 1 min	30 cycles

*94 °C/5 min for initial denaturation and 72 ºC/7 min for extension final

agr type was developed according to Shopsin et al. (2003) and the amplification of *spa* region was carried out following the website http://www.ridom.com/, the repeats were identified for *spa* types detection after sequencing by Sanger method.

2.2.3 Antibiotic resistance detection

Antibiotic resistance of each isolate was tested using the agar diffusion method following the Clinical and Laboratory Standards Institute guidelines (CLINICAL LABORATORY INSTITUTE, 2015). The antibiotics tested included penicillin, cefoxitin, oxacillin, erythromycin, clindamycin, chloramphenicol, ciprofloxacin, vancomycin, tobramycin, tetracycline and gentamicin. The *tet*K, *tet*L, *tet*M (GÓMEZ-SANZ et al., 2010), *ant(4')-Ia* (van de KLUNDERT; VLIEGENTHART,1993), *erm*A, *erm*B, *erm*C (GÓMEZ-SANZ et al., 2010), *mec*A

(MOON et al., 2007) and *mec*C (CUNY et al., 2011) genes were detected by PCR, and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) types I to V in methicillin-resistant *S. aureus* were identified as described by Kondo et al. (2007).

2.3 Results

Of the total isolates, 46 (93.9%) were confirmed as coagulase-positive through amplification of *coa* gene (*S. aureus* and *S. hyicus*). The species observed were *S. aureus* (42 strains, 85.7% of isolates), *S. hyicus* (4, 8.2%), *S. xylosus* (2, 4.1%) and *S. chromogenes* (1, 2.0%), Table 3.

Table 3 - Species, typing, virulence factor genes, and antibiotic resistance genes detected by farm

(to be continued)

Farm	Species (n)	*spa* type (n)	*agr* type (n)	Enterotoxin gene (n)	Hemolysin gene (n)	Antibiotic resistance gene (n)
A						
	S. aureus (28)	t605 (28)	II (28)	*seb* (2) *seg* (18) *seh* (18) *sel**k*** (2) *sel**l*** (5) *sel**m***(2) *sel**o*** (6) *sel**q*** (1) *ser* (13) *sel**u*** (13) *sel**x***(17)	*hla* (10) *hlb* (17) *hld* (10) *hlg* (15) *hlg-v* (15)	*mec*A (2)
	S. hyicus (2)					*erm*A (1) *erm*C (1)
	S. xylosus (2)			*sel**l*** (1) *sel**p*** (1)		*tet*K (2) *tet*M (1)

Table 3 - Species, typing, virulence factor genes, and antibiotic resistance genes detected by farm

(conclusion)

Farm	Species (n)	*spa* type (n)	*agr* type (n)	Enterotoxin gene (n)	Hemolysin gene (n)	Antibiotic resistance gene (n)
B						
	S. aureus(10)	t605 (5) t267(4) t9129 (1)	I (5) II (5)	*seg* (7) *seh* (7) *selj* (1) *sell*(2) *seln* (2) *selo* (1) *ser* (8) *selu* (6) *selx* (9)	*hla* (7) *hlb* (8) *hld* (4) *hlg* (4) *hlg-v* (9)	*erm*C (1) *tet*M (1)
	S. hyicus (2)			*seh*(1) *selj*(1) *sell* (2) *selp*(1)		
C						
	S. aureus (4)	t605 (2) t521 (2)	II (4)	*seh* (3) *sell* (2) *seln* (1) *selo* (2) *ser* (2) *selx* (2)	*hla* (2) *hlb* (2) *hld* (2) *hlg* (2) *hlg-v* (2)	
	S. chromogenes (1)			*selp*(1)		

In multiplex PCR optimization, a total of eleven multiplex PCR (Table 2) to detect virulence genes (*sea, seb, sec, sed, see, seg, seh, seli, selj, selk, sell, selm,*

seln, selo, selp, selq, ser, selu, pvl, tst, hla, hlb, hld, hlg, hlg-v) were performed. Multiplex PCR for 25 genes were evaluated across 11 reactions, which permitted optimization of the analyses and reducing costs.

Forty-two isolates (85.7% of isolates) were positive for one or more enterotoxin gene. The enterotoxin genes observed were *seh* (59.2%) and *selx* (57.1%) followed by *seg* (51.0%), *ser* (46.9%), *selu* (38.8%), *selI* (24.5%), *selo* (18.4%), *seln* and *selp* (6.1% each one), *seb*, *selj*, *selk* and *selm*, (4.1% each one) and *selq* (2,0%). *sea*, *sec*, *sed*, *see*, *sei*, *ses*, *set* and *selv* genes were not detected. In this study 30 profiles were observed across 49 isolates. Among the profiles identified in this study, *seg+seh+ser+seu+selx,* was the most abundant (10.2% of strains), followed by *seg+seh*, *seg+seh+sem+seo+ser+seu+selx*, *seg+seh+seo+ser+seu+selx*, *seh*, *seh+seo+ser+selx*, *seh+ser+selx* and *sel* (4.1% each profile). In seven isolates enterotoxin genes were not identified. All hemolysin genes were detected i.e. *hla* (38.8%), *hlb* (55.1%), *hld* (32.7%), *hlg* (42.9%) and *hlg-v* (53.1%). The presence of all hemolysins was the most frequent profile (28.6%), and 19 of the strains (38.7%) did not carry hemolysin genes. Genes encoding exfoliative toxins, *pvl* and *tst* were not identified.

Regarding antibiotic resistance, isolates were resistant to penicillin (69.4 % of isolates), cefoxitin (8.2%), erythromycin, chloramphenicol, tetracycline (4.1% to each antibiotic), tobramycin, clindamycin, oxacillin (2.0% to each antibiotic). One isolate demonstrated intermediate resistance to gentamicin, erythromycin, clindamycin, while all strains were sensitive to vancomycin and ciprofloxacin. Across all strains, three isolates were multi-drug resistant. Herein, *erm*A, *erm*C, *tet*K and *tet*M genes were detected in few isolates (Table 3). Of the isolates positive for *mec*A (*S. aureus*, 4.1%), one was identified as SCC*mec* type IVa and another was non-typeable. In addition, the methicillin-resistant *S. aureus* (MRSA) strains belong to *spa* typing t605 and *agr* type II, and the absence of a novel *mec*A homologue was observed. In *spa* typing detected across 42 *S. aureus* (Table 3), the type most frequent was t605 (83.3%), also it was present on all farms, followed by t267 (9.5%), t521 (4.8%) and t9129 (2.4%). The *agr* types detected were I (11.9%) and II (88.1%).

2.4 Discussion

In this study, we identified in a limited group of staphylococci that *S. aureus* was the dominant species; however, CNS was also present. In addition, several

virulence factor genes were identified in the majority of isolates by multiplex PCR as well as antibiotic resistance to one or more antibiotics tested by diffusion method. Regarding SEs, the importance of *seh* and *selx* genes corresponding to SEH and SElX is emphasized due their high incidence, while low frequency or absence of classical SEs was observed. The *mec*A positive isolates detected were *spa* type 605,and *agr* type II, which were also identified in the majority isolates.

Herein, high frequency of *S. aureus* was detected, this species has been identified as the primary pathogen associated with mastitis (OTE et al., 2011; SILVA et al., 2014) and previous studies have identified a high frequency of this pathogen in Brazil (LANGE et al., 2015; SILVA et al., 2013). Giannechini et al. (2002) also detected high frequency of *S. aureus*, and low frequency of *S. hyicus* coagulase-positive among isolates from sub-clinical mastitis cases. The *coa* gene amplification also showed that the minority of the isolates belonged to CNS; which are capable of causing opportunistic mastitis (MOON et al., 2007). Lange et al. (2015) reported *S. chromogenes* at a frequency of 38.5%, which highlights the importance of coagulase-negative strains; however, in our study the detection of CNS was low. *S. xylosus*, coagulase-negative, were also detected, within this species there are strains that can potentially be hazardous, and they are related to animal opportunistic infections (DORDET-FRISONI et al., 2007).

The low frequency of classical SEs is in agreement with a previous study in which *S. aureus* associated with bovine mastitis were analyzed (OTE et al., 2011). In this study, classical SEs were not identified in *S. chromogenes*, *S. xylosus* and *S. hyicus*; however, classical SEs have been reported in these species (PARK et al., 2011). Among the other SEs, the frequency of *seh* was highly detected. SEH has emetic activity and staphylococcal food poisoning associated with *S. aureus* carrying the *seh* gene has been reported (ARGUDÍN et al., 2010; JORGENSEN et al., 2005). Considering the potential of SEH to cause foodborne disease, strains from our collection that carry the *seh* gene should be tested for enterotoxin protein expression in further investigations. The staphylococcal enterotoxin-like toxin X (SElX) also demonstrated a high frequency. The *selx* gene is encoded in the core genome of *S. aureus*, which explains the frequency of *selx*. However, its emetic activity has not yet been tested (HU; NAKANE, 2014). In addition, we suggest in this case to further studies the research of allelic diversification. Other genes (*seb*, *seg*, *sej* and *ser*) that encode for SEs with emetic activity were detected; it shows that milk quality control

needs to be strict in order to avoid the pathogen or significant count of it, and consequently the possibility of milk contamination with SEs. Several SEs profiles were identified, this finding demonstrates the high distribution of SEs genes in the species studied; for example, 32 superantigenic toxin genotypes were observed across 166 isolates (69 food poisoning isolates, and 97 healthy human nasal swab isolates) in the study performed by Omoe et al. (2005). All hemolysin genes were identified, *hla*, *hlb*, *hld* and *hlg*AC also were detected by Ote et al. (2011), and they identified frequencies between 78.6%-100% in strains. In our study, hemolysin gene frequencies were between 32.7%-55.1%. The most prevalent was *hlb*, which is in agreement with other study that assessed isolates from raw milk products (MORANDI et al., 2009). Genes encoding exfoliative toxins, *pvl* and *tst* were not identified; previously Ote et al. (2011) identified *eta* and *tst* genes in isolates associated with bovine mastitis.

Regarding antibiotic resistance, penicillin resistance is commonly detected in *Staphylococcus* spp. (GÓMEZ-SANZ et al., 2010; MOON et al., 2007), and this was demonstrated in the present study. Silva et al.(2013) did not detect resistance to erythromycin in their isolates, although they detected one strain of *S. aureus* with resistance to chloramphenicol. Erythromycin and tetracycline resistance genes were observed (Table 3); these genes have been detected in *Staphylococcus* sp. (GÓMEZ-SANZ et al., 2010; SILVA et al., 2014). It is important to highlight that all isolates were tested for the presence of *mec*A and *mec*C genes as well as other resistance genes. Our results on *mec*A, and SCC*mec* type are in line with Silva et al. (2014), where they assessed methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. Meanwhile, the absence of a novel *mec*A homologue could be expected because it is of rare occurrence (CUNY et al., 2011). The absence of *mec* genes in most cefoxitin and oxacillin resistant strains indicates the possible presence of modified *S. aureus* (MODSA), MODSA possesses a modification of its penicillin-binding proteins (PBPs), which become it different of mechanism classical of MRSA (BHUTIA et al., 2012).

Few *spa* types were detected, and on the farm A only one *spa* type (t605) was observed. This suggests that the *spa* type t605 is common in bovine clones, and it can be endemic in the region causing subclinical bovine mastitis. The t605 type was initially detected in Austria, France, Germany, Netherlands, Norway, Spain, Sweden and United Kingdom, and represents 0.1% of relative global frequency of

spa type occurrences in accordance with the website http://www.ridom.com/ (http://spaserver.ridom.de - data collected on June 2015). Other studies in Brazil also detected this *spa* type in strains isolated from milk from bovine and others animals (AIRES-SOUSA, 2007; SILVA et al., 2013). On the other hand, the *spa* type t127 were the most detected by Silva et al. (2013). The *agr* types detected were I and II, which were also detected in a previous study with isolates from bovine mastitis (SILVA et al., 2013).

2.5 Conclusion

The majority of isolates were identified as *S. aureus*. Other isolates also identified were *S. hyicus*, *S. xylosus*, and *S. chromogenes*. The majority virulence factor genes identified using multiplex PCR, in total eleven different multiplex reactions were successfully optimized and applied in this study. The most isolates carried virulence factor genes; *seh* and *selx* were the most detected among SEs. Hemolysins genes were widely identified, presenting several profiles as well as SEs. The antibiotic resistance was widely detected for penicillin and the identification of MRSA strains was observed which presents a concern. The *spa* types t605, t521, t267 and t9121 and the *agr* types I and II were detected. The most prevalent *spa* type was t605, which suggests that this could be an endemic *spa* type in the herds sampled. In summary, we showed data regarding molecular variability and antibiotic resistance for a small group of staphylococci isolated from mastitic milk, which confirms that more studies should be completed to identify and understand strains/clones in specifics regions, and thus to help prevent *Staphylococcus* infection in dairy cows.

References

AARESTRUP, F.M.; DANGLER, C.A.; SORDILLO, L.M. Prevalence of coagulase gene polymorphism in *Staphylococcus aureus* isolates causing bovine mastitis. **Canadian Journal of Veterinary Research**, Ottawa, v. 59, n. 2, p. 124-128, Apr. 1995.

AIRES-DE-SOUSA, M.; PARENTE, C.E.S.R.; VIEIRA-DA-MOTTA, O.; BONNA, I.C.F.; SILVA, D.A.; LENCASTRE, H. Characterization of *Staphylococcus aureus* isolates from buffalo, bovine, ovine, and caprine milk samples collected in Rio de Janeiro State, Brazil. **Applied and Environmental Microbiology**, Washington, v. 73, n. 12, p. 3845-3849, June 2007.

ARGUDÍN, M.A.; MENDONZA, M.C.; RODICIO, M.R. Food poisoning and *Staphylococcus aureus* enterotoxins. **Toxins**, Basel, v. 2, n. 7, p. 1751-1773, July 2010.

BHUTIA, K.O.; SINGH, T.S.; BISWAS, S.; ADHIKARI, L. Evaluation of phenotypic with genotypic methods for species identification and detection of methicillin resistant in *Staphylococcus aureus*. **International Journal of Applied & Basic Medical Research**, Mumbai, v. 2, n. 2, p. 84-91, July 2012.

CLINICAL LABORATORY STANDARDS INSTITUTE. Performance standards for antimicrobial susceptibility testing. In: _____. **National Committee for Clinical Laboratory standards.** Wayne, 2015. p. 146-156. (Twenty-fifth Informational Supplement, M100–S25).

CREMONESI, P.; LUZZANA, M.; BRASCA, M., MORANI, S.; LODI, R.; VIMERCATI, C.; AGNELLINI, D.; CARAMENTI, G.; MORONI, P.; CASTIGLIONI, B. Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. **Molecular and Cell Probes**, London, v. 19, n. 5, p. 299-305, Oct. 2005.

CUNY, C.; LAYER, F.; STROMMENGER, B.; WITTE, W. Rare occurrence of methicillin-resistant *Staphylococcus aureus* CC130 with a novel *mec*A homologue in humans in Germany. **PLoS One**, San Francisco, v. 6, n. 9, p.1-5, Sept. 2011.

DORDET-FRISONI, E.; DORCHIES, G.; DE ARAUJO, C.; TALON, R.; LEROY, S. Genomic diversity in *Staphylococcus xylosus*. **Applied and Environmental Microbiology**, Washington, v. 73, n. 22, p. 7199–7209, Nov. 2007.

FISCHER, A.; FRANCOIS, P.; HOLTFRETER, S.;BROEKER, B.; SCHRENZEL, J. Development and evaluation of a rapid strategy to determine enterotoxin gene content in *Staphylococcus aureus*. **Journal of Microbiological Methods**, Amsterdam, v. 77, n. 2, p. 184-190, May 2009.

GIANNECHINI, R.; CONCHA, C.; RIVERO, R.; DELUCCI, I.; LÓPEZ, M.J. Occurrence of clinical and sub-clinical mastitis in dairy herds in the west littoral region in Uruguay. **Acta Veterinaria Scandinavica**, London, v. 43, n. 4, p. 221–230, Dec. 2002.

GÓMEZ-SANZ, E.; TORRES, C.; LOZANO, C.; FERNÁNDEZ-PÉREZ, R.; ASPIROZ, C.; RUIZ-LARREA, F.; ZARAZAGA, M. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. **Foodborne and Pathogens Disease**, Larchmont, v. 7, n. 10, p. 1269-1277, Oct. 2010.

HU, D.L.; NAKANE, A. Mechanisms of staphylococcal enterotoxins-induced emesis. **European Journal of Pharmacology**, Amsterdam, v. 722, p. 95-107, Jan. 2014.

JARRAUD, S.; MOUGEL, C.; THIOULOUSE, J.; LINA, G.; MEUGNIER, H.; FOREY, F.; NESME, X.; ETINNE, J.; VANDENESCH, F. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. **Infection and Immunity**, Washington, v. 70, n. 2, p.631-641, Feb. 2002.

JOHNSON, W.M.; TYLER, S.D.; EWAN, E.P.; ASHTON, F.E.; POLLARD, D.R.; ROZEE, K.R. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. **Journal of Clinical Microbiology**, Washington, v. 29, n. 3, p. 426–430, Mar. 1991.

JORGENSEN, H.J.; MATHISEN, T.; LOVSETH, A.; OMOE, K.; QVALE, K.S.; LONCAREVIC, S. An outbreak of *Staphylococcus* food poisoning cause by enterotoxin H in mashed potato made with raw milk. **FEMS Microbiology Letters**, Amsterdam, v. 252, n. 2, p. 267-272, Nov. 2005.

KONDO, Y.; ITO, T.; MA, X.X.; WATANABE, S.; KREISWIRTH, B.N.; ETIENNE, J.; HIRAMATSU, K. Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. **Antimicrobial Agents and Chemotherapy**, Washington, v. 51, n. 1, p. 264–274, Jan. 2007.

LANGE, C.C.; BRITO, M.A.V.P.; REIS, D.R.L.; MACHADO, M.M; GUIMARÃES, A.S.; AZEVEDO, A.L.; SALLES, E.B.; ALVIM, M.C; SILVA, F.S.; MEURER, I.R. Species-level identification of staphylococci isolated from bovine mastitis in Brazil using partial 16S rRNA sequencing. **Veterinary Microbiology**, Amsterdam, v. 176, n. 3/4, p. 382-388, Apr. 2015.

LINA, G.; PIÉMONT, Y.; GODAIL-GAMOT, F.; BES, N.M.; PETER, M.O.; GAUDUCHON, V.; VANDENESCH, F.; ETIENNE, J. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. **Clinical Infectious Diseases**, Chicago, v. 29, n. 5, p. 1128-1132, Nov. 1999.

MEHROTRA, M.; WANG, G.; JOHNSON, W.M. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. **Journal of Clinical Microbiology**, Washington, v. 38, n. 3, p.1032–1035, Mar. 2000.

MOON, J.S.; LEE, A.R.; KANG, H.M.; LEE, E.S.; KIM, M.N.; PAIK, Y.H.; PARK, Y.H.; JOO, Y.S.; KOO, H.C. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. **Journal of Dairy Science**, Champaign, v. 90, n. 3, p. 1176-1185, Mar. 2007.

MORANDI, S.; BRASCA, M.; ANDRIGHETTO, C.; LOMBARDI, A.; LODI, R. Phenotypic and genotypic characterization of *Staphylococcus aureus* strains from Italian dairy product. **International Journal of Food Microbiology**, Amsterdam, v. 2009, n. 2009, p. 1-7, Oct. 2009.

NASHEV, D.; TOSHKOVA, K.; SALASIA, S.I.; HASSAN, A.A.; LAMMLER, C.; ZSCHÖCK, M. Distribution of virulence genes of *Staphylococcus aureus* isolated from stable nasal carriers. **FEMS Microbiology Letters**, Amsterdam, v. 233, n. 1, p. 45–52, Apr. 2004.

OMOE, K.; ISHIKAMA, M.; SHIMODA, Y.; HU, D.L.; UEDA, S.; SHINAGAWA, K. Detection of *seg, seh* and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolate harboring *seg*, *seh* or *sei* genes. **Journal of Clinical Microbiology**, Washington, v. 40, n. 3, p. 857–862, Mar. 2002.

OMOE, K.; HU, D.L.; TAKAHASHI-OMOE, H.; NAKANE, A.; SHINAGAWA, K. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. **FEMS Microbiology Letters**, Amsterdam, v. 246, n. 2, p. 191-198, May 2005.

ONO, H.K.; OMOE, K.; IMANISHI, K.; IWAKABE, K.; HU, D.L.; KATO, H.; SAITO, N.; NAKANE, A.; UCHIVAMA, T.; SHINAGAWA, K. Identification and characterization of two novel staphylococcal enterotoxins, types S and T*.* **Infection and Immunity**, Washington, v. 76, n. 11, p. 4999-5005, Nov. 2008.

OTE, I.; TAMINIAU, B.; DUPREZ, J.N.; DIZIER, I.; MAINIL, J.G. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. **Veterinary Microbiology**, Amsterdam, v.153, n. 3/4, p. 285-292, Dec. 2011.

PARK, J.Y.; FOX, L.K.; SEO, K.S.; MCGUIRE, M.A.; PARK, Y.H.; RURANGIRWA, F.R.; SISCHO, W.M.; BOHACH, G.A. Detection of classical and newly described staphylococcal superantigen genes in coagulase-negative staphylococci isolated from bovine intramammary infections. **Veterinary Microbiology**, Amsterdam, v. 147, n. 1/2, p. 149-154, Jan. 2011.

RAJIC-SAVIC, N.; KATIC, V.; VELEBIT, B.; COLOVIC, S. Characteristics of enterotoxigenic coagulase positive staphylococci isolated from bovine milk in cases of subclinical mastitis. **Procedia Food Science**, Amsterdam, v. 5, p. 250-253, 2015.

SASAKI, T.; TSUBAKISHITA, S.; TANAKA, Y.; SAKUSABE, A.; OHTSUBA, M.; HIROTAKI, S.; KAWAKAMI, T.; FUKATA, T.; HIRAMATSU, K. Multiplex-PCR method species identification of coagulase-positive staphylococci. **Journal of Clinical Microbiology**, Washington, v. 48, n. 3, p. 765-769, Mar. 2010.

SHOPSIN, B.; MATHEMA, B.; ALCABES, P.; SAID-SALIM, B.; LINA, G.; MATSUKA, A.; MARTINEZ, J.; KREISWIRTH, B.N. Prevalence of *agr* specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. **Journal of Clinical Microbiology**, Washington, v. 41, n. 1, p. 456-459, Jan. 2003.

SILVA, N.C.C.; GUIMARÃES, F.F.; MANZI, M.P.; BUDRI, P.E.; GÓMEZ-SANZ, E.; BENITO, D.; LANGONI, H.; RALL, V.L.; TORRES, C. Molecular characterization and clonal diversity of methicillin susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil. **Journal of Dairy Science**, Champaign, v. 96, n. 11, p. 6856-6862, 2013.

SILVA, N.C.C.; GUIMARÃES, F.F.; MANZI, M.P.; GÓMEZ-SANZ, E.; GÓMEZ, P.; ARAÚJO-JÚNIOR, J.P.; LANGONI, H.; RALL, V.L.; TORRES, C. Characterization of methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. **Antonie van Leeuwenhoek**, Dordrecht, v. 106, n. 2, p. 227–223, Aug. 2014.

THOMAS, D.; DAUWALDER, O.; BRUN, V.; BADIOU, C.; FERRY, T.; ETIENNE, J.; VANDENESCH, F.; LINA, G. *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. **Infection and Immunity**, Washington, v. 77, n. 5, p. 2043–2050, May 2009.

van de KLUNDERT, J.A.M.; VLIEGENTHART, J.S. PCR detection of genes for aminoglycoside-modifying enzymes. In: PERSING, D.H.; SMITH, T.F.; TENOVER, F.C.; WHITE, T. J. **Diagnostic molecular microbiology**. Washington: American Society for Microbiology, 1993. p. 547–552.

WILSON, G.J.; SEO, K.S., CARTWRIGHT, R.A.; CONNELLEY, T.; CHUANG-SMITH, O.N.; MERRIMAN, J.A.; GUINANE, C.M.; PARK, J.Y.; BOHACH, G.A.; SCHLIEVERT, P.M.; MORRISON, W.I.; FITZGERALD, J.R. A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. **PLoS Pathogens**, San Francisco, v. 7, p. 1-16, June 2011.

YAMAGUCHI, T.; NISHIFUJI, K.; SASAKI, M.; FUDABA, Y.; AEPFELBACHER, M.; TAKATA, T.; OHARA, M.; KOMATSUZAWA, H.;AMAGAI, M.; SUGAI, M. Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. **Infection and Immunity**, Washington, v. 70, n. 10, p. 5835–5845, Oct. 2002.

3 THE *Lactococcus* GENUS AS A POTENTIAL EMERGING MASTITIS PATHOGEN GROUP: a report on an outbreak investigation

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., *L. lactis* and *L. 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 high similarity among the *L. lactis* isolates as well as between the isolates and reference sequences. DNA fingerprinting analysis based on random amplified polymorphic DNA (RAPD) results of the 27 *L. lactis* isolates indicated different RAPD profiles; however, dominant RAPD profiles were identified. Thus, apparently identical strains of *L. lactis* were present in different cows. 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 area potential etiological agent in the mastitis outbreak studied. Further studies should be conducted to understand the importance of *Lactococcus*, especially *L. lactis*, as pathogenic microbes in veterinary medicine and food safety.

Keywords: Mastitis; *Lactococcus*; Microbiome; Next-generation sequencing

3.1 Introduction

Mastitis is an important disease in dairy cows causing reproduction problems (HERTL et al., 2010), anticipated 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 is essential (ROYSTER; 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, non-motile cocci, homofermentative, poorly α-hemolytic, and exclusively produce L(+) lactic acid. They are members of the group Lactic Acid Bacteria (LAB)and are routinely found on animal skin and plants (CASALTA; MONTEL, 2008). LAB are generally not considered harmful to humans (MOFREDJ; BAHLOUL; CHANUT, 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; BAHLOUL; CHANUT, 2007) and animal diseases (KLOSTERMANN et al., 2008; ESPECHE et al., 2012; BOUCHARD et al., 2015). LAB have been reported to produce proteins, chemical mediators, and other molecules that stimulate local immune responses (MOFREDJ; BAHLOUL; CHANUT, 2007).

Interestingly, in dairy cows, the potential of LAB for treatment and/or prevention 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 *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 had potentially a similar level of efficacy as common antibiotics (KLOSTERMANN et al., 2008).

Lactococcus lactis, in particular, is of considerable economic importance (CAVANAGH; FITZGERALD; McAULIFFE, 2015) and is widely used as a starter culture in dairy fermentation (CASALTA; MONTEL, 2008). *L. 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 (QPS) list of the European Food Safety Authority (PLUMED-FERRER et al., 2013) and is accepted as Generally Recognized as Safe (GRAS) (CASALTA; MONTEL, 2008). However, some species of *Lactococcus* have been reported to be the cause of human (DAVIES; BURKITT; WATSON, 2009; HADJISYMEOU; LOIZOU; KOTHARI, 2013; INOUE et al., 2014) and animal infections (PLUMED-FERRER et al., 2013; KHOO et al., 2014; PLUMED-FERRER et al., 2015a). 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 Streptococci-like genera such as *Enterococccus* and *Aerococcus* (WERNER et al., 2014). 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 16SrRNA gene, random amplified polymorphic DNA-PCR (RAPD-PCR), and phylogenetic techniques.

3.2 Materials and Methods

3.2.1 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).

3.2.2 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 of 2015, the farm milked approximately 1,200 cows. Primiparous and multiparous cows were housed separately in free-stall barns bedded with sand. 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

(NATIONAL RESEARCH COUNCIL, 2001). Cows were milked thrice daily in a double-20 milking parlor.

The target length of the dry period was 55 days. Cows were dried by abrupt interruption of milking and dry cow therapy was equally performed for all quarters of all cows and consisted of intramammary infusion of with Ceftiofur Hydrochloride (Spectramast® DC, Zoetis, Madison, NJ) followed by the administration of an internal teat sealant (Orbeseal®, Zoetis, Madison, NJ). Before the outbreak *Lactococcus* spp. the common mastitis pathogens encountered based on aerobic mastitic milk culture was represented by around 33% Gram-negative microbes, mainly *E. coli* and *Klebsiella*, 33% Gram-positive microbes, mainly *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus* spp., and *Staphylococcus* spp., and 33% were culture negative. The historical clinical cure rates following intramammary antibiotic therapy of clinical mastitis was around 70%. The bulk tank somatic cell count ranged from 150,000-250,000. The herd is 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.

3.2.3 Enrollment criteria, on farm culture, and sample collection

Clinical mastitis was identified by the presence of abnormal changes in the udder and 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, USA). The Accumast® system consists of a single petri dish with three 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 against the results of a referral laboratory and also against 16SrRNA gene sequencing. Unfortunately, no *Lactococcus* mastitis cases were identified on 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).

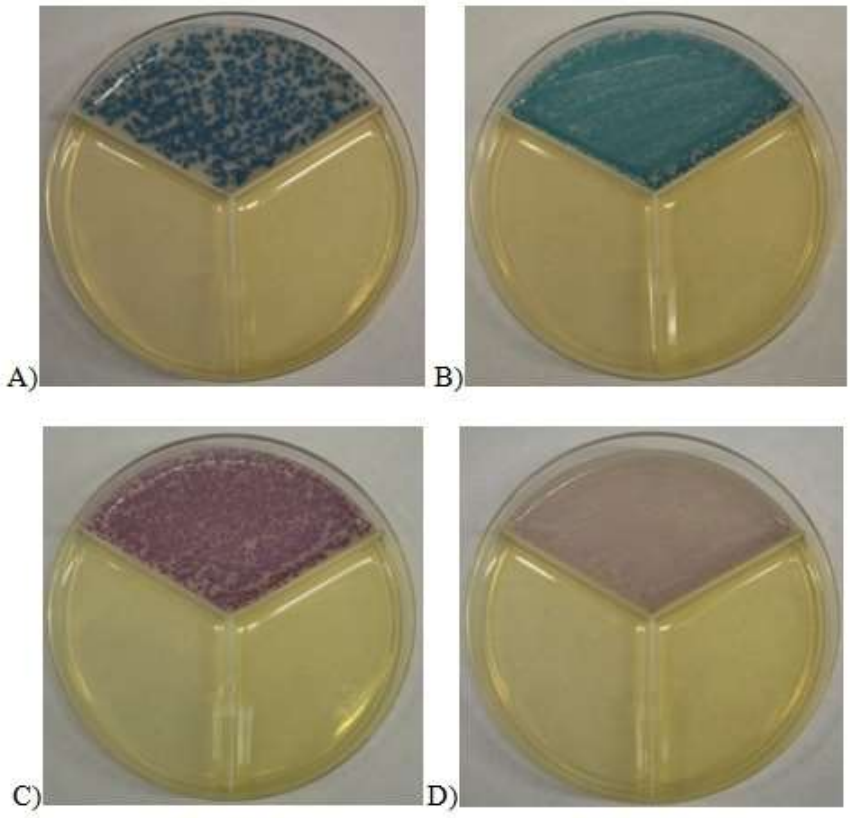


Figure 1 -Growth characteristics of different bacteria on the second section of the AccuMast-Mastitis Culture System®. A) Growth of *Streptococcus uberis* (ATCC 700407). B) Growth of *Streptococcus agalactiae* (ATCC 27956). C) Growth of *Enterococcus faecalis* (ATCC 29212). D) Growth of *Lactococcus lactis* isolated from mastitic milk in this study

Plates were incubated aerobically at 37 ºC for a period of 16-20 hours. 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 1-D) were eligible to enter the study. Before intramammary antibiotic treatment was started and within 24 hours of the initial diagnosis, a fresh milk sample was aseptically collected from cows that were considered *Lactococcus* positive by 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 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, during approximately 2 hours, and were 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 ºC until further processing.

3.2.4 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 ºC for up to 24 hours. 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 first inoculation, thus this step was repeated three 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, USA) and incubated overnight at 37 ºC; these cultures were used to prepare stocks (bacterial cultures stored in 50% glycerol at -20 ºC for further use) and to extract DNA.

DNA was extracted from each bacterial isolate using InstaGene™ Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The 16S ribosomal DNA gene was amplified using 10 pmol of each fD1forward and rP2 reverse primers (WEISBURG et al., 1991), EconoTaq Plus Green 1x Master Mix (Lucigen®, Middleton, WI, USA), 280-350 ng of template DNA, and ultrapure distilled water to bring the final reaction volume to 100µL. The conditions for amplification were: 94 ºC for 5 minutes, 57 ºC for 2 minutes, and 72 ºC for 2 minutes followed by 29 cycles of 94 ºC for 2 minutes, 57 ºC for 30 seconds and 72 ºC for 2 minutes, with a final extension of 72 ºC for 10 minutes (WOOD et al., 1998). Agarose gel electrophoresis (1.2% wt/vol) was used to verify the presence of PCR products. DNA was visualized with 0.5 µg/ml ethidium bromide.

PCR products were purified using a Gel/PCR Fragments Extraction Kit (IBI Scientific, Peosta, IA, USA) 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).

3.2.5 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): *L. garvieae* (JCM8735; AB012306), *L. lactis* (MRS1; AJ488173), *L. lactis* subsp. *cremoris* (CF4; AB181302), *L. lactis* subsp. *lactis* (MR26, AF493057), *L. piscium* (fish isolate HR1A-68.; X53905), *L. raffinolactis* (NCDO617; X54261), *Streptococcus agalactiae* ((T); JCM 5671; AB023574), *Streptococcus dysagalactiae* (ATCC 35666; AJ319643), and *Streptococcus 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; NEI, 1993) and the phylogenetic tree was constructed by applying the Neighbor-Joining method (SAITOU; NEI, 1987).

3.2.6 RAPD-PCR

The random amplified polymorphic DNA-PCR was performed as previously described by Akopyanz et al. (1992) using primer 1254. The reactions were performed using 10 pmol of 1254 primer, 1x EconoTaq Plus Green Master Mix (Lucigen®, Middleton, WI, USA), 280-350 ng of template DNA, and ultrapure distilled water was used to bring the final reaction volume to 50 µL. The cycling program was: 4 cycles of 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min, followed by 30 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, and then a final extension at72°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 µg/ml ethidium

bromide. The profiles were compared visually and clustered according to suggested similarity.

3.2.7 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. DNA was extracted from all samples by using PowerSoil® DNA Isolation Kit (MO BIO Laboratory Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The 515f-806rB region of the 16S rRNA gene was amplified according to a previous study (CAPORASO et al., 2012). 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 1x Master Mix (Lucigen®, Middleton, WI, USA), 10 - 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 minutes; 35 cycles of 94°C for 45 seconds, 50°C for 1 minute, 72 °C for 90 seconds, and 72 °C for 10 minutes. 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, Peosta, IA, USA).The amplicons were quantified using a Qubit® 2.0 Fluorometer (Invitrogen™ by Life Technologies™, Carlsbad, CA, USA), standardized to the same concentration and pooled for sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). A final equimolar library was sequenced using the MiSeq Reagent Nano Kit V2 (300 cycles) (Illumina, Inc., San Diego, CA, USA).

3.2.8 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). ANOVA, implemented in JMP, was used to compare the relative abundances of *Lactococcus* between milk from mastitic and healthy cows.

3.3 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 1-D. 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 the16S 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* subsp. *cremoris* (CF4; AB181302) compared to the *L. lactis* subsp. *lactis* (MR26, AF493057).

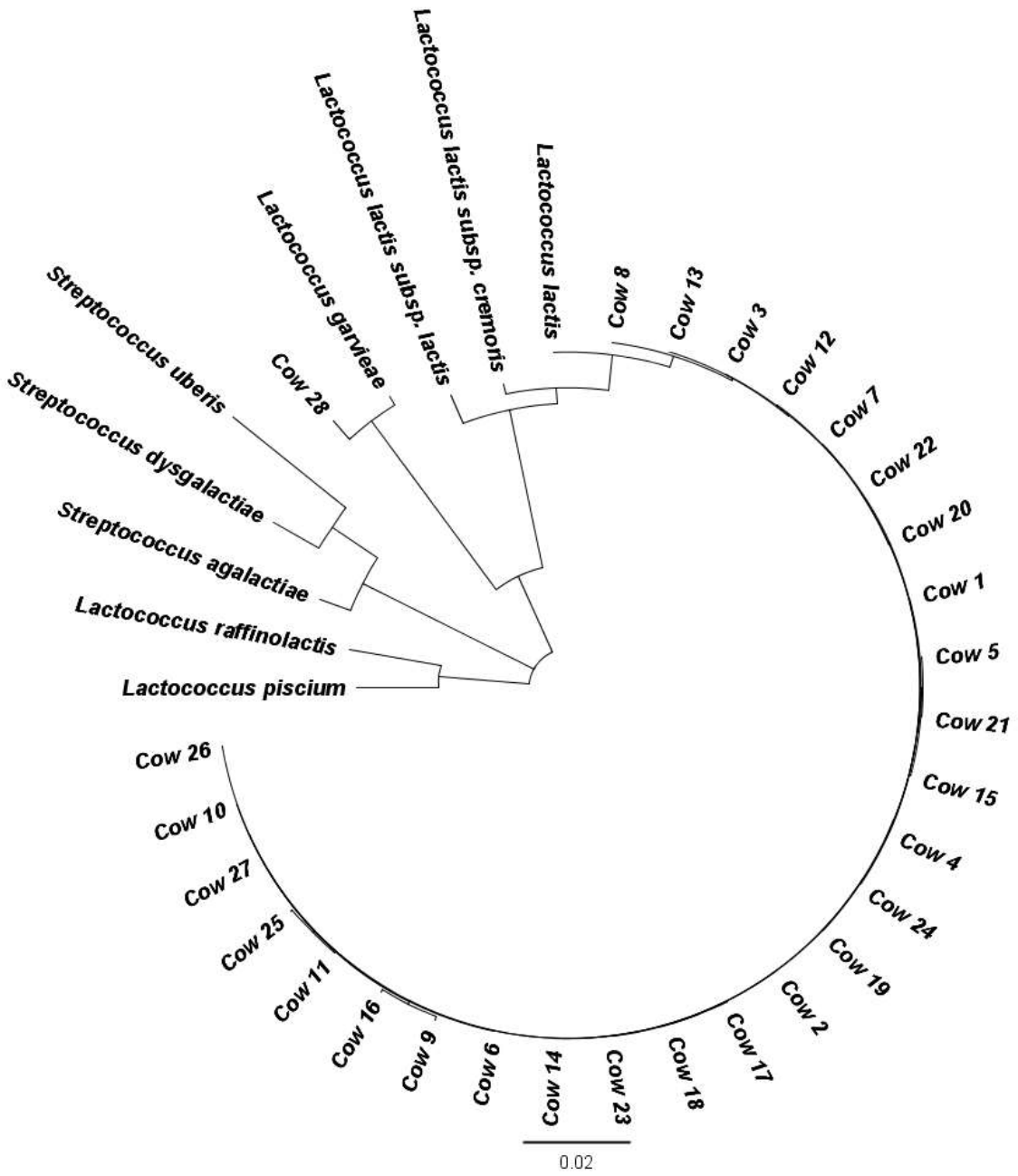


Figure 2 -Phylogenetic tree based on the sequences of 28 isolates from mastitic milk samples (where each isolate is represented by a single cow) and the reference sequences used, i.e., *L. garvieae* (JCM8735; AB012306), *L. lactis* (MRS1; AJ488173), *L. lactis* subsp*. cremoris* (CF4; AB181302), *L.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

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 a number of distinct RAPD profiles was observed, some of isolates had a similar pattern (Figure 3).

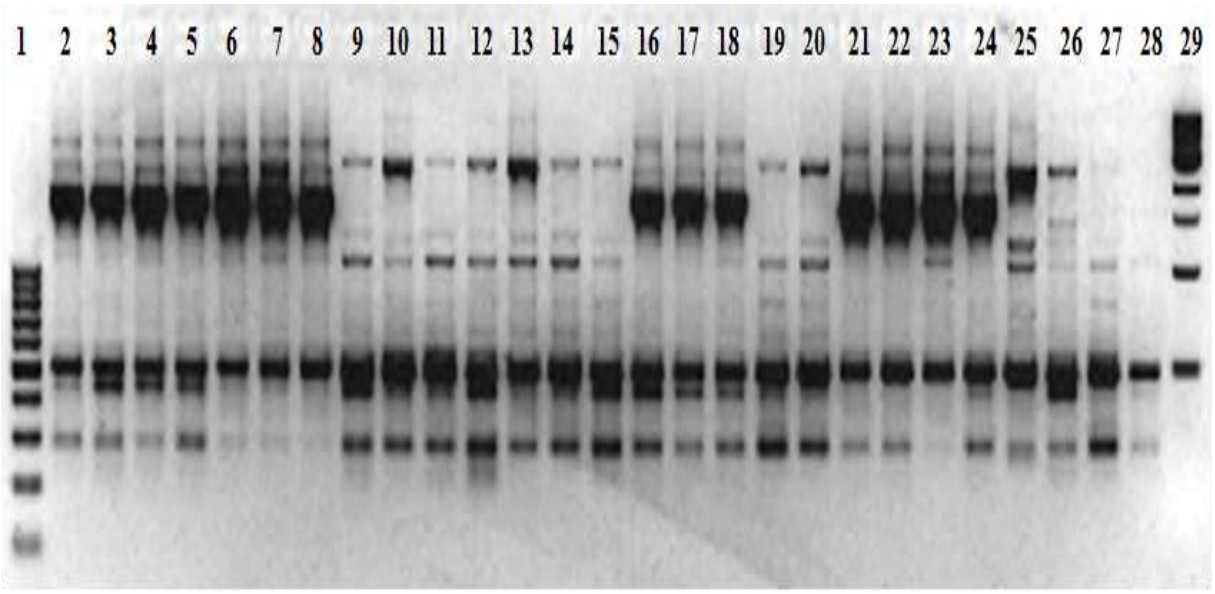


Figure 3 -RAPD-PCR analysis using primer 1254 to fingerprint 27 *Lactococcus lactis* strains isolated from mastitic milk. Lane 1: 100 bp DNA Ladder (Omega Bio-tek, Norcross, GA, USA), lanes 2-28: isolates from cow 3, 17, 20, 4, 21, 5, 2, 23, 9, 7, 25, 15, 19, 14, 22, 24, 1, 8, 12, 18, 16, 6, 10, 26,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

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 the17 most abundant are shown in Figure 4.

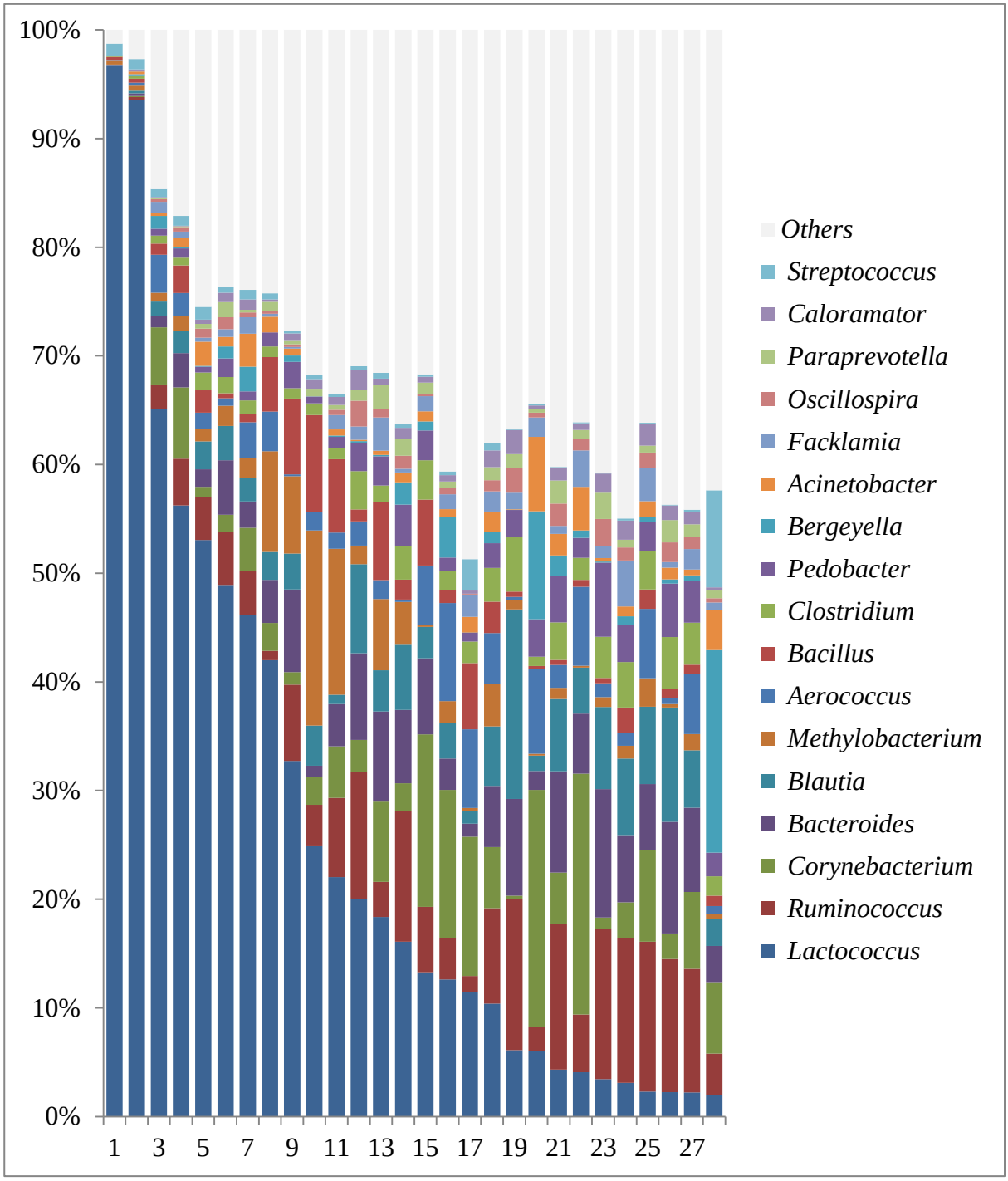


Figure 4 -Mean relative abundance of the ten most prevalent bacterial genera identified in mastitic milk samples

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 to the microbiome of healthy milk samples (Figure 5).

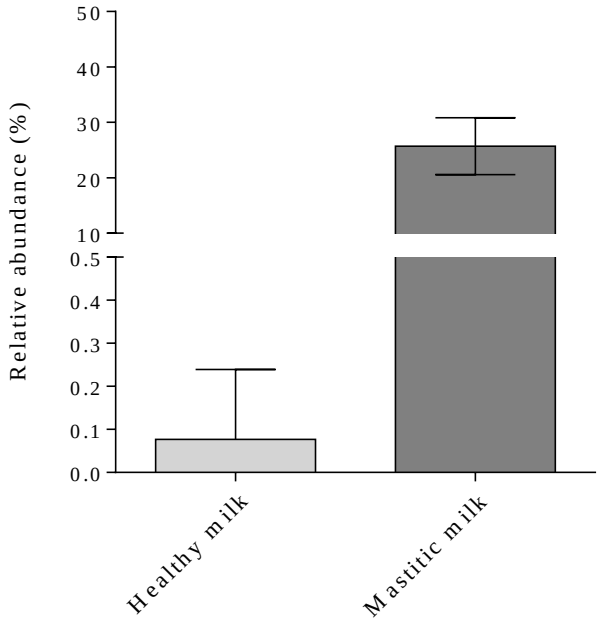


Figure 5 -Mean of relative abundance of *Lactococcus* in healthy milk versus mastitic milk. Error bars represent the standard deviation of the mean relative abundance

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 (Figure 6).

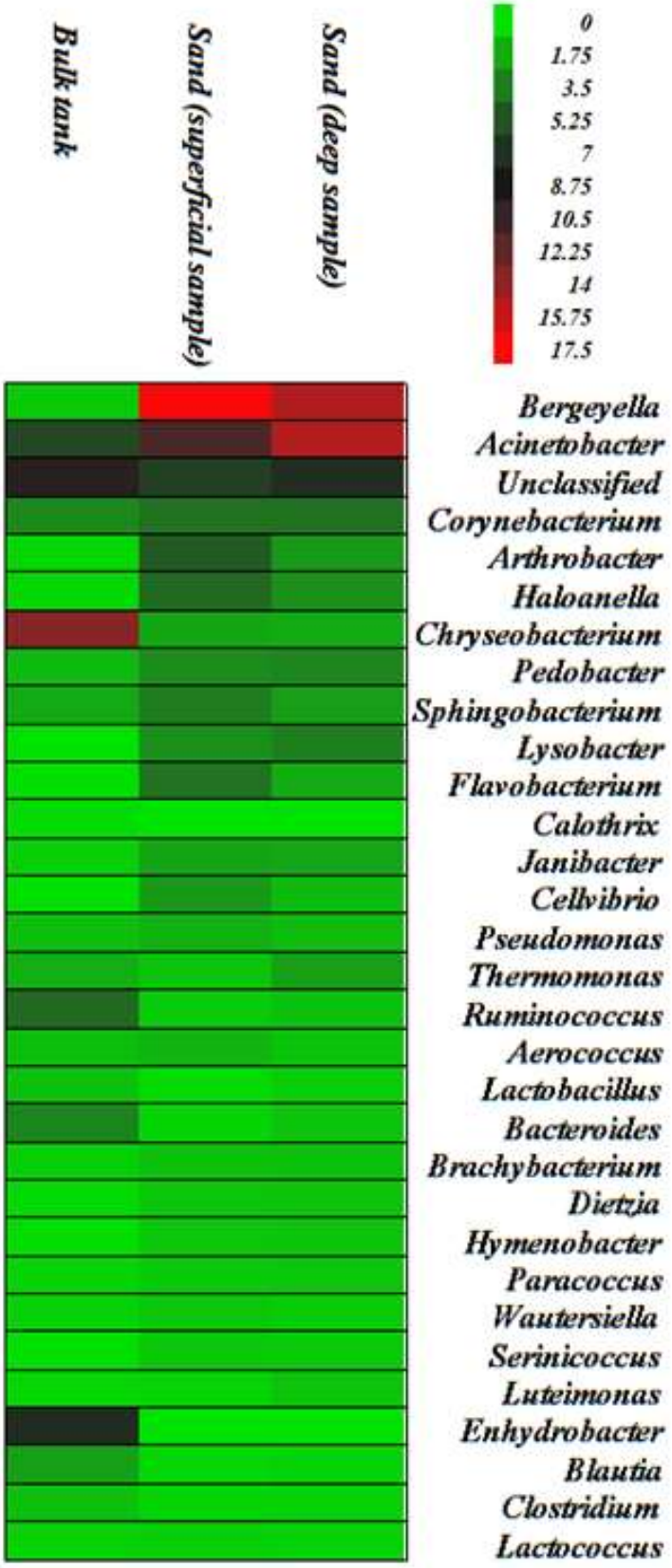


Figure 6 -Mean relative abundance of the 30 most prevalent bacterial genera identified in the sand bedding and bulk tank milk samples

3.4 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. *L. lactis* was the dominant species detected and *L. garvieae* was the only other *Lactococcus* species identified. Phylogenetic tree analysis showed high similarity among the *L. lactis* isolates, and RAPD profiles revealed that some isolates from different cows shared the same profile. Microbiome analysis determined the *Lactococcus* genus to be dominant in most mastitic samples, with a high relative abundance, and it was present in all 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 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.

PCR-based methods 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; 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 *Streptococcus uberis* and failed to identify *L. lactis* ssp. *lactis*. Similarly, Plumer-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* subsp. *lactis* and *L. garvieae* (WYDER et al., 2011).

In the phylogenetic tree (Figure 2), we observed highest similarity between the isolates and *L. lactis*, followed by the isolates and *L. lactis* subsp. *cremoris* (CF4; AB181302). In humans, infections caused by *Lactococcus*, specifically *L. lactis* subsp. *cremoris*, are generally rare (HADJISYMEOU; LOIZOU; KOTHARI, 2013);

however, during the past 50 years numerous case reports of infection have been published (DAVIES; BURKITT; WATSON, 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; LOIZOU; KOTHARI, 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; LOIZOU; KOTHARI, 2013). The pathogenicity of *L. lactis* subsp. *cremoris* is unknown and its route of infection is not well-understood (INOUE et al., 2014). Both *L. lactis* subsp. *cremoris* and *L. garvieae* have been isolated from bovine mastitis (PLUMED-FERRER et al., 2013, 2015b). *L. garvieae* is a known important pathogen in aquaculture, and in some human cases of *L. garvieae* infection there was 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. Different RAPD profiles were present and two distinct strains of *L. lactis* appeared to be dominant. The transmission of strains can occur from the same source, e.g. milking machine and bedding, or from animal to animal in a contagious way. The distribution of environmental and contagious mastitis-causing bacteria is a concern. *Streptococcus agalactiae* and *Staphylococcus aureus* are known important pathogens causing contagious mastitis (BOSS et al., 2016; JØRGENSEN et al., 2016). The environmental transmission potential of *S. agalactiae* was recently discussed (JØRGENSEN et al., 2016). On the other hand, the contagious transmission potential of *Streptococcus dysagalactiae* and *Streptococcus uberis* was described years ago (FOX; GAY, 1993; ZADOCKS et al., 2003).

Munoz et al. (2007) studied two outbreaks caused by *Klebsiella* spp., they found in an outbreak a single strain in milk from several cows and the same strain type was also isolated from rubber liners of milking machine (after milking infected cows) and bedding corresponding to the outbreak pen, which could indicate a contagious transmission or exposure to different cows to the same environmental source. Intervention measures were implemented in the farm and no new case caused by that strain was identified. However, weeks later a new outbreak caused by *Klebsiella* spp. was identified; several RAPD types were detected indicating opportunist infection from the environment (MUNOZ et al., 2007). Herein, we suggested that *Lactococcus* was behaving as a contagious pathogen based on the results of RAPD-PCR. However, we should also consider the situation exposed by

Munoz et al. (2007), it could be that different cows were being infected by a common source which could explain the fact that several *Lactococcus* isolates had identical RAPD-PCR agarose gel profiles. Daly et al. (1999), for example, reported that findings suggested that the infection by *Pseudomonas aeruginosa* was spread to herds by teat wipes contaminated with this microorganism, they also reported that the same *Pseudomonas aeruginosa* was responsible for the mastitis outbreak. There are only a few studies that have used RAPD-PCR for DNA fingerprinting of *Lactococcus* isolates (TAILLIEZ et al., 1998; MAGIN et al., 1999; PLUMED-FERRER et al., 2015a) and further studies are still needed to validate this technique for differentiation of *Lactococcus* isolates. Therefore, further studies are warranted for to elucidate the transmission of *Lactococcus* between cows and also to evaluate the short and long term impact of *Lactococcus* mastitis on cow health, milk production, quality and composition.

Transmission of lactococci maybe aided by the administration of whey to feeding cows; in the dairy farm in study this practice is not used. Whey (a liquid by-product of cheese production) from the food industry is composed of lactose (approximately 70% of dry matter), protein, minerals (DeFRAIN et al., 2004) and starter culture, e.g. *Lactococcus lactis* subp. *cremoris* (DABOUR et al., 2005). The use of whey is practiced on some farm due to its chemical and physical properties, and it is an inexpensive ingredient to include in the diet of dairy cattle (DeFRAIN et al., 2004). However, it must be considered that the starter culture added may cause changes in the cow microbiota.

We assessed the microbial diversity in mastitic milk, sand bedding, and bulk tank milk. 16S rRNA gene sequencing of microbes in mastitic milk identified *Lactococcus* in all samples; in fact, it was the most abundant genus in the majority of samples (Figure 4). While 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 non-mastitic 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 psychrotropic bacteria frequently detected in dairy products (DELBÈS; ALI-MANDJEE; MONTEL, 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 were not the primary source of contamination but was simply contaminate by milk leakage from infected cows while they were lying down; samples analyzed on 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. It has been reported that environmental mastitis is difficult to control because the disease-causing bacteria are nearly ubiquitous in cattle housing systems, e.g. in bedding, soil and feces (SMITH; TODHUNTER; SCHOENBERGER, 1985). Zdanowicz et al. (2004) highlighted the importance of understanding that the lying surface promotes bacterial proliferation. In that work they assessed the bacterial population on teat ends of dairy cows bedded on different materials. The data showed that sand bedding produced 1 log unit more streptococci on teat ends compared with sawdust bedding (ZDANOWICZ et al., 2004).

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 opportunist 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 what importance they may have to the dairy industry.

3.5 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 high similarity based on phylogenetic tree and RAPD-PCR showed different RAPD patterns. The microbiome was assessed for a number of 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.

References

AKOPYANZ, N.; BUKANOV, N.O.; WESTBLOM, T.U.; KRESOVICH, S.; BERG,D.E. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. **Nucleic Acids Research**, London, v. 20, n. 19, p. 5137-5142, Oct. 1992.

BAR, D.; TAUER, L.W.; BENNETT, G.; GONZÁLEZ, R.N.; HERTL, J.A.; SCHUKKEN, Y.H.; SCHULTE, H.F.; WELCOME, F.L.; GRÖHN,Y.T. The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. **Journal of Dairy Science**, Champaign, v. 91, n. 6, p. 2205-2214, June 2008.

BENSON, D.A.; KARSCH-MIZRACHI, I.; LIPMAN, D.J.; OSTELL, J.; SAYERS, E.W. GenBank. **Nucleic Acids Research**, London, v. 37, D26-D31, Jan. 2009.

BOSS, R.; COSANDEY, A.; LUINI, M.; ARTURSSON, K.; BARDIAU, M.; BREITENWIESER, F.; HEHENBERGER, E.; LAM, T.; MANSFELD, M.; MICHEL, A.; MÖSSLACHER, G.; NASKOVA, J.; NELSON, S.; PODPEČAN, O.; RAEMY, A.; RYAN, E.; SALAT, O.; ZANGERL, P.; STEINER, A.; GRABER,H.U. Bovine *Staphylococcus aureus*: subtyping, evolution, and zoonotic transfer. **Journal of Dairy Science**, Champaign, v. 99, n. 1, p. 515-528, Jan. 2016.

BOUCHARD, D.S.; SERIDAN, B.; SARAOUI, T.; RAULT, L.; GERMON, P.; GONZALEZ-MORENO, C.; NADER-MACIAS, F.M.; BAUD, D.; FRANÇOIS, P.; CHUAT, V.; CHAIN, F.; LANGELLA, P.; NICOLI, J.; LE LOIR, Y.; EVEN, S. Lactic acid bacteria isolated from bovine mammary microbiota: potential allies against bovine mastitis. **PLoS One**, San Francisco, v. 10, n. 12, p.1-18, Dec. 2015

CAPORASO, J.G.; LAUBER, C.L.; WALTERS, W.A.; BERG-LYONS, D.; HUNTLEY, J; FIERRER, N.; OWENS, S.M.; BETLEY, J.; FRASER, L.; BAURER, M.; GORMLEY, N.; GILBERT, J.A.; SMITH, G.; KNIGHT, R. Ultra-high-throughput microbial comminuty analysis on the Illumina HiSeq and MiSeq platforms. **The ISME Journal**, London, v. 6, n. 8, p. 1621-1624, Aug. 2012.

CASALTA, E.; MONTEL, M.C. Safety assessment of dairy microorganisms: the *Lactococcus* genus. **International Journal of Food Microbiology**, Amsterdam, v. 126, n. 1, p. 271-273, Sept. 2008.

CAVANAGH, D.; FITZGERALD, G.F.; McAULIFFE, O. From field to fermentation: the origins of *Lactococcus lactis* and its domestication to the dairy environment. **Food Microbiology**, London, v. 47, p. 45-61, May 2015.

COLE, J.R.; WANG, Q.; FISH, J.A.; CHAI, B.; MCGARRELL, D.M.; SUN, Y.; BROWN, C.T.; PORRAS-ALFARO, A.; KUSKE, C.R.; TIEDJE;J.M. Ribosomal Database Project: data and tools for high throughput rRNA analysis. **Nucleic Acids Research**, London, v. 42, D633-D642, Jan. 2014.

DABOUR, N.; KHEADR, E.E.; FLISS, I.; LAPOINTE, G. Impact of ropy and capsular exopolysaccharide-producing strains of *Lactococcus lactis* subsp. *cremoris* on reduced-fat Cheddar cheese production and whey composition. **International Dairy Journal**, Amsterdam, v. 15, n. 5, p. 459-471, May 2005.

DALY, M.; POWER, E.; BJÖRKROTH, J.; SHEEHAN, P.; O'CONNELL, A.; COLGAN, M.; KORKEALA, H.; FANNING, S. Molecular analysis of *Pseudomonas aeruginosa*: epidemiological investigation of mastitis outbreak in Irish dairy herds.**Applied and Environmental Microbiology**, Washington,v. 65, n. 6, p. 2723-2729, Jun. 1999.

DAVIES, J.; BURKITT, M.D.; WATSON, A. Ascending cholangitis presenting with *Lactococcus lactis cremoris* bacteraemia: a case report. **Journal of Medical Case Reports**, London, v. 3, n. 3, p.1-4, Jan. 2009.

DeFRAIN, J.M.; HIPPEN, A.R.; KALSCHEUR, K.F.; SCHINGOETHE,D.J. Feeding lactose increases ruminal butyrate and plasma beta-hydroxybutyrate in lactating dairy cows. **Journal of Dairy Science**, Champaign, v. 87, n. 8, p. 2486-2494, Aug. 2004.

DELBÈS, C.; ALI-MANDJEE, L.; MONTEL, M.C. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. **Applied and Environmental Microbiology**, Washington, v. 73, n. 6, p. 1882-1891, Jan. 2007.

ESPECHE, M.C.; PELLEGRINO, M.; FROLA, I.; LARRIESTRA,A.; BOGNI, C.; NADER-MACÍAS, M.E.F. Lactic acid bacteria from raw milk as potentially beneficial strains to prevent bovine mastitis. **Anaerobe**, v. 18, n. 1, p.103-109, Feb. 2012.

FORTIN, M.; MESSIER, S.; PARÉ, J.; HIGGINS, R. Identification of catalase-negative, non-beta-hemolytic, gram-positive cocci isolated from milk samples. **Journal of Clinical Microbiology**, Washington, v. 41, n. 1, p. 106-109, Jan. 2003.

FOX, L.K; GAY, J.M. Contagious mastitis. **Veterinary Clinics North America: Food Animal Practice**, Philadelphia, v. 9, n. 3, p. 475-487, Nov.1993.

FURTADO, D.N.; TODOROV, S.D.; LANDGRAF, M.; DESTRO, M. T.; FRANCO, B.D. Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* DF04Mi isolated from goat milk: characterization of the bacteriocin. **Brazilian Journal of Microbiology**, São Paulo,v. 45, n. 4, p.1541-1550, Mar. 2014.

GANDA, E.K.; BISINOTTO, R.S.; DECTER, D.H.; BICALHO, R.C. Evaluation of an on-farm culture system (accumast) for fast identification of milk pathogens associated with clinical mastitis in dairy cows. **PLoS One**, San Francisco, v. 11, n. 5, p.1-16, May 2016.

GRÖHN, Y.T.; GONZÁLEZ, R.N.; WILSON, D.J.; HERTL, J.A.; BENNETT,G.; SCHULTE, H.; SCHUKKEN, Y.H. Effect of pathogen-specific clinical mastitis on herd life in two New York State dairy herds. **Preventive Veterinary Medicine**, Amsterdam, v. 71, n.1/2, p. 105-125, Sept. 2005.

HADJISYMEOU, S.; LOIZOU,P.; KOTHARI, P. *Lactococcus lactis cremoris* infection: not rare anymore? **BMJ Case Reports**, London, p.1-3, May 2013.

HAN, Y.W.; IKEGAMI, A.; BISSADA, N.F.; HERBST,M.; REDLINE, R.W.; ASHMEAD, G.G. Transmission of an uncultivated *Bergeyella* strain from the oral cavity to amniotic fluid in a case of preterm birth. **Journal of Clinical Microbiology**, Washington, v. 44, n. 4, p. 1475-1483, Apr. 2006.

HERTL, J. A.; GRÖHN, Y.T.; LEACH, J.D.G.; BAR, D.; BENNETT, G.J.; GONZÁLEZ, R.N.; RAUCH, B.J.; WELCOME, F.L.; TAUER, L.W.; SCHUKKEN, Y.H. Effects of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on the probability of conception in New York State Holstein dairy cows. **Journal of Dairy Science**, Champaign, v. 93, n. 4, p. 1551-1560, Apr. 2010.

INOUE, M.; SAITO, A.; KON, H.; UCHIDA,H.; KOYAMA, S.; HARYU,S.; SASAKI, T.; NISHIJIMA, M. Subdural empyema due to *Lactococcus lactis cremoris*: case report. **Neurologia Medico-Chirurgica**,Tokyo, v. 54, n. 4, p.341-347, Nov. 2014.

JØRGENSEN, H.J.; NORDSTOGA, A.B.; SVILAND,S.; ZADOKS, R.N.; SØLVERØD, L.; KVITLE, B.; MØRK, T. *Streptococcus agalactiae* in the environment of bovine dairy herds: rewriting the textbooks? **Veterinary Microbiology**, Amsterdam, v. 184, p. 64-72, Feb. 2016.

KAWAMURA, Y.; FUJIWARA, N.; NAKA, T.; MITANI, A.; KUBOTA, H.; TOMIDA, J.; MORITA, Y.; HITOMI, J. Genus *Enhydrobacter* Staley et al. 1987 should be recognized as a member of the family *Rhodospirillaceae* within the class *Alphaproteobacteria*. **Microbiology and Immunology**, Tokyo, v. 56, n. 1, p. 21-26, Jan. 2012.

KHOO, L.H.; AUSTIN,F.W.; QUINIOU, S.M.; GAUNT, P.S.; RIECKE,D.K.; JACOBS, A.M.; MEALS, K.O.; DUNN,A.W.; GRIFFIN, M.J. Lactococcosis in silver carp. **Journal of Aquatic Animal Health**, Bethesda, v. 26, n. 1, p.1-8, Mar. 2014.

KLOSTERMANN, K.; CRISPIE,F.; FLYNN, J.; ROSS, R.P.; HILL,C.; MEANEY, W. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. **Journal of Dairy Research**, Cambridge, v. 75, n. 3, p. 365-373, Aug. 2008.

KUANG, Y.; TANI, K.; SYNNOTT, A.J.; OHSHIMA, K.; HIGUCHI, H.; NAGAHATA, H.; TANJI, Y. Characterization of bacterial population of raw milk from bovine mastitis by culture-independent PCR–DGGE method. **Biochemical Engineering Journal**, Amsterdam, v. 45, n. 1, p. 76-81, June 2009.

KÄMPFER, P. *Acinetobacter*. In: BATT, C.A.; TORTORELLO, M.L. **Encyclopedia of food microbiology**. Oxford: Academic Press, 2014. p. 11-17.

LARKIN, M.A.; BLACKSHIELDS,G.; BROWN, N.P.; CHENNA,R.; MCGETTIGAN, P.A.; MCWILLIAM,H.; VALENTIN,F.; WALLACE, I.M.; WILM, A.; LOPEZ, R. THOMPSON, J.D.; GIBSON, T.J.; HIGGINS, D.G. Clustal W and Clustal X version 2.0. **Bioinformatics**, Oxford, v. 23, n. 21, p. 2947-2948, Nov. 2007.

LIMA, F.S.; OIKONOMOU, G.; LIMA, S.F.; BICALHO, M.L.; GANDA, E.K.; FILHO, J.C.; LORENZO, G.; TROJACANEC, P.; BICALHO, R.C. Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows. **Applied and Environmental Microbiology**, Washington, v. 81, n. 4, p. 1327-1337, Feb. 2015.

MAGIN, I.; CORROLER, E.; REINHARDT, A.; GUEGUEN, M. Genetic diversity among dairy lactococcal strains investigated by polmerase chain reaction with three arbitrary primers. **Journal of Applied Microbiology**, Oxford, v. 86, n. 3, p. 514-520, Mar. 1999.

MEDRANO-GALARZA, C., GIBBONS, J.; WAGNER, S.; DE PASSILLÉ, A.M.; RUSHEN. J. Behavioral changes in dairy cows with mastitis. **Journal of Dairy Science**, Champaign, v. 95, n. 12, p. 6994-7002, Dec. 2012.

MOFREDJ, A.; BAHLOUL, H.; CHANUT, C. *Lactococcus lactis*: an opportunistic bacterium?. **Médecine et Maladies Infectieuses**, Paris, v. 37, n. 4, p. 200-207, Apr. 2007.

MUNOZ, M.A.; WELCOME, F.L.; SCHUKKEN, Y.H.; ZADOCKS, R.N. Molecular epidemiology of two *Klebsiella pneumoniae*mastitis outbreaks on a dairy farm in New York State. **Journal of Clinical Microbiology**, Washington, v. 45, n. 12, p. 3964-3971, Dec. 2007.

MUNSCH-ALATOSSAVA, P.; GAUCHI, J.P.; CHAMLAGAIN, B.; ALATOSSAVA, T. Trends of antibiotic resistance in mesophilic and psychrotrophic bacterial populations during cold storage of raw milk. **ISRN Microbiology**, Cairo, v. 2012, p.1-13, 2012.

NATIONAL RESEARCH COUNCIL. **Nutrient requirements of dairy cattle**. 7thed. Washington: The National Academies Press, 2001. 381 p.

NAVAS, M.E.; HALL, G.; EL BEJJANI, D. A case of endocarditis caused by *Lactococcus garvieae* and suggested methods for identification. **Journal of Clinical Microbiology**, Washington, v. 51, n. 6, p. 1990-1992, 2013.

OIKONOMOU, G.; MACHADO, V.S.; SANTISTEBAN, C.; SCHUKKEN, Y.H.; BICALHO, R.C. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. **PLoS One**, San Francisco, v. 7, n. 10, p.1-14, Oct. 2012.

OIKONOMOU, G.; BICALHO, M.L.; MEIRA, E.; ROSSI, R.E.; FODITSCH, C.; MACHADO, V.S.; TEIXEIRA, A.G.; SANTISTEBAN, C.; SCHUKKEN, Y.H.; BICALHO, R.C. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. **PLoS One**, San Francisco, v. 9, n. 1, p. 1-11, Jan. 2014.

OKI, K.; DUGERSUREN, J.; DEMBEREL, S.; WATANABE,K. Pyrosequencing analysis of the microbial diversity of airag, khoormog and tarag, traditional fermented dairy products of mongolia. **Bioscience of Microbiota, Food and Health**, Tokyo, v. 33, n. 2, p. 53-64, Apr. 2014.

PLUMED-FERRER, C.; UUSIKYLÄ,K.; KORHONEN,J.; von WRIGHT, A. Characterization of *Lactococcus lactis* isolates from bovine mastitis. **Veterinary Microbiology**, Amsterdam, v. 167, n. 3/4, p. 592-599, Dec. 2013.

PLUMED-FERRER, C.; BARBERIO, A.; FRANKLIN-GUILD, R.; WERNER, B.; MCDONOUGH, P.; BENNETT, J.; GIOIA, G.; ROTA, N.; WELCOME, F.; NYDAM, D.V.; MORONI, P. Antimicrobial susceptibilities and random amplified polymorphic DNA-PCR fingerprint characterization of *Lactococcus lactis* ssp. *lactis* and *Lactococcus garvieae* isolated from bovine intramammary infections. **Journal of Dairy Science**, Champaign, v. 98, n. 9, p. 6216-6225, Sept. 2015a.

PLUMED-FERRER, C.; GAZZOLA,S.; FONTANA,C.; BASSI,D.; COCCONCELLI,P.S.; von WRIGHT, A. Genome sequence of *Lactococcus lactis* subsp. *cremoris* Mast36, a strain isolated from bovine mastitis. **Genome Announcements**, Washington, v. 3, n. 3, p.1-2, 2015b.

QUIGLEY, L.; McCARTHY, R.; O'SULLIVAN, O.; BERESFORD, T.P.; FITZGERALD, G.F.; ROSS, R.P.; STANTON, C.; COTTER,P.D. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. **Journal of Dairy Science**, Champaign, v. 96, n. 8, p. 4928-4937, Aug. 2013.

ROYSTER, E.; WAGNER, S. Treatment of mastitis in cattle. **Veterinary Clinics North America: Food Animal Practice**, Philadelphia, v. 31, n. 1, p. 17-46, Mar. 2015.

SAITOU, N.; NEI, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. **Molecular Biologyand Evolution**, Chicago, v. 4, n. 4, p. 406-425, July 1987.

SAYERS, E.W.; BARRETT,T.; BENSON, D.A.; BRYANT,S.H.; CANESE,K.; CHETVERNIN,V.; CHURCH,D.M.; DICUCCIO, M.; EDGAR,R.; FEDERHEN,S.; FEOLO,M.; GEER, L.Y.; HELMBERG, W.; KAPUSTIN, Y.; LANDSMAN, D.; LIPMAN, D.J.; MADDEN, T.L.; MAGLOTT, D.R.; MILLER, V.; MIZRACHI,I.; OSTELL,J.; PRUITT, K.D.; SCHULER, G.D.; SEQUEIRA, E.; SHERRY, S.T.; SHUMWAY,M.; SIROTKIN, K.; SOUVOROV, A.; STARCHENKO,G.; TATUSOVA, T.A.; WAGNER,L.; YASCHENKO,E.; YE, J. Database resources of the National Center for Biotechnology Information. **Nucleic Acids Research**, London, v. 37, D5-D15, Jan. 2009.

SMITH, K.L.;TODHUNTER,D.A.; SCHOENBERGER, P.S. Environmental mastitis: cause, prevalence, prevention. **Journal of Dairy Science**, Champaign, v. 68, n. 6, p. 1531-1553, June 1985.

TAILLIEZ, P.; TREMBLAY, J.; EHRLICH,S.D.; CHOPIN, A. Molecular diversity and relationship within *Lactococcus lactis*, as revealed by randomly amplified polymorphic DNA (RAPD). **Systematic and Applied Microbiology**, Jena, v. 21, n. 4, p. 530-538, Dec. 1998.

TAMURA, K.; NEI, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. **Molecular Biologyand Evolution**, Chicago, v. 10, n. 3, p. 512-526, May 1993.

TOUCHON, M.; CURY,J.; YOON, E.J.; KRIZOVA, L.; CERQUEIRA,G.C.; MURPHY,C.; FELDGARDEN,M.; WORTMAN,J.; CLERMONT,D.; LAMBERT, T.; GRILLOT-COURVALIN,C.; NEMEC,A.; COURVALIN, P.; ROCHA, E.P. The genomic diversification of the whole *Acinetobacter* genus: origins, mechanisms, and consequences. **Genome Biology and Evolution**, Oxford, v. 6, p. 2866-2882,Oct. 2014.

WEISBURG, W.G.; BARNS,S.M.; PELLETIER, D.A.; LANE, D.J.16S ribosomal DNA amplification for phylogenetic study. **Journal of Bacteriology**, Washington, v. 173, n. 2, p. 697-703, Jan. 1991.

WERNER, B., MORONI,P.; GIOIA, G.; LAVÍN-ALCONERO, L.; YOUSAF, A.; CHARTER, M.E.; CARTER, B.M.; BENNETT, J.; NYDAM, D.V.; WELCOME, F.; SCHUKKEN,Y.H. Short communication: genotypic and phenotypic identification of

environmental streptococci and association of *Lactococcus lactis* ssp. *lactis* with intramammary infections among different dairy farms. **Journal of Dairy Science**, Champaign, v. 97, n. 11, p. 6964-6969, Nov. 2014.

WOOD, J.; SCOTT, K.P.; AVGUSTIN, G.; NEWBOLD, C.J.; FLINT, H.J. Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. **Applied and Environmental Microbiology**, Washington, v. 64, n. 10, p. 3683-3689, Oct. 1998.

WYDER, A.B.; BOSS, R.; NASKOVA, J.; KAUFMANN,T.; STEINER, A.; GRABER, H.U. *Streptococcus* spp. and related bacteria: their identification and their pathogenic potential for chronic mastitis; a molecular approach. **Research in Veterinary Science**, London, v. 91, n. 3, p. 349-357, Dec. 2011.

ZADOKS, R.N.;GILLESPIE, B.E.; BARKEMA, H.W.; SAMPIMON, O.C.; OLIVER, S.P.; SCHUKKEN, Y.H. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. **Epidemiology and Infection**, Cambrigde, v. 130, n. 2, p. 335-349, Apr. 2003.

ZDANOWICZ, M.; SHELFORD,J.A.; TUCKER, C.B.; WEARY, D.M.;von KEYSERLINGK, M.A. Bacterial populations on teat ends of dairy cows housed in free stalls and bedded with either sand or sawdust. **Journal of Dairy Science**, Champaign, v. 87, p. 1694-1701, June 2004.

4 THE MICROBIOME OF BULK TANK MILK: characterization and associations with somatic cell count and bacterial count

Abstract

Numerous studies have evaluated associations between bacterial groups or specific bacteria and milk quality parameters. However, to our knowledge, there is no published research that has analyzed associations between the microbiome and quality parameters of bulk tank milk (BTM). Thus, the aims of this study were to identify the core microbiome of BTM, and to examine associations between the microbiome and milk quality parameters. Four-hundred and seventy-two BTM samples from 19 different dairy farms located in New York State were analyzed by next-generation sequencing and quantitative polymerase chain reaction (qPCR) of the 16S rRNA gene to assess the milk microbiome and measure total bacterial load, respectively. Additionally, flow-cytometry was used to determine bacterial and somatic cell counts. Heatmaps were constructed and simple linear regressions and response screening analysis were performed. To facilitate data analysis and interpretation of the results, we dichotomized the BTM samples into high somatic cell count (SCC) (HSCC>200,000) and low SCC (LSCC≤200,000) and into high log10-standard plate count (SPC) (HSPC>3.6) and low log10-SPC (LSPC≤ 3.6). Spoilage-causing, spore-forming, and pathogenic bacteria of importance to the dairy industry were identified in the core microbiome. In addition, the genera *Thermoanaerobacterium* and *5-7N15* were identified in the core microbiome; to our knowledge, these genera have not been previously identified in milk samples. Several bacterial genera were encountered in significantly higher relative abundances in the HSCC group when compared to the LSCC group, including *Corynebacterium, Streptococcus, Lactobacillus, Coxiella, Arthrobacter,* and *Lactococcus.* Additionally, several bacterial taxa were found in significantly higher relative abundances in the HSPC groups versus the LSPC groups: *Acinetobacter,* Enterobacteriaceae, *Corynebacterium,* and *Streptococcus*. In addition, *Streptococcus* was highly correlated to HSPC, and this genus was the second most abundant bacterial taxon detected in samples classified as HSCC. Bacterial diversity (Shannon index) was negatively correlated with bacterial load, suggesting that the microbiomes of high bacterial load BTM samples are dominated by smaller groups of bacterial taxa. In conclusion, the associations described corroborated current knowledge about pathogens and spoilage bacteria in relationship to milk quality, and also indicated that other bacterial taxa should be a focus of further investigations.

Keywords: Milk; Microbiome; Quality; Next-generation sequencing; *Streptococcus*

4.1 Introduction

Ensuring the safety and quality of raw milk is a challenge worldwide (GSCHWENDTNER et al., 2016). Nonetheless, raw milk quality, the health status of cows, particularly mammary gland health, and the hygiene of milking procedures can be assessed by bulk tank milk (BTM) analysis (GILLESPIE et al., 2012). Since the 1990s, BTM has been used to diagnose current and potential problems in dairy herds

related to milk quality and mastitis (JAYARAO et al., 2004). In addition, the food industry and cooperatives have been using BTM analysis to identify higher quality milk for which they pay premium prices (JAYARAOet al., 2004; BARBANO; MA; SANTOS, 2006) based on one or more parameters (GILLESPIE et al., 2012). Nightingale et al. (2008) evaluated the impact of a premium program for high-quality milk in a United States cooperative focused on BTM somatic cell count (SCC). The findings showed that farmers responded to high premiums, and the impact on milk quality was significant; the authors also noted that penalties provide a good incentive to reach the quality expected (NIGHTINGALE et al., 2008).

A widely used criterion for milk quality premium payments is SCC (BARBANO; MA; SANTOS, 2006). Somatic cells are naturally present in milk (LI et al., 2014); commonly, SCC has been used to indicate the prevalence of intramammary infection in dairy herds (GILLESPIE et al., 2012) and overall milk quality (LI et al., 2014). Somatic cells are a source of protein, e.g. endogenous enzymes, which are exposed after lysis of these cells(LI et al., 2014). Such enzymes promote production of off-flavors due to the degradation of milk protein and fat during storage, and the heat-resistant enzyme load in milk is determined by somatic cell and microbial counts (BARBANO; MA; SANTOS, 2006). Associations among milk components, presence of bacteria, and milk quality parameters have been evaluated elsewhere (PARK et al., 2007; KATHOLM et al., 2012).

The quality and safety of dairy products are influenced by the bacterial community present in BTM (LI et al., 2014). Microbial contamination of BTM can occur through several sources and by distinct microorganisms (ELMOSLEMANY et al., 2009). Furthermore, high levels of psychrotrophic bacteria contribute to the presence of increased proteases and lipases after pasteurization (BARBANO; MA; SANTOS, 2006). A high standard plate count (SPC) in raw milk indicates mastitis occurrence, and problems in milking and/or general hygiene (GILLESPIE et al., 2012). Moreover, raw milk is a source of endospores produced by mesophilic, thermophilic, and psychrotolerant spore-forming bacteria (MILLER et al., 2015). Spore-forming bacteria are a concern (BARBANO; MA; SANTOS, 2006), and standard laboratory analysis (based on traditional culturing) following pasteurization is a good technique to indicate the level of spores (BARBANO; MA; SANTOS, 2006). The majority of microbiological analyses of raw milk microbiota have been based on culture-dependent methods (FRICKER et al., 2011). However, culture-dependent

methods have several limitations when compared to culture-independent methods, e.g. viable but non-culturable cells cannot be identified by culturing (WEBER et al., 2014). Consequently, the use of different culture-independent methods to describe the bacterial composition has emerged. The composition of the bacterial community of raw milk has been described using different culture-independent methods (KUANG et al., 2009; OIKONOMOU et al., 2014; WEBER et al., 2014); however, it is believed that a knowledge gap still exists in our understanding of natural bacterial communities in raw milk (FRICKER et al., 2011). Moreover, to our knowledge, there is no published research that has analyzed associations between the microbiome and quality parameters of BTM. Therefore, the aims of this study were to identify the core microbiome of BTM and to examine associations between the microbiome and milk quality parameters.

4.2 Material and Methods

4.2.1 Bulk tank milk samples collection

Dairy farms associated with Cayuga Milk Ingredients (Finger Lakes region, New York), which regularly send BTM samples to Dairy One Co-op, Inc. (Ithaca, NY) for milk analyses, were invited to participate in this study. Letters of consent were sent to the dairy farmers requesting permission to collect BTM samples to perform milk microbiome analyses, and to use data from milk analyses carried out by Dairy One. From 29 members contacted, 19 dairy farms agreed to participate in the research. Therefore, dairy farms within New York State were included in this study, from which 472 BTM samples were obtained during the study period, September to October 2015. Trained employees collected approximately 10 mL of milk samples from bulk tanks aseptically into vials and these were stored at 4^{o}C during transport to Dairy One Milk Laboratory. The samples submitted to Dairy One were subjected to milk quality analysis, and aliquots of those samples were sent to our laboratory at Cornell University (Ithaca, NY). The samples were received within 48 hours after collection, and farm, tank, and date were recorded on a spreadsheet. Samples were stored at -20ºC for downstream molecular analysis.

4.2.2 Somatic cell count and total bacteria count

SCC and SPC were carried out at Dairy One Milk Laboratory. SCC was determined by flow-cytometry using a Fossomatic™ FC Somatic Cell Counter (Foss, Hillerod, Denmark). Briefly, a mixture of each sample and staining solution was prepared, and passed through a flow cell in which somatic cells emit fluorescent light pulses, and the fluorescent light pulses were counted (cells/mL). SPC was determined using a BactoScan™ FC+ instrument (Foss, Hillerod, Denmark), which is also based on flow-cytometry (International Dairy Federation, IDF Standard 100B: 1991, the reference or anchor method applied in this technology). The results for individual bacterial count (IBC) were converted to CFU by BactoScan™ FC+ software (Foss, Hillerod, Denmark).

4.2.3 Next-generation sequencing of the bacterial 16S rRNA gene

DNA was extracted from all samples using a PowerFood® Microbial DNA Isolation Kit (MO BIO Laboratory Inc., Carlsbad, CA) following the manufacturer's protocol. The V4 hypervariable region of the bacterial/archaeal 16S rRNA gene was amplified by PCR according to a previously described protocol and optimized for the Illumina MiSeq platform (CAPORASO et al., 2012) using different 12-bp error-correcting Golay barcodes for the 16S rRNA gene PCR (LIMA et al., 2015). The PCRs were performed using 10 µM of each primer (515F and 806R), EconoTaq Plus Green 1x Master Mix (Lucigen®, Middleton, WI), 5 ng to 50 ng of individual metagenomic DNA samples, and ultrapure water to bring the final reaction volume to 25 µL. Blank controls in which no DNA was added to the reaction were also performed. All reactions were set up in triplicate, and the PCR conditions for amplification included an initial denaturing step of 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 1 min and 72°C for 90 s, and a final elongation step of 72°C for 10 min. Replicates were pooled and the amplified DNA visualized by electrophoresis using 1.2% (wt/vol) agarose gels stained with 0.5 mg/ml ethidium bromide. The DNA was purified using a Gel/PCR Fragments Extraction Kit (IBI Scientific, Peosta, IA). Quantification of purified DNA was carried out using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). All samples were standardized to the same concentration and pooled for sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) in two different runs according to individual barcode primers. Final equimolar libraries were prepared and

sequenced using the MiSeq Reagent Kit V2-300 cycles (Illumina, Inc., San Diego, CA).

4.2.4 Quantitative polymerase chain reaction

For assessment of the total bacterial load by quantitative polymerase chain reaction (qPCR), a sub-sample of 120 samples was randomly selected. For determination of bacterial load, a plasmid containing the amplified V6 hypervariable region was cloned into TOP10 cells using a Zero Blunt® TOPO® PCR cloning kit (Life Technologies, Darmstadt, Germany). Plasmid was purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and quantified using Quant-iT™ PicoGreen® and a dsDNA Broad Range Assay Kit (Life Technologies Corporation, Carlsbad, CA). Insertion of the DNA fragment was confirmed by agarose gel electrophoresis, and by sequencing at the Cornell University Life Science Core Laboratories Center. The 16S rRNA copy numbers were measured by qPCR using forward 5' TGG AGC ATG TGG TTT AAT TCG A 3' and reverse 5' TGC GGG ACT TAA CCC AAC A 3' primers previously described (NONNENMACHER et al., 2004). Reactions were performed in 15 µL volumes composed of 1X iQ^{TM}Sybr Green Supermix (BIO-RAD Laboratories, Hercules, CA), 300 nM of each primer and 5 pg to 50 ng of genomic DNA (or plasmid DNA standards). The thermal cycler conditions were as follows: denaturation at 95°C for 3 min, 40 amplification cycles (95°C for 10 s, 55°C for 30 s) and two final steps at 95°C for 1 min and 55°C for 1 min followed by melting curve analysis. All reactions were performed in duplicate (plasmid standards, BTM samples, and blank control) using a MyiQ^{TM} Real-Time PCR Detection System (BIO-RAD Laboratories, Hercules, CA). Quantification of 16S rRNA target DNA was achieved by 10-fold serial dilutions ranging from 10^0 to 10^7 plasmid copies of the previously quantified plasmid standard. The average of the cycle threshold value was used for calculation of the bacterial load.

4.2.5 Bioinformatics and statistical analysis

The 16S rRNA sequences obtained were processed through the open-source pipeline Quantitative Insights into Microbial Ecology (QUIIME) version 1.7.0-dev (CAPORASO et al., 2010). Sequences were filtered for quality using established guidelines (BOKULICH et al., 2013), and were binned into operational taxonomic units (OTUs) based on 97% identity using UCLUST (EDGAR, 2010) against the

Greengenes reference database (McDONALD et al., 2012) (May 2013 release). Chimeric sequences were removed, and low-abundance clusters were filtered using USEARCH (EDGAR, 2010). The representative sequences for each OTU were compared against the Greengenes database for taxonomy assignment, and only full-length, high-quality reads (-r = 0) were used for analysis. Shannon diversity index output was generated by the QIIME pipeline. Before estimating the Shannon index, all sample libraries were rarefied to an equal depth of 10,000 sequences using QIMME.

The OUT data obtained from bioinformatics analyses were used to describe the most abundant phyla of BTM samples in terms of relative abundance (%), and the data also were used to generate heatmaps to describe the BTM core microbiome, and the 30 most abundant bacterial taxa. Analysis of associations between bacterial taxon and milk quality parameters were first performed using the heatmaps according to quartiles of SCC (Quartile 1: 54,000 – 120,000 cells/mL; Quartile 2: 130,000 – 150,000cells/mL; Quartile 3: 160,000 – 170,000 cells/mL; Quartile 4: 180,000 – 450,000cells/mL), and log10 of the standard plate count (log10-SPC) (Quartile 1: 3.0 log CFU/mL; Quartile 2: 3.30 log CFU/mL; Quartile 3: 3.47 – 3.60 log CFU/mL; Quartile 4: 3.69 – 5.07 log CFU/mL). The heatmaps were plotted using Cell Plot in JMP Pro 11 (SAS Institute Inc., Cary, NC). Using JMP Pro 11, mean, standard deviation, and range of SCC and of log10-SPC were calculated per dairy farm. Response screening analysis was performed in JMP Pro 11 to determine which bacterial taxa were most associated to high SCC (HSCC>200,000 cells/mL), and low SCC (LSCC≤200,000 cells/mL), and associated to high log10-SPC (HSPC>3.6 log CFU/mL), and low log10-SPC (LSPC≤3.6 log CFU/mL). The 200 most prevalent OTUs were added as outcome variables and the dichotomized SCC variable was added as the dependent variable; the same procedure was performed using log10-SPC. *P*-values were adjusted for false discovery rate (FDR) (BENJAMINI; HOCHBERG, 1995) and presented as FDR LogWorth (i.e. $-\log_{10}$ *P*-value). The mean relative abundance for each bacterial taxon observed in the BTM samples was used as a reference for calculation of fold-changes. The effect size was explored because it shows the magnitude of the difference found. OTUs statistically significant on the response screening analysis (log10 of the FDR≥1.3) are presented; bacterial taxa with higher relative abundance in HSCC BTM samples compared to LSCC samples, and bacterial taxa with higher relative abundance in LSCC BTM samples compared

to HSCC samples, shown for LSCC samples and HSCC samples (mean relative abundance ± SEM); the same procedure was also applied to log10-SPC. Simple linear regressions between log10-SPCand Shannon diversity index were assessed by using simple linear regression in JMP Pro 11 software (*P*-value < 0.001). Simple linear regressions were also fitted in JMP software to assess the association between the total bacterial load (log10 copy numbers of the 16S rRNA gene) and four factors evaluated: Shannon index, log10-SPC, relative abundance of Enterobacteriaceae, and relative abundance of *Acinetobacter* (an initial screening was performed and these two bacterial taxa presented the highest associations with total bacterial load).

4.3 Results

4.3.1 Sequencing results, prevalence of bacterial taxa, and the core microbiome

Quality filtered reads for the 16S rRNA sequences were demultiplexed, yielding 24,509,428raw sequences in total (median= 45,821; range = 6,977 - 158,311) with a median length of 301 bases per read, and an average of 51,965 sequences per sample. The most prevalent bacterial phyla were Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria, Spirochaetes, and Tenericutes; the mean relative abundance of each phylum by farm is depicted in Figure 1.

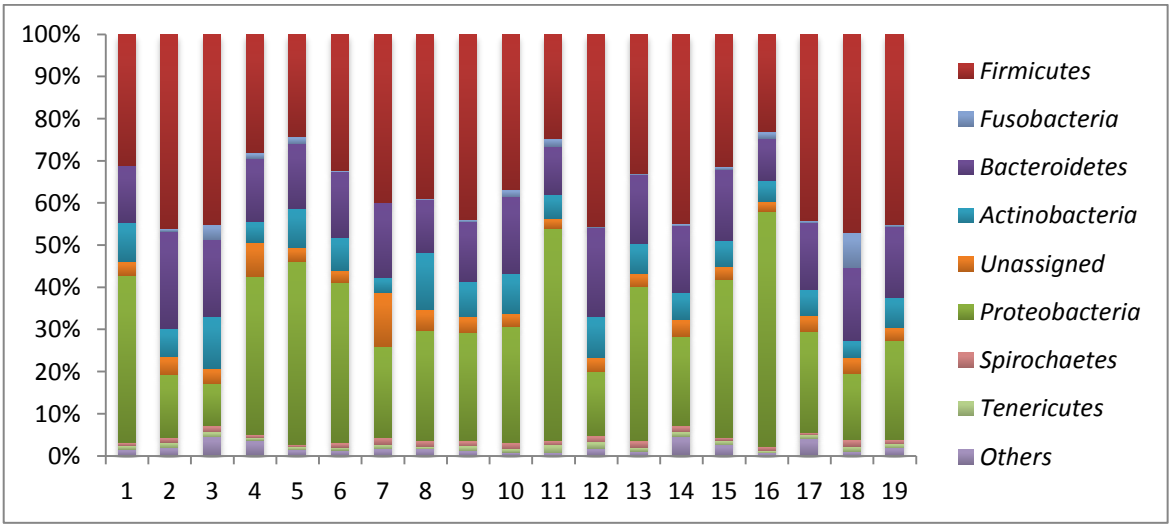


Figure 1 – Mean relative abundance of the most prevalent bacterial phyla identified in milk samples of 19 farms studied

The 30 most common OTUs identified in the BTM samples from each farm are shown in Figure 2.

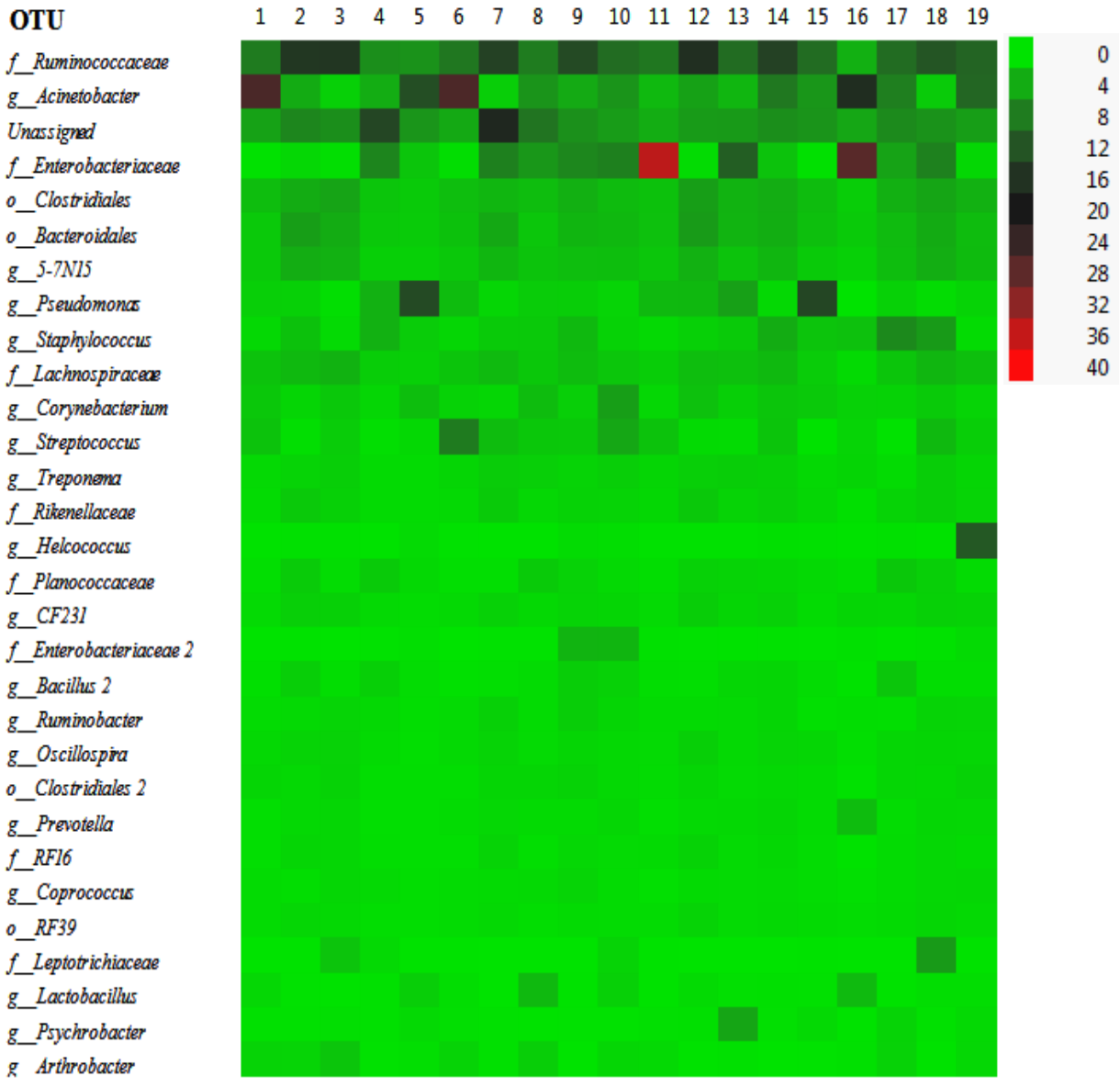


Figure 2 –Heatmap illustrating the relative abundance of the 30 most prevalent bacterial taxa in BTM from the 19 dairy farms enrolled in this study. Each square in the heat map represents the abundance level of a single category. Small relative abundance values are represented by light green, progressing to higher values as black and dark red. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus)

The core microbiome across the 19 farms studied is composed of Ruminococcaceae, *Acinetobacter*, Clostridiales, Bacteroidales, 5-7N15, *Pseudomonas*, *Staphylococcus*, Lachnospiraceae, *Corynebacterium*, Planococcaceae, *Bacillus* 2, and *Thermoanaerobacterium* (Figure 3).

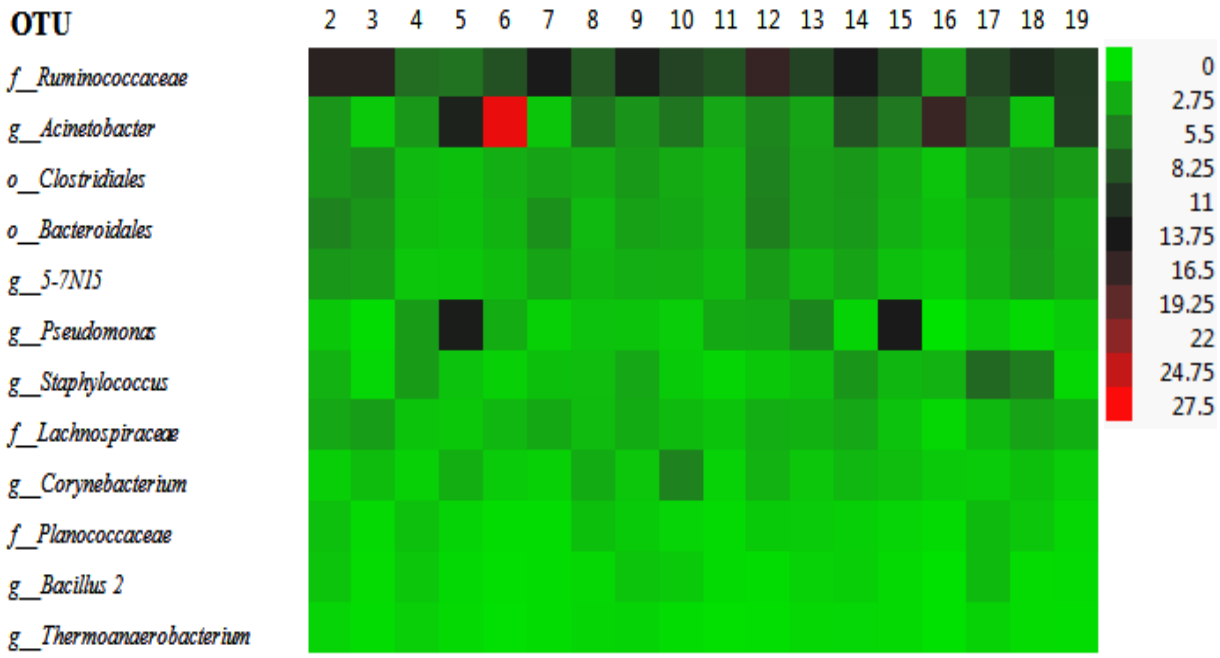
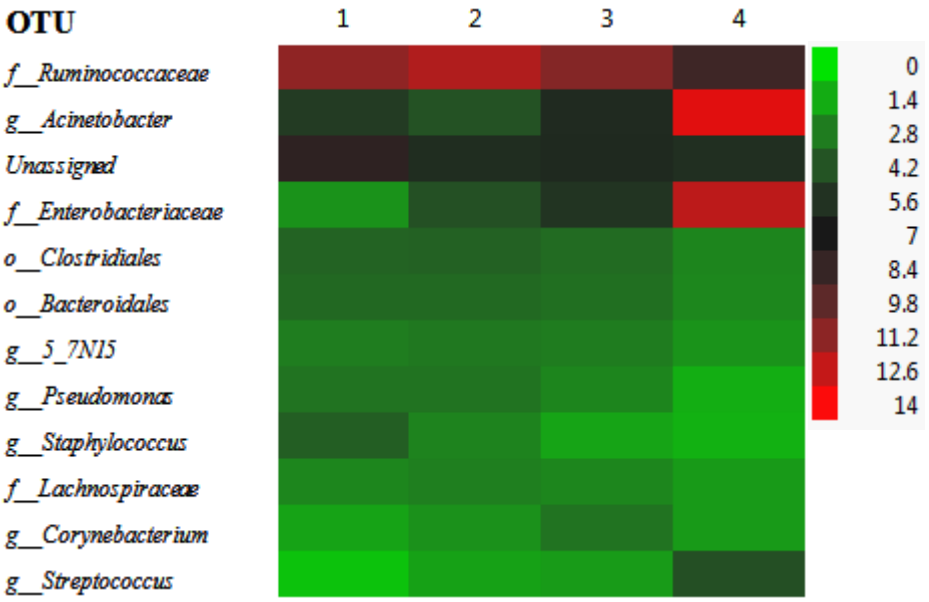


Figure 3 – The BTM core microbiome, defined as the bacterial taxa detected in all bulk tank milk samples across all 19 farms enrolled in this study. Each square in the heat map represents the abundance level of a single category. Small relative abundance values are represented by light green, progressing to higher values as black and dark red. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f= family, and g= genus)

4.3.2 Milk quality parameters, bacterial load by qPCR, and bacterial taxa associations

The mean, standard deviation, and range of SCC, and log10-SPC are shown in Table 1. Associations between SCC quartiles or log10-SPC quartiles and mean relative abundance of the 12 most common OTUs identified in the BTM samples were assessed (Figure 4).

(A)



(B)

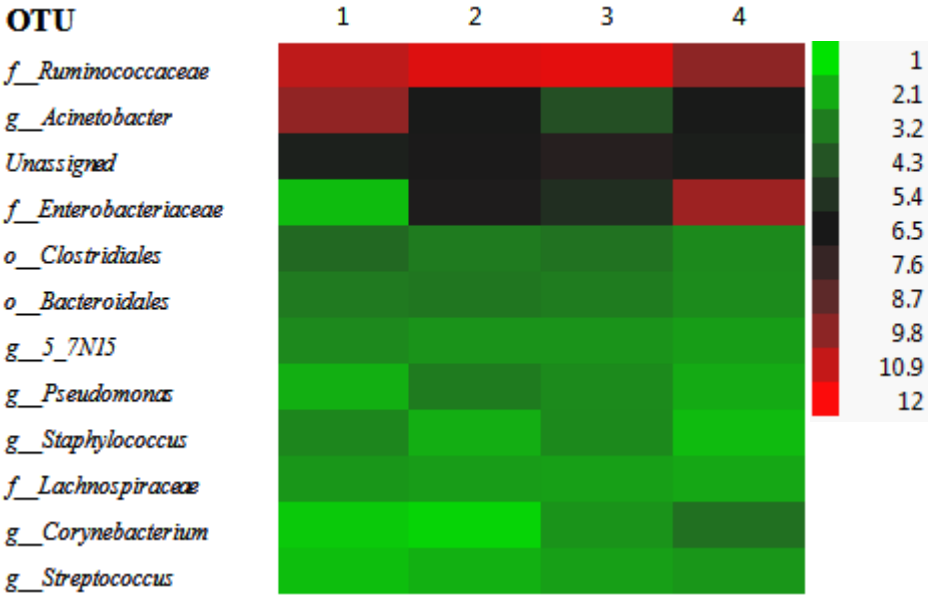


Figure 4 –Heat maps illustrating the mean relative abundance of the 12 most common bacterial taxa relative to log10 of the Standard Plate Count (A) quartiles and Somatic Cell Count (B) quartiles for all milk samples evaluated in this study. Each square in the heat map represents the abundance level of a single category. Small relative abundance values are represented by light green, progressing to higher values as black and dark red. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus)

The relative abundances of *Acinetobacter*, Enterobacteriaceae, and *Streptococcus* were higher in the highest quartile of log10-SPC in comparison to the lower log10-SPC groups (Figure 4A). Regarding SCC, Enterobacteriaceae was identified with a higher relative abundance in the fourth quartile than in the other quartiles, as was *Corynebacterium*, and the mean relative abundance of *Streptococcus* also increased with the increase in SCC (from the first quartile to the fourth quartile).

Screening analysis was performed to identify the main microbial types distinguishing the microbiomes in samples classified as high and low SSC, and log10-SPC. Figure 5 shows the OTUs significantly associated with HSCC and LSCC. In HSCC, *Coxiella*, *Dermacoccus*, and *Lactococcus* had higher effect sizes whereas *Corynebacterium* and *Streptococcus* were the most abundant (Figure 5). The relative abundances among the OTUs that were associated with the lower SCC category were higher than those of the OTUs in samples classified as HSCC. Ruminococcaceae, Clostridiales, and Bacteroidales were the bacterial taxa with the highest abundances in the HSCC category.

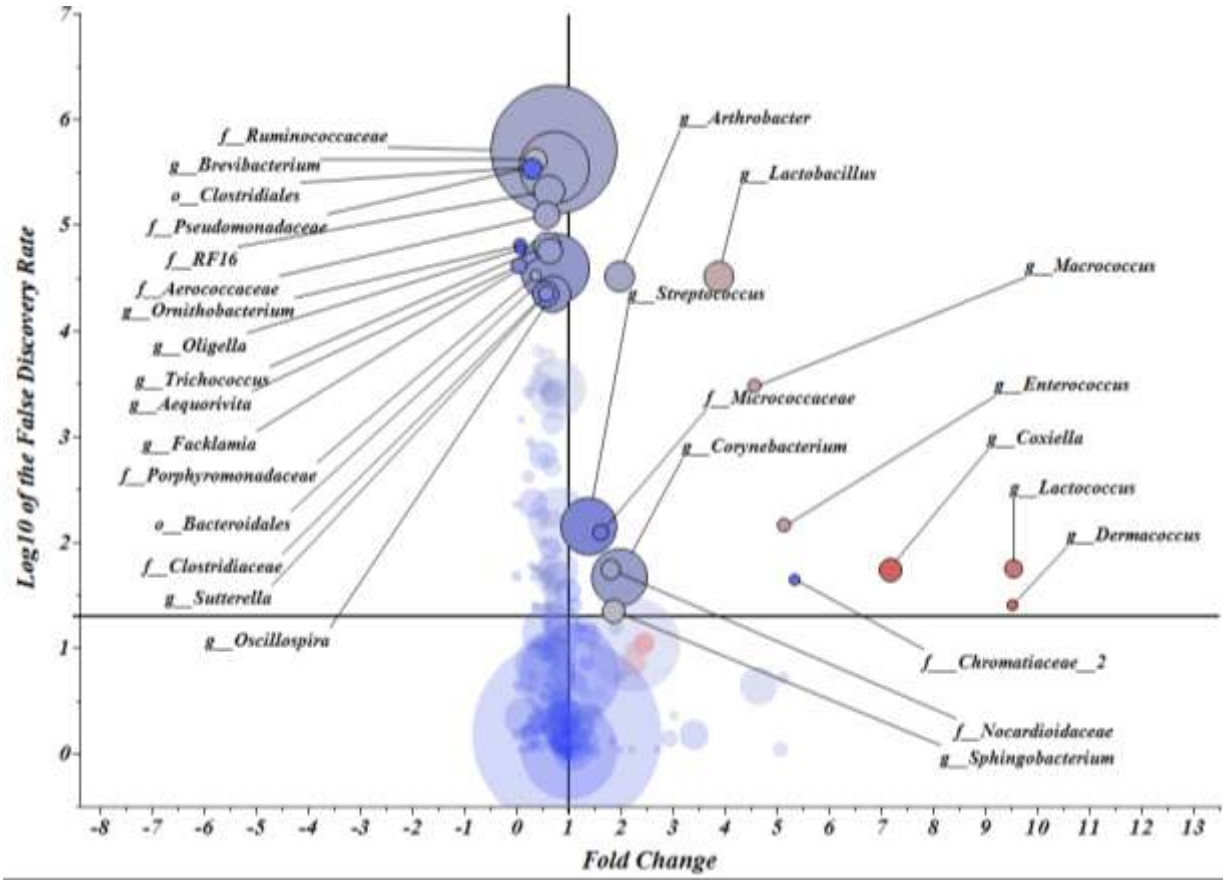


Figure 5 –Bubble graph illustrating the operational taxonomic units (OTUs) that were significantly associated with Somatic Cell Count (SCC). BTM samples were divided into high SCC (HSCC > 200,000) and low SCC (LSCC ≤ 200,000). The graph illustrates the log10 of the false discovery rate along the Y axis, and the fold change of the relative abundance of the each specific OTU comparing high versus low SCC samples along the X axis. The size of the bubbles represents the overall abundance of each OTU and the colors represent the size of the detected effect (blue = small effect and red = large effect). OTUs with X axis values greater than 1 were more abundant in high SCC milk, and OTUs with X axis values below 1 were more abundant in low SCC milk

More detailed analyses were performed using OTUs classified as statistically significant (log10 of the FDR ≥ 1.3) in the response screening analysis for the HSCC (Figure 6) and LSCC categories (Figure 7).

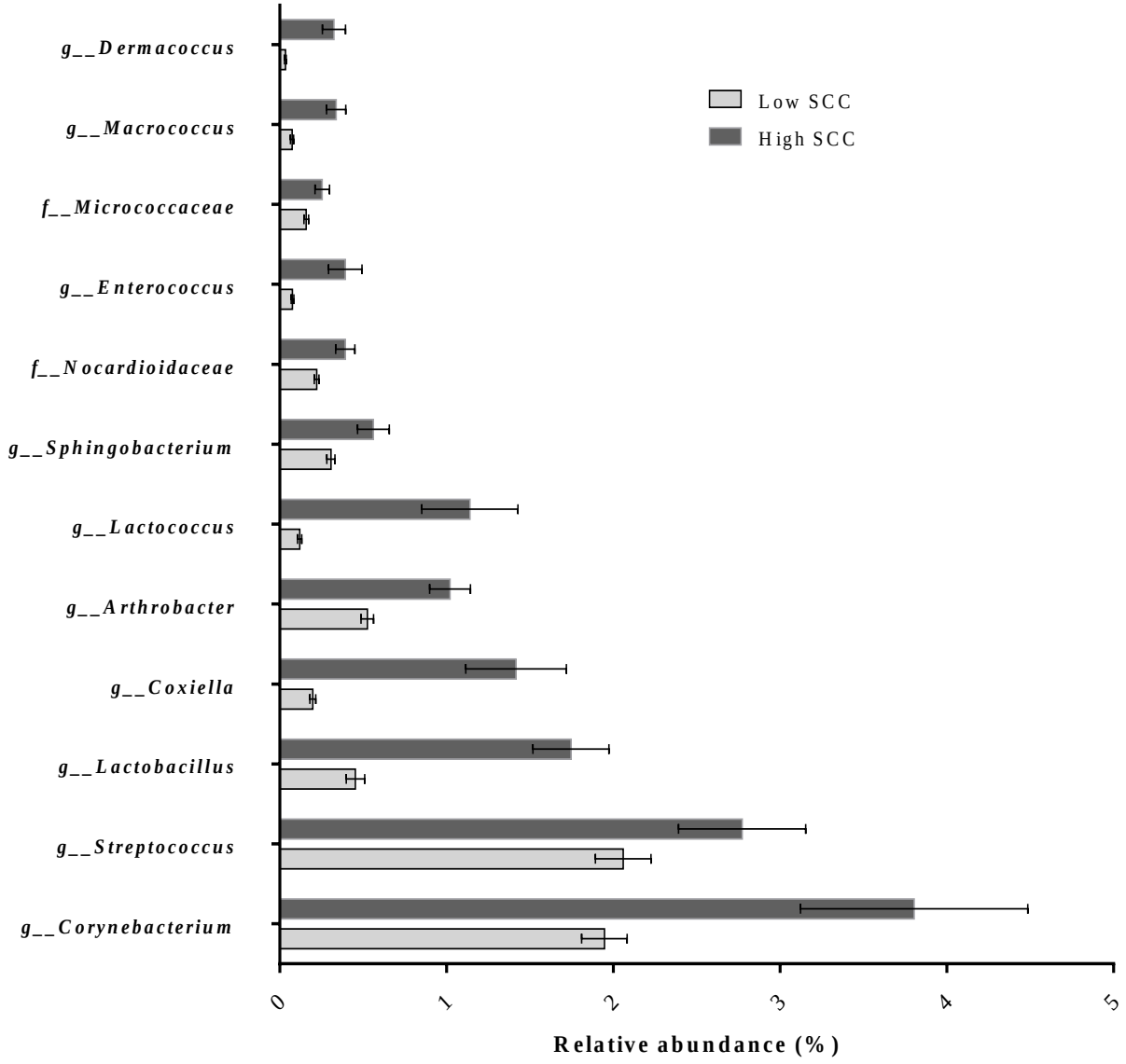


Figure 6 – Bar graph depicting the difference of relative abundances between the BTM samples that were classified as high and low Somatic Cell Count (HSCC > 200,000; LSCC ≤ 200,000). This graph depicts the taxa that were found in significantly higher relative abundance in high SCC samples compared to low SCC samples. The mean relative abundances (±SEM) are shown for each operational taxonomic unit (OTU) for low SCC samples (light gray bars) and high SCC samples (dark gray bars)

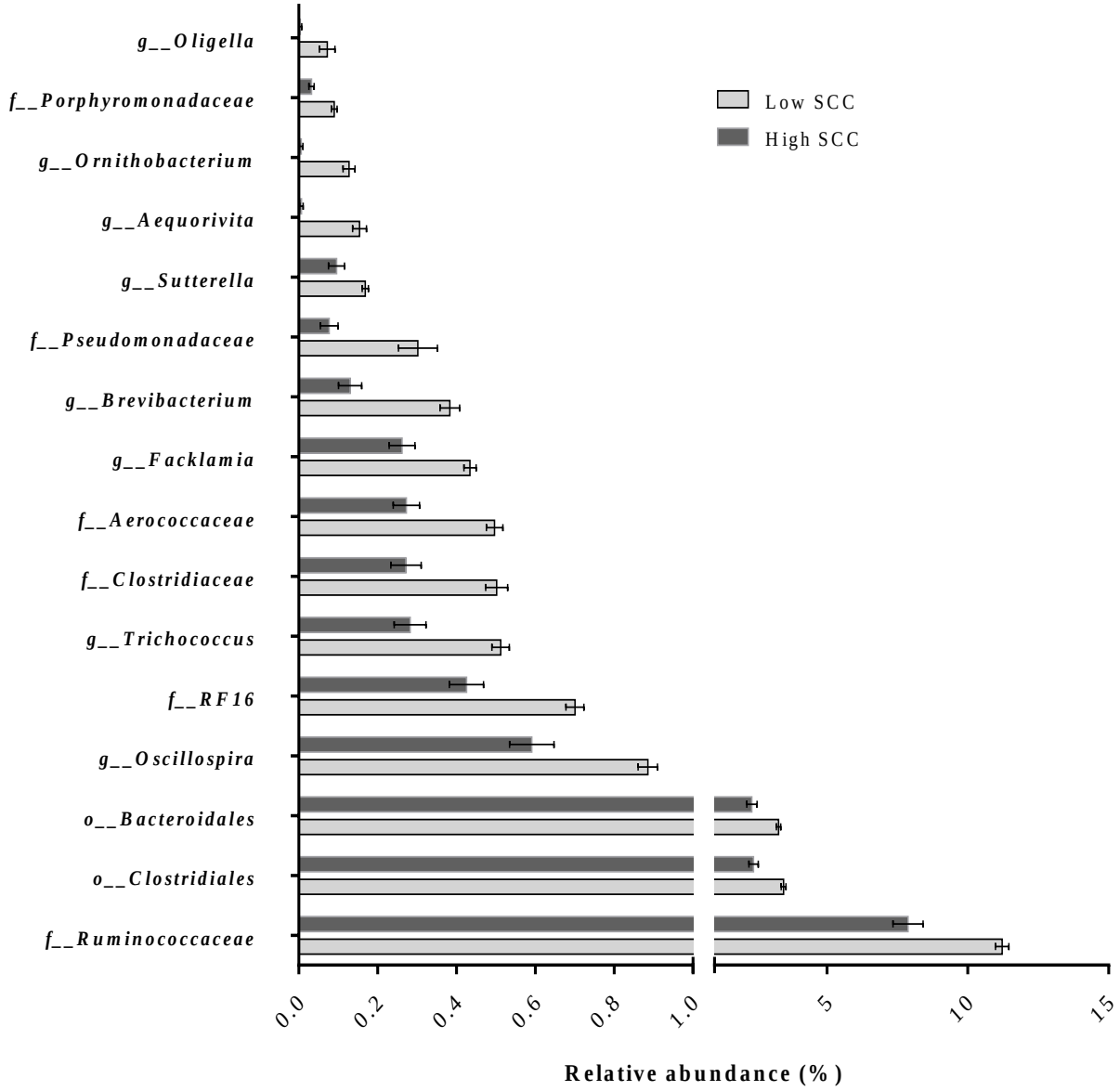


Figure 7 – Bar graph depicting the difference of relative abundances between the BTM samples that were classified as high and low Somatic Cell Count (HSCC > 200,000; LSCC ≤ 200,000). This graph depicts the taxa that were found in significantly higher relative abundance in low SCC samples compared to high SCC samples. The mean relative abundances (±SEM) are shown for each operational taxonomic unit (OTU) for low SCC samples (light gray bars) and high SCC samples (dark gray bars)

The effect of milk microbiome on log10-SPC was also evaluated using response screening analysis (Figure 8). In HSPC, the *Streptococcus* genus had the

highest effect size while the most abundant bacterial taxa were *Acinetobacter* and Enterobacteriaceae (Figure 8). In LSPC, *Thermoanaerobacterium* and *Bacillus* had the highest effect sizes, while Ruminococcaceae, Clostridiales, Bacteroidales, and *Staphylococcus* were the most abundant bacterial taxa.

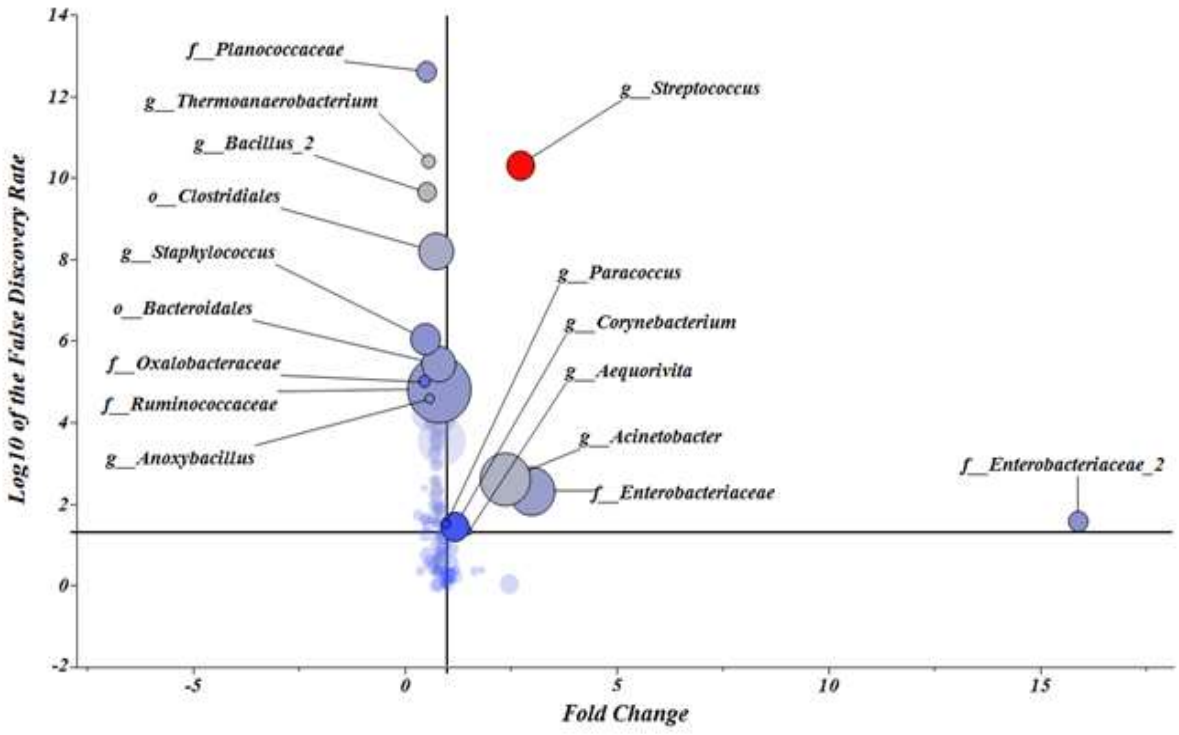


Figure 8 – Bubble graph illustrating the operational taxonomic units (OTUs) that were significantly associated with the log10 of the Standard Plate Count (log10-SPC). BTM samples were divided into high log10-SPC (HSPC > 3.6) and low log10-SPC (LSPC ≤ 3.6). The graph illustrates the log10 of the false discovery rate along the Y axis and the fold change of the relative abundance of each specific OTU in comparisons of high versus low log10-SPC samples along the X axis. The size of the bubbles represents the overall abundance of each OTU and the colors represent the size of the detected effect (blue = small effect and red = large effect). OTUs with X axis values greater than 1 were more abundant in high log10-SPC milk and OTUs with X axis values below 1 were more abundant in low log10-SPC milk

Detailed analysis was performed using bacterial taxa statistically significant in the response screening in the HSPC (Figure 9) and LSPC categories (Figure 10).

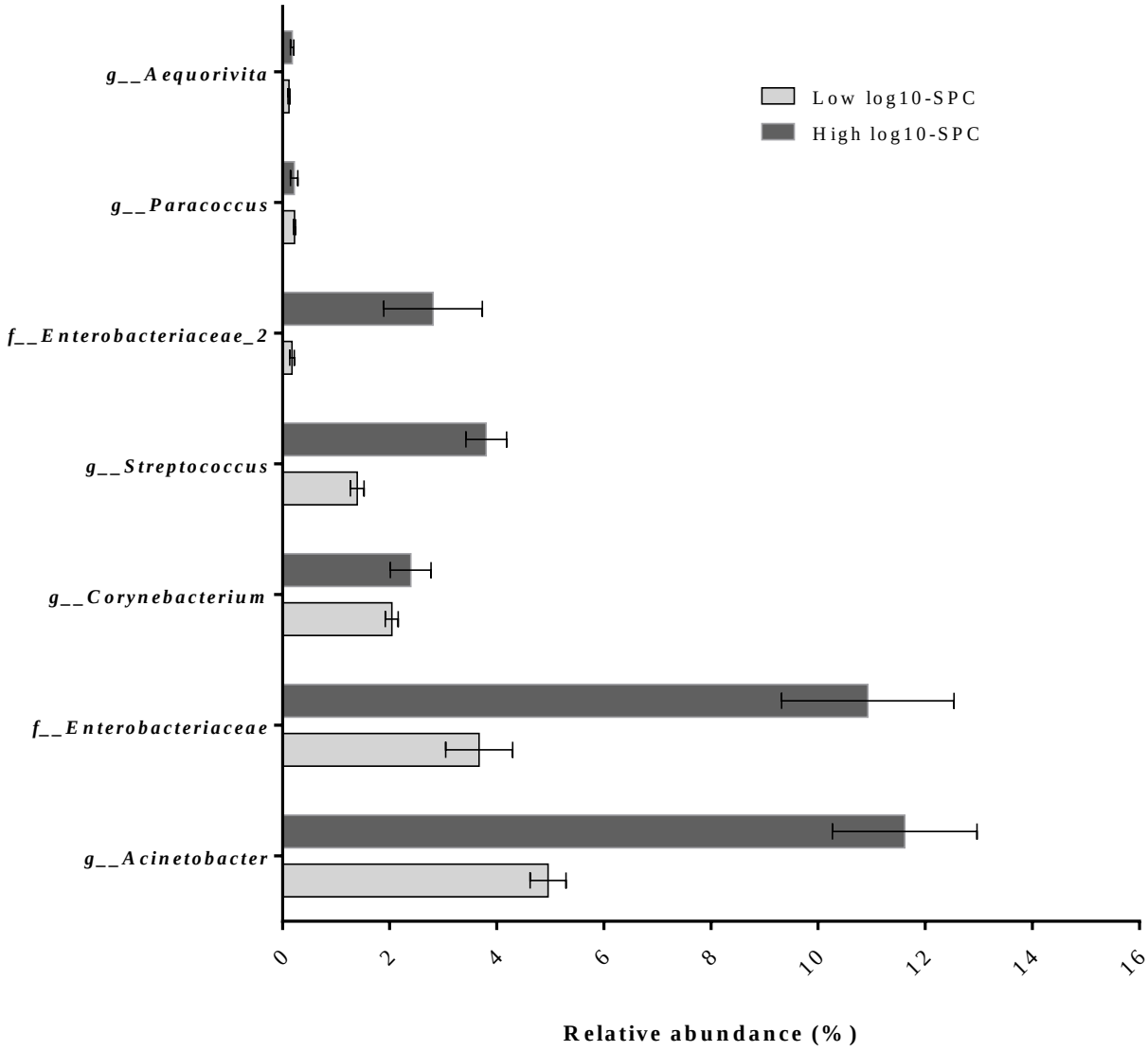


Figure 9 – Bar graph depicting differences of relative abundances between the BTM samples that were classified as high and low log10 of the Standard Plate Count (HSPC > 3.6; LSPC ≤ 3.6). This graph depicts the bacteria that were found in significantly higher relative abundance in high log10-SPC BTM samples when compared to low log10-SPC samples. The mean relative abundances (±SEM) are shown for each operational taxonomic unit (OTU) for low log10-SPC samples (light gray bars) and high log10-SPC samples (dark gray bars)

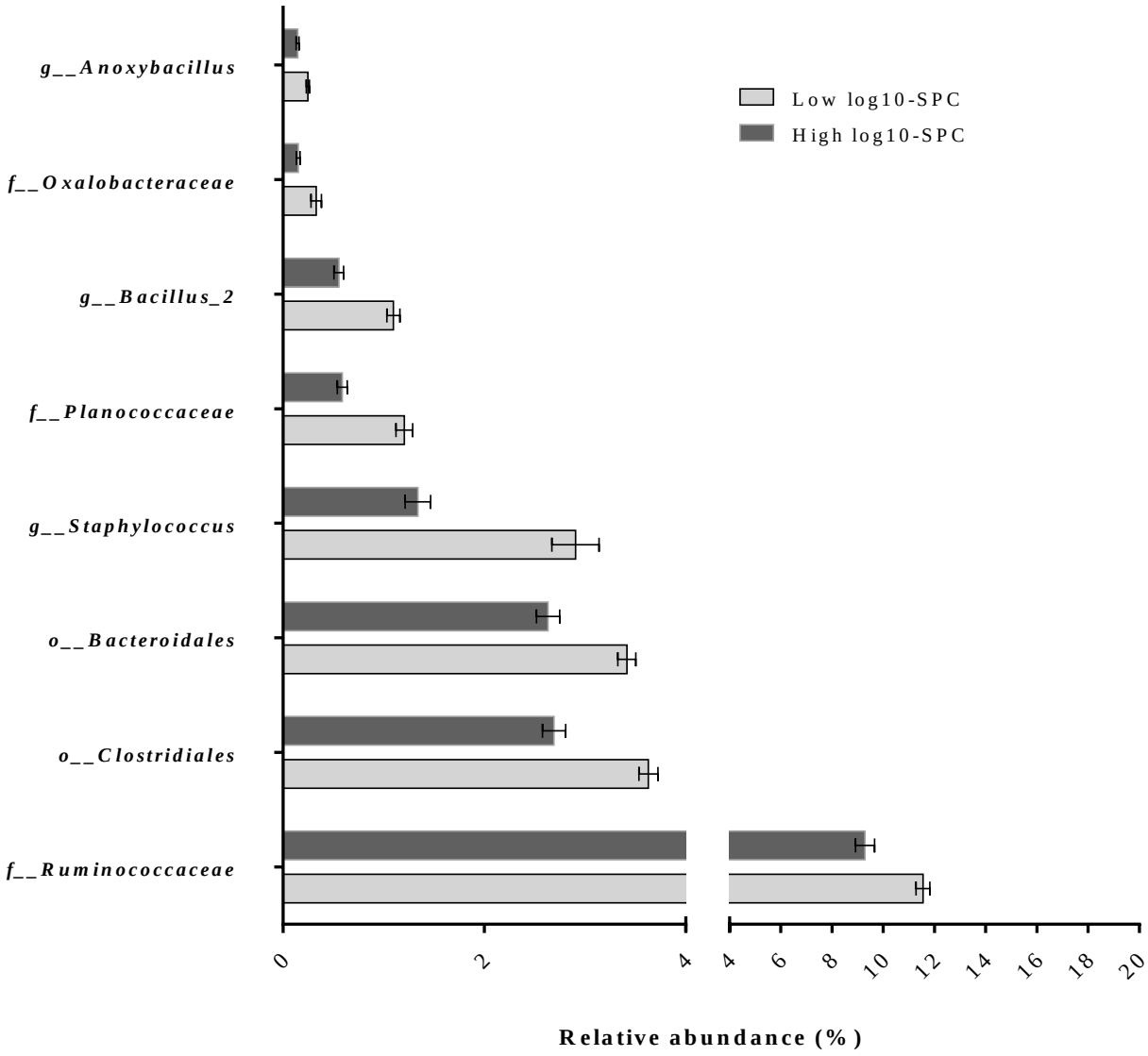


Figure 10 – Bar graph depicting differences of relative abundances between the BTM samples that were classified as high and low log10 of the Standard Plate Count (HSPC > 3.6; LSPC ≤ 3.6). This graph depicts the bacteria that were found in significantly higher relative abundance in low log10-SPC BTM samples when compared to high log10-SPC samples. The mean relative abundances (±SEM) are shown for each operational taxonomic unit (OTU) for low log10-SPC samples (light gray bars) and high log10-SPC samples (dark gray bars)

Log10-SPC and Shannon index were linearly correlated (Figure 11); bacterial taxa diversity decreased with higher values of log10-SPC. Using qPCR, we

demonstrated that the total bacterial load (log10 of the number of 16S rRNA gene copies) was positively and linearly correlated with the relative abundances of *Acinetobacter* and Enterobacteriaceae as well as with log10-SPC (Figure 12A, B, and D). Additionally, a negative correlation was detected between the total bacterial load and the Shannon diversity index (Figure 12C).

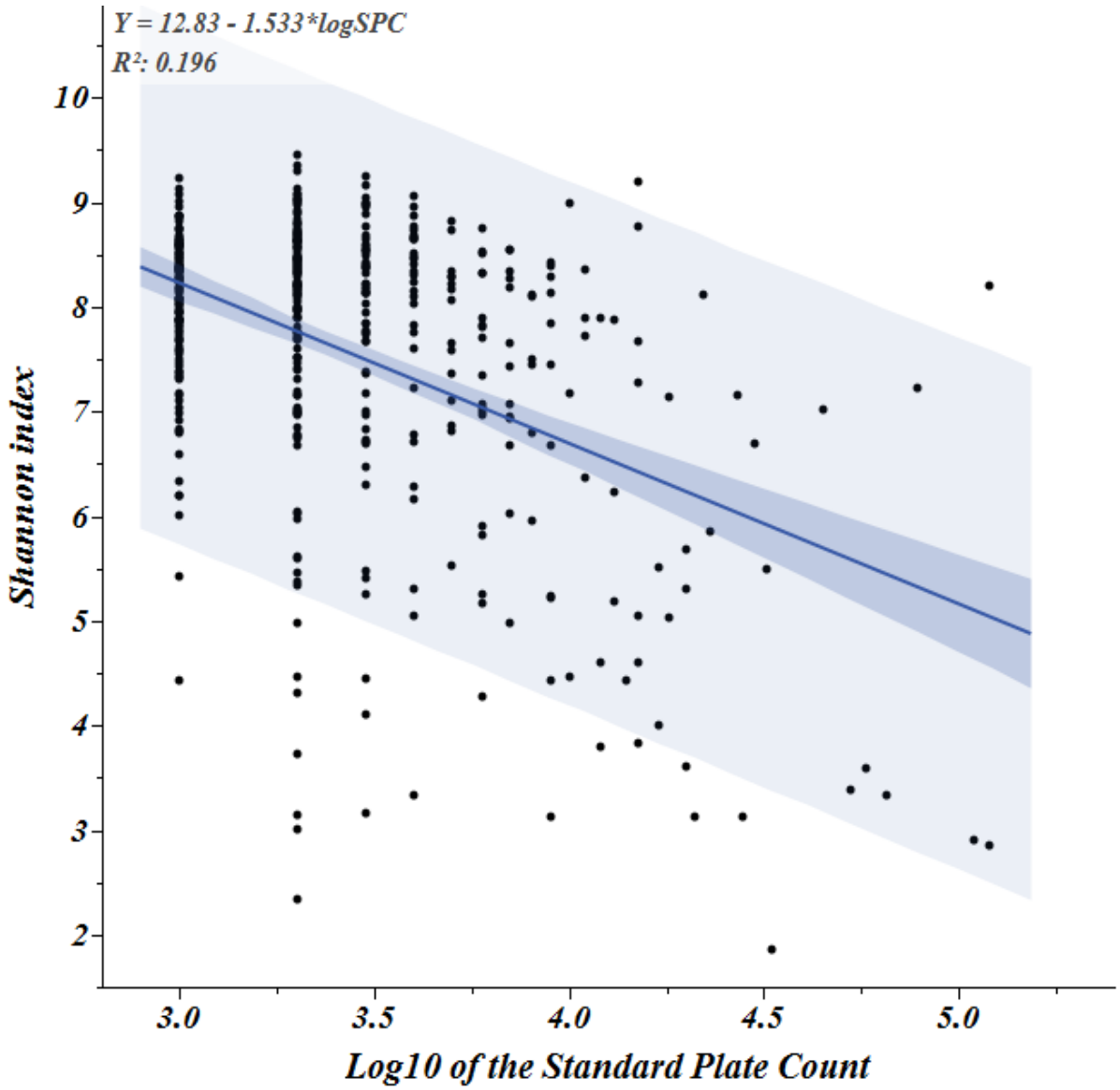


Figure 11 – Simple linear regression illustrating the relationship between the Shannon index and log10 of the Standard Plate Count

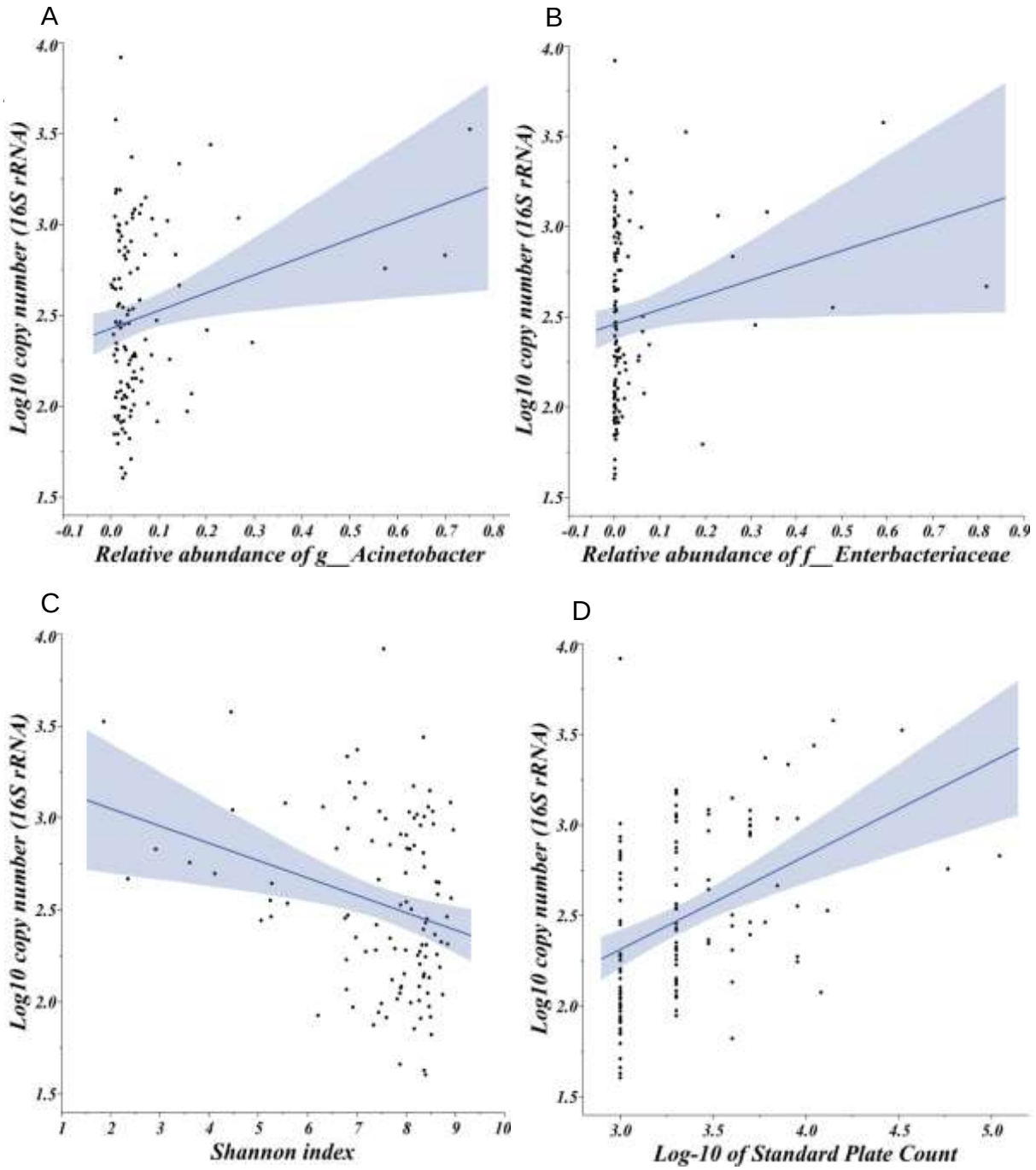


Figure 12 – Simple linear regression illustrating the relationship between the log10 copy number (16S rRNA) and relative abundance of *g_Acinetobacter* (A), relative abundance of *f*_Enterobacteriaceae (B), Shannon index (C), and log10 of the Standard Plate Count (D)

4.4 Discussion

To our knowledge, this is the first report to have described associations between the BTM bacterial community and milk quality parameters (SSC and SPC)

based on high-throughput sequencing of the 16S rRNA gene. Previous studies focused only on identification of specific bacterial species and their relationships to milk quality parameters, or conducted a simple characterization of the raw milk microbiome. In this study, important spoilage (e.g. *Acinetobacter*), spore-forming (e.g. *Bacillus*), and pathogenic (e.g. *Staphylococcus*) bacteria were identified in the core microbiome of BTM, and these are all bacterial groups of known importance in the dairy industry. We also observed that *Streptococcus* was the second most abundant bacterial genus detected in samples classified as high SCC, and it was highly correlated to high log10-SPC (highest size effect), which corroborates the existing knowledge that species of *Streptococcus* are important mastitis pathogens. A negative correlation between the Shannon diversity index and total bacterial load (log10 of the copy number of the 16S rRNA gene) was observed, suggesting that a BTM environment with high bacterial count may be dominated by a smaller group of bacterial taxa.

The phylum-level structure of the BTM microbiome detected in the present study is similar to those of previous studies that described the microbial communities of raw milk (QUIGLEY et al., 2013) and BTM (RAATS et al., 2011). Furthermore, the common genera previously detected in raw milk, such as *Acinetobacter* (GURUNG et al., 2013), *Pseudomonas* (von NEUBECK et al., 2015), *Corynebacterium* (QUIGLEY et al., 2013), *Staphylococcus*, and *Streptococcus* (PARK et al., 2007; ZANARDI et al., 2014), were also identified in our samples. Additionally, the bacterial taxa that presented the highest abundance in BTM samples were Ruminococcaceae, *Acinetobacter* and Enterobacteriaceae. Our results are in line with studies that previously reported Ruminococcaceae in the milk microbiome (OIKONOMOU et al., 2014; YOUNG et al., 2015). Members of this family have been widely identified in samples across the different anatomical parts of the gastrointestinal tract of dairy cattle (LIMA et al., 2015; MAO et al., 2015). Therefore, it has been suggested that microorganisms from maternal gastrointestinal microbiota reach the mammary secretions, contributing to the bacterial components in milk (YOUNG et al., 2015). The existence of the entero-mammary pathway in humans and mice has been described, where microbes from the gut lumen travel through the mesenteric lymph nodes and then reach the mammary gland (PEREZ et al., 2007; DONNET-HUGHES et al., 2010; FERNÁNDEZ et al., 2013). The mechanism by which microorganisms cross body sites is not fully understood and may involve immune cells (PEREZ et al.,

2007; DONNET-HUGHES et al., 2010). Young et al. (2015) investigated if a similar mechanism was evident in cows, and found support for the presence of an endogenous entero-mammary pathway during lactation in the cow, when the same bacterial taxon (i.e. *Ruminococcus* genus) was found in milk, blood, and feces of healthy lactating cows. Furthermore, contamination by this bacterial taxon during milking and milk storage should be also considered.

Acinetobacter, a psychrotrophic bacterial genus, are tolerant against drying, disinfectants, heat, and antibiotics. They adapt to a variety of environmental conditions, and emerging pathogens have been reported in this genus (GURUNG et al., 2013). In the milk production process, the lipolytic and proteolytic activities of *Acinetobacter* contribute to spoilage of dairy products (HANTSIS-ZACHAROV; HALPERN, 2007). Another important group of bacteria related to food safety and spoilage are members of the Enterobacteriaceae family, which can be detected in almost all natural environments and are often associated with intestinal infections (ANAND; GRIFFITHS, 2011). Contamination of raw milk by cow feces during milking is common, and thus Enterobacteriaceae spp. have been used as indicators of hygiene and microbial quality (ANAND; GRIFFITHS, 2011). Additionally, in the present study, we observed by using qPCR that the relative abundances of *Acinetobacter* and Enterobacteriaceae increase with increasing total bacterial load in BTM samples. As mentioned above, *Acinetobacter* are psychrotolerant bacteria (FRICKER et al., 2011), and within the Enterobacteriaceae, although coliforms cannot grow and compete well at refrigeration temperatures, some members of the family such as *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia* do grow very well in refrigerated milk (ANAND; GRIFFITHS, 2011).

An interesting finding from our study was the identification of 12 bacterial taxa common to all 19 dairy farms enrolled, suggesting that a core BTM microbiome may exist. Several bacterial taxa identified in the core microbiome have been previously described in raw milk (HANTSIS-ZACHAROV; HALPERN, 2007; OIKONOMOU et al., 2014; WEBER et al., 2014; MILLER et al., 2015). To our knowledge, ours is the first report describing *5-7N15* and *Thermoanaerobacterium* in raw milk samples, both in the milk core microbiome described. *5-7N15* has been identified in fecal samples from dairy cows (YOUNG et al., 2015); however, information regarding this genus is limited. The *Thermoanaerobacterium* genus has generated interest within the food sector in the last decade due its potential as a

contaminant during manufacturing involving high temperatures, because the genus contains thermophilic spore-forming bacteria (MTIMET et al., 2016). Spores present in raw milk can survive pasteurization and other conditions during dairy-product processing (MILLER et al., 2015), and the spores can germinate post-processing to cause dairy-product spoilage (MASIELLO et al., 2014) and/or foodborne disease (BENNETT; WALSH; GOULD, 2013). Raw milk has been implicated as an important source of spores produced by psychrotolerant (MASIELLO et al., 2014; MILLER et al., 2015), mesophilic, and thermophilic spore-forming bacteria (MILLER et al., 2015). We identified a high abundance of Ruminococcaceae in all SCC and log10-SPC quartiles; therefore, further studies should be conducted to understand the origin and the role of Ruminococcaceae in milk samples. The observed high abundance of Enterobacteriaceae in the highest quartiles of log10-SPC and SCC can indicate poor hygiene and/or a high prevalence of mastitis in the herd. In addition, the abundance of *Acinetobacter* was clearly higher in the fourth quartile of log10-SPC, and the positive correlation between *Acinetobacter* and total bacterial load that we observed suggests that bacteria within the *Acinetobacter* genus are an important environment contaminant. This genus is known to cause spoilage (HANTSIS-ZACHAROV; HALPERN, 2007) and is also a rare primary cause of mastitis (OLIVER; MURINDA, 2012). The mean relative abundances of *Streptococcus* and *Corynebacterium* increased from the first to the fourth quartiles of SCC, consistent with their importance as etiological agents of mastitis.

The bacterial genera associated with samples classified as high SCC were *Macrococcus*, *Enterococcus*, *Coxiella*, *Lactococcus*, *Dermacoccus*, and *Lactobacillus*. *Enterococcus*, *Coxiella*, and *Lactococcus* are known mastitis pathogens; however, *Macrococcus*, *Dermacoccus*, and *Lactobacillus* are not associated with bovine mastitis. *Dermacoccus* was first isolated from human skin and described as *Micrococcus* (STACKEBRANDT et al., 1995); *Macrococcus* is phylogenetically close to the genus *Staphylococcus* (BABA et al., 2009); and *Lactobacillus* spp. are known for their beneficial properties (BOUCHARD et al., 2015). In addition, foodborne illness can be caused by *Enterococcus* (GIRAFFA, 2002) and *Coxiella* spp. (HILBERT et al., 2015). The highest relative abundances of *Corynebacterium* and *Streptococcus* were encountered in samples classified as HSCC, and both are known to be important mastitis pathogens (SMITH; TODHUNTER; SCHOENBERGER, 1985; JAYARAO et al., 2004; GONÇALVES et

al., 2016). Species within the genus *Streptococcus* have been previously associated with increased SCC in BTM samples (JAYARAO et al., 2004). *Corynebacterium bovis*, a contagious pathogen, has been associated with increased SCC in milk samples from dairy cows with subclinical mastitis (GONÇALVES et al., 2016). On the other hand, in samples classified as LSCC, Ruminococcaceae, Clostridiales, and Bacteroidales were the most abundant taxa. These groups, mainly Ruminococcaceae and Bacteroidales, have been previously detected during cold storage of bovine raw milk (GSCHWENDTNER et al., 2016).

In samples classified as HSPC, our microbiome analysis showed HSPC to be associated with a small group of bacterial taxa. In most cases the increase of bacterial count is correlated with unsanitary conditions (JAYARAO et al., 2004), which may explain the high prevalence of Enterobacteriaceae in BTM samples classified as HSPC, and the positive correlation between relative abundance of Enterobacteriaceae and total bacterial load described in the present study. Presence of coliforms, an important group within this family, is suggestive of fecal contamination in BTM samples or contamination of water used in the cleaning process (JAYARAO et al., 2004). Furthermore, some Enterobacteriaceae are environmental mastitis pathogens that access BTM not only from intramammary infections, but also from cow skin, teat ends and skin, bedding, manure, and water (JAYARAO et al., 2004). *Streptococcus* was the bacterial taxon with the highest effect size in samples classified as HSPC. This genus is frequently detected in BTM samples (ZADOKS et al., 2004), and it includes contagious and environmental mastitis pathogens (JAYARAO et al., 2004; JØRGENSEN et al., 2016). Similarly, Gillespie et al. (2012) reported the highest correlation between SPC and *Streptococcus* spp. count. In another study, Hayes et al. (2001) found *Streptococcus uberis* to be associated with increased SPC in BTM samples (HAYES et al., 2001). In summary, *Streptococcus* abundance was highly correlated with both SCC and SPC, suggesting that this genus remains a very significant problem to the dairy industry and that more preventive actions should be implemented to reduce its incidence. Nonetheless, although many of the bacterial taxa described in this study have the ability to cause foodborne illness, most of these microorganisms are destroyed during processing (e.g. pasteurization). However, an important remaining concern is the presence of spore-forming bacteria in milk (ANAND; GRIFFITHS, 2011) which may

survive pasteurization; indeed, in the present study, we identified spore-forming spp. in low SPC groups of BTM.

4.5 Conclusions

Our description of the microbiome of BTM samples and its association with milk quality parameters corroborate the current knowledge regarding the bacterial community of raw milk, and the effects on milk quality parameters. Bacterial taxa widely studied in milk were among the most abundant identified in our BTM samples, with Ruminococcaceae being the most abundant. Spoilage bacteria such as *Acinetobacter* and *Pseudomonas* were ubiquitous in BTM for all studied dairy farms. In samples classified as HSPC, *Acinetobacter* and Enterobacteriaceae presented higher relative abundances, and both increased as bacterial load increased. Spore-forming bacteria (*Thermoanaerobacterium* and *Bacillus*) were identified in the core microbiome of BTM, which is a concern to the dairy industry. Among the pathogens associated with SCC and SPC, *Streptococcus* was highlighted; it was abundant in samples classified as HSCC, and was the most significant taxon in samples classified as HSPC. Other pathogens (e.g. *Corynebacterium*) were observed at significantly higher relative abundances in the HSCC group when compared with the LSCC group; a similar result was obtained in the analysis of SPC. Also, we found that the BTM environment is dominated by a smaller group of bacterial taxa in cases where there is a higher bacterial load. For further investigation, we suggest studying the bacterial taxa that were detected at significantly higher relative abundance in BTM samples classified as HSCC (e.g. *Lactobacillus*, *Arthrobacter*, and *Lactococcus*) in order to verify the influence of these genera on general milk quality and herd health.

References

ANAND, S.K.; GRIFFITHS, M.W. Pathogens in milk. Enterobacteriaceae. In: SMITHERS, G.W. **Encyclopedia of dairy sciences**. 2nd ed. Cambridge: Cambridge Academic Press, 2011. p. 67-71

BABA, T.; KUWAHARA-ARAI, K.; UCHIYAMA, I.; TAKEUCHI, F.; ITO, T.; HIRAMATSU, K. Complete genome sequence of *Macrococcus caseolyticus* strain JCSCS5402, [corrected] reflecting the ancestral genome of the human-pathogenic staphylococci. **Journal of Bacteriology**, Washignton, v. 191, n. 4, p. 1180-1190, Feb. 2009.

BARBANO, D.M.; MA, Y.; SANTOS, M.V.Influence of raw milk quality on fluid milk shelf life. **Journal of Dairy Science**, Champaign, v. 89, suppl. 1, E15-E19, Mar. 2006.

BENJAMINI, Y.; HOCHBERG, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. **Journal of Royal Statistical Society**, Series B. Methodological, London, v. 57, n. 1, p. 289-300,1995.

BENNETT, S.D.; WALSH, K.A.; GOULD,L.H. Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*: United States, 1998-2008. **Clinical InfectiousDiseases**, Chicago, v. 57, n. 3, p. 425-433, Aug. 2013.

BIANCHINI, V.; BORELLA, L.; BENEDETTI, V.; PARISI, A.; MICCOLUPO, A.; SANTORO, E.; RECORDATI, C.; LUINI, M. Prevalence in bulk tank milk and epidemiology of *Campylobacter jejuni* in dairy herds in Northern Italy. **Applied and Environmental Microbiology**, Washington, v. 80, p. 1832-1837, 2014.

BOKULICH, N.A.; SUBRAMANIAN, S.;FAITH, J.J.; GEVERS, D.; GORDON, J.I.; KNIGHT, R.; MILLS, D.A.; CAPORASO, J.G. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. **Nature Methods**, New York, v. 10, n. 1, p. 57-59, Jan. 2013.

BOUCHARD, D.S.; SERIDAN, B.; SARAOUI, T.; RAULT, L.; GERMON, P.; GONZALEZ-MORENO, C.; NADER-MACIAS, F.M.; BAUD, D.; FRANÇOIS, P.; CHUAT, V.; CHAIN, F.; LANGELLA, P.; NICOLI, J.; LE LOIR, Y.; EVEN,S. Lactic Acid Bacteria isolated from bovine mammary microbiota: potential allies against bovine mastitis. **PLoS One**, San Francisco, v. 10, n. 12, p. 1-18, Dec. 2015.

BOUTIN, S.; GRAEBER, S.Y.; WEITNAUER, M.; PANITZ, J.; STAHL, M.; CLAUSZNITZER, D.; KADERALI, L.; EINARSSON, G.; TUNNEY, M.M.; ELBORN, J.S.; MALL, M.A.; DALPKE, A.H. Comparison of microbiomes from different niches of upper and lower airways in children and adolescents with cystic fibrosis. **PLoS One**, San Francisco, v. 10, n. 1, p. 1-19, Jan. 2015.

CAPORASO, J.G.; LAUBER, C.L.; WALTERS, W.A.; BERG-LYONS, D.; HUNTLEY, J.; FIERER,N.; OWENS, S.M.; BETLEY, J.; FRASER, L.; BAUER, M.; GORMLEY, N.; GILBERT, J.A.; SMITH, G.; KNIGHT, R. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. **The ISME Journal**, London, v. 6, n. 8, p. 1621-1624, Aug. 2012.

CAPORASO, J.G.; KUCZYNSKI, J.; STOMBAUGH,J.; BITTINGER, K.; BUSHMAN, F.D.; COSTELLO, E.K.; FIERER,N.; PEÑA, A.G.; GOODRICH, J.K.; GORDON, J.I.; HUTTLEY, G.A.; KELLEY, S.T.; KNIGHTS, D.; KOENIG, J.E.; LEY, R.E.; LOZUPONE, C.A.; MCDONALD,D.; MUEGGE, B.D.; PIRRUNG, M.; REEDER,J.; SEVINSKY, J.R.; TURNBAUGH, P.J.; WALTERS, W.A.; WIDMANN, J.; YATSUNENKO, T.; ZANEVELD,J.; KNIGHT, R. QIIME allows analysis of high-throughput community sequencing data. **Nature Methods**, New York, v. 7, n. 5, p. 335-336, May 2010.

DECIMO, M.; MORANDI, S.; SILVETTI, T.; BRASCA, M. Characterization of Gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. **Journal of Food Science**, Champagin, v. 79, n. 10, p. 20081-2090, Oct. 2014.

DONNET-HUGHES, A.; PEREZ, P.F.; DORÉ, J.; LECLERC, M.; LEVENEZ, F.; BENYACOUB, J.; SERRANT, P.; SEGURA-ROGGERO, I.; SCHIFFRIN, E.J. Potential role of the intestinal microbiota of the mother in neonatal immune education. **Proceedings of the Nutrition Society**, Cambridge, v. 69, n. 3, p. 407-415, Aug. 2010.

EDGAR, R.C. Search and clustering orders of magnitude faster than BLAST. **Bioinformatics**, Oxford, v. 26, n. 19, p. 2460-2461, Oct. 2010.

ELMOSLEMANY, A.M.; KEEFE, G.P.; DOHOO, I.R.; JAYARAO, B.M. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 2: bacteria count-specific risk factors. **Journal of Dairy Science**, Champaign, v. 92, n. 6, p. 2644-2652, June 2009.

FERNÁNDEZ, L.; LANGA, S.; MARTÍN, V.; MALDONADO, A.; JIMÉNEZ, E.; MARTÍN, R.; RODRÍGUEZ, J.M. The human milk microbiota: origin and potential roles in health and disease. **Pharmacological Research**, London, v. 69, n. 1, p. 1-10, Mar. 2013.

FRICKER, M.; SKÅNSENG, B.; RUDI,K.; STESSL, B.; EHLING-SCHULZ, M. Shift from farm to dairy tank milk microbiota revealed by a polyphasic approach is independent from geographical origin. **International Journal of Food Microbiology**, Amsterdam, v. 145, suppl. 1, S24-S30, Mar. 2011.

GILLESPIE, B.E.; LEWIS, M.J.; BOONYAYATRA, S.; MAXWELL, M.L.; SAXTON,A.; OLIVER,S.P.; ALMEIDA, R.A. Short communication: evaluation of bulk tank milk microbiological quality of nine dairy farms in Tennessee. **Journal of Dairy Science**, Champaign, v. 95, n. 8, p. 4275-4279, Aug. 2012.

GIRAFFA, G. Enterococci from foods. **FEMS Microbiology Reviews**, Amsterdam, v. 26, n. 2, p.163-171, June 2002.

GONÇALVES, J.L.; TOMAZI, T.; BARREIRO, J.R.; BEURON, D.C.; ARCARI, M.A.; LEE, S.H.; MARTINS, C.M.; ARAÚJO JUNIOR,J.P.; SANTOS, M.V. Effects of bovine subclinical mastitis caused by *Corynebacterium* spp. on somatic cell count, milk yield and composition by comparing contralateral quarters. **The Veterinary Journal**, London, v. 209, p. 87-92, Mar. 2016.

GSCHWENDTNER, S.; ALATOSSAVA, T.; KUBLIK, S.; FUKA, M.M.; SCHLOTER, M.; MUNSCH-ALATOSSAVA, P. N_2 gas flushing alleviates the loss of bacterial diversity and inhibits psychrotrophic pseudomonas during the cold storage of bovine raw milk. **PLoS One**, San Francisco, v. 11, n. 1, p. 1-17, Jan. 2016.

GURUNG, M.; NAM, H.M.; TAMANG, M.D.; CHAE, M.H.; JANG,G.C.; JUNG, S.C.; LIM, S.K. Prevalence and antimicrobial susceptibility of *Acinetobacter* from raw bulk tank milk in Korea. **Journal of Dairy Science**, Champaign, v. 96, n. 4, p. 1997-2002, Apr. 2013.

HANTSIS-ZACHAROV, E.; HALPERN, M. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. **Applied and Environmental Microbiology**, Washignton, v. 73, n. 22, p. 7162-7168, Sept. 2007.

HAYES, M.C.; RALYEA, R.D.; MURPHY, S.C.; CAREY,N.R.; SCARLETT, J.M.; BOOR, K.J. Identification and characterization of elevated microbial counts in bulk tank raw milk. **Journal of Dairy Science**, Champaign, v. 84, n. 1, p. 292-298, Jan. 2001.

HILBERT, A.; ANDRES,T.; WERNER,R.; WEHR, R.; FRÖHLICH,A.; CONRATHS, F.J.; HENNING, K. Detection of *Coxiella burnetii* in dairy cattle bulk tank milk and single tank milk samples by confirmatory testing. **Berliner und Münchener tierärztliche Wochenschrift**, Hannover, v. 128, n. 7/8, p. 271-277, July/Aug. 2015.

JAYARAO, B.M.; PILLAI,S.R.; SAWANT, A.A.; WOLFGANG, D.R.; HEGDE, N.V. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. **Journal of Dairy Science**, Champaign, v. 87, n. 10, p. 3561-3573, Oct. 2004.

JØRGENSEN, H.J.; NORDSTOGA, A.B.; SVILAND,S.; ZADOKS, R.N.; SØLVERØD, L.; KVITLE, B.; MØRK, T. *Streptococcus agalactiae* in the environment of bovine dairy herds: rewriting the textbooks? **Veterinary Microbiology**, Amsterdam, v. 184, p. 64-72, Feb. 2016.

KATHOLM, J.; BENNEDSGAARD, T.W.; KOSKINEN, M.T.; RATTENBORG, E. Quality of bulk tank milk samples from Danish dairy herds based on real-time polymerase chain reaction identification of mastitis pathogens. **Journal of Dairy Science**, Champaign, v. 95, n. 10, p. 5702-5708, 2012.

KUANG, Y.; TANI,K.; SYNNOTT, A.J.; OHSHIMA,K.; HIGUCHI, H.; NAGAHATA,H.; TANJI, Y. Characterization of bacterial population of raw milk from bovine mastitis by culture-independent PCR–DGGE method. **Biochemical Engineering Journal**, Amsterdam, v. 45, n. 1, p. 76-81, June 2009.

LI, N.; RICHOUX,R.; BOUTINAUD,M.; MARTIN, P.; GAGNAIRE, V. Role of somatic cells on dairy processes and products: a review. **Dairy Science & Technology**, Les Ulis, v. 94, n. 6, p. 517-538, July 2014.

LIMA, F.S.; OIKONOMOU, G.; LIMA, S.F.; BICALHO, M.L.; GANDA, E.K.; FILHO, J.C.; LORENZO, G.; TROJACANEC, P.; BICALHO, R.C. Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows. **Applied and Environmental Microbiology**, Washington, v. 81, n. 4, p. 1327-1337, Feb. 2015.

MAO, S.; ZHANG,M.; LIU,J.; ZHU, W. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. **Scientific Reports**, London, v. 5, p.1-14, Nov. 2015.

MASIELLO, S.N.; MARTIN, N.H.; WATTERS, R.D.; GALTON, D.M.; SCHUKKEN, Y.H.; WIEDMANN, M.; BOOR, K.J. Identification of dairy farm management practices associated with the presence of psychrotolerant sporeformers in bulk tank milk. **Journal of Dairy Science**, Champaign, v. 97, n. 7, p. 4083-4096, July 2014.

McDONALD, D.; PRICE, M.N.; GOODRICH,J.; NAWROCKI, E.P.; DESANTIS, T.Z.; PROBST,A.; ANDERSEN, G.L.; KNIGHT,R.; HUGENHOLTZ, P. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea.**The ISME Journal**, London, v. 6, n. 3, p. 610-618, Mar. 2012.

MILLER, R.A.; KENT, D.J.; BOOR, K.J.; MARTIN, N.H.; WIEDMANN, M. Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. **Journal of Dairy Science**, Champaign, v. 98, n. 7, p. 4338-4351, July 2015.

MTIMET, N.; GUÉGAN, S.; DURAND, L.; MATHOT, A.G.; VENAILLE, L.; LEGUÉRINEL, I.; COROLLER,L.; COUVERT, O. Effect of pH on *Thermoanaerobacterium thermosaccharolyticum* DSM 571 growth, spore heat resistance and recovery. **Food Microbiology**, London, v. 55, p. 64-72, May 2016.

NIGHTINGALE, C.; DHUYVETTER,K.; MITCHELL, R.; SCHUKKEN, Y. Influence of variable milk quality premiums on observed milk quality. **Journal of Dairy Science**, Champaign, v. 91, n. 3, p. 1236-1244, Mar. 2008.

NONNENMACHER, C.; DALPKE,A.; MUTTERS,R.; HEEG, K. Quantitative detection of periodontopathogens by real-time PCR. **Journal of Microbiological Methods**, Amsterdam, v. 59, n. 1, p. 117-125, Oct. 2004.

OIKONOMOU, G.; BICALHO, M.L.; MEIRA, E.; ROSSI, R.E.; FODITSCH, C.; MACHADO, V.S.; TEIXEIRA, A. G.; SANTISTEBAN, C.; SCHUKKEN, Y.H.; BICALHO, R.C. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. **PLoS One**, San Francisco, v. 9, n. 1, p. 1-11, Jan. 2014.

OLIVER, S.P.; MURINDA, S.E. Antimicrobial resistance of mastitis pathogens.**TheVeterinary Clinics of North America: Food Animal Practice**, Philadelphia, v. 28, n. 2, p. 165-185, July 2012.

PARK, Y.K.; KOO,H.C.; KIM, S.H.; HWANG, S.Y.; JUNG, W.K.; KIM, J.M.; SHIN,S.; KIM, R.T.; PARK, Y.H. The analysis of milk components and pathogenic bacteria isolated from bovine raw milk in Korea. **Journal of Dairy Science**, Champaign, v. 90, n. 12, p. 5405-5414, Dec. 2007.

PEREZ, P.F.; DORÉ, J.; LECLERC, M.; LEVENEZ, F.; BENYACOUB, J.; SERRANT, P.; SEGURA-ROGGERO, I.; SCHIFFRIN, E.J.; DONNET-HUGHES, A. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? **Pediatrics**, São Paulo, v. 119, n. 3, p. 724-732, Mar. 2007.

QUIGLEY, L.; McCARTHY, R.; O'SULLIVAN, O.; BERESFORD, T.P.; FITZGERALD, G.F.; ROSS, R.P.; STANTON, C.; COTTER,P.D. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. **Journal of Dairy Science**, Champaign, v. 96, n. 8, p. 4928-4937, Aug. 2013.

RAATS, D.; OFFEK,M.; MINZ, D.; HALPERN, M. Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. **Food Microbiology**, London, v. 28, n. 3, p. 465-471, Mar. 2011.

SMITH, K.L.; TODHUNTER,D.A.; SCHOENBERGER, P.S. Environmental mastitis: cause, prevalence, prevention. **Journal of Dairy Science**, Champaign, v. 68, n. 6, p.1531-1553, June 1985.

STACKEBRANDT, E.; KOCH, C.; GVOZDIAK,O.; SCHUMANN, P. Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. **International Journal of Systematic and Evolutionary Microbiology**, London, v. 45, n. 4, p. 682-692, Oct. 1995.

von NEUBECK, M.; BAUR, C.; KREWINKEL, M.; STOECKEL, M.; KRANZ, B.; STRESSLER, T.; FISCHER,L.; HINRICHS, J.; SCHERER, S.; WENNING, M. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. **International Journal of Food Microbiology**, Amsterdam, v. 211, p. 57-65, Oct. 2015.

WEBER, M.; GEIßERT, J.; KRUSE,M.; LIPSKI, A. Comparative analysis of bacterial community composition in bulk tank raw milk by culture-dependent and culture-independent methods using the viability dye propidium monoazide. **Journal of Dairy Science**, Champaign, v. 97, 11, p. 6761-6776, Nov. 2014.

YOUNG, W.; HINE, B.C.; WALLACE, B.C.; CALLAGHAN, M.; BIBILONI, R. Transfer of intestinal bacterial components to mammary secretions in the cow. **PeerJ**, Corte Madera, v. 3, p. 1-17, Apr. 2015.

ZADOKS, R.N.; GONZÁLEZ, R.N.; BOOR, K.J.; SCHUKKEN, Y.H. Mastitis-causing streptococci are important contributors to bacterial counts in raw bulk tank milk. **Journal of Food Protection**, Ames, v. 67, n. 12, p. 2644-2650, Dec. 2004.

ZANARDI, G.; CAMINITI, A.; DELLE DONNE, G.; MORONI, P.; SANTI,A.; GALLETTI,G.; TAMBA,M.; BOLZONI,G.; BERTOCCHI, L. Short communication: comparing real-time PCR and bacteriological cultures for *Streptococcus agalactiae* and *Staphylococcus aureus* in bulk-tank milk samples. **Journal of Dairy Science**, Champaign, v. 97, n. 9, p. 5592-5598,Sept. 2014.

5 MOLECULAR CHARACTERIZATION AND ANTIBIOTIC RESISTANCE PHENOTYPES OF *Staphylococcus* spp. ISOLATED FROM BRAZILIAN CHEESE PROCESSING PLANTS

Abstract

The aim of this study was to characterize coagulase-positive and negative staphylococci from raw milk, *Minas* cheese, and production lines of *Minas* cheese processing. One hundred isolates from three different cheese producers were characterized using molecular approaches, such as polymerase chain reaction, molecular typing, and DNA sequencing. *S. aureus* (88% of the isolates) were the most abundant followed by *S. epidermidis*, *S. hyicus*, and *S. warneri*. Among the twenty-two enterotoxin genes tested, the most frequent was *seh* (62% of the isolates), followed by *sel*x, and *ser*. Hemolysin genes were widely distributed across isolates, *hlb* gene was the most detected (58.0% of isolates was positive for *hlb*),Panton-Valentine leukocidin (2% of isolates) and toxic shock syndrome toxin (1% of isolates) genes also were identified. Methicillin-Resistant *S. aureus* (6.0% of isolates), were SCC*mec* III, IVa, IVd, and others nontypeable. In the phenotypic antibiotic resistance, multiresistant isolates were detected and resistance to penicillin was the most observed (48% of isolates). Using *spa* typing, 19 types were identified and a new type described, t14969 isolated from cheese.

Keywords: *Minas* cheese; Staphylococci; Antibiotic resistance; Virulence factor; Molecular typing

5.1 Introduction

Staphylococcus spp. are known worldwide as a cause of human and animal infections, such as bacteremia, wound infections (PODKOWIK et al., 2013) and mastitis (LUINI et al., 2015; PODKOWIK et al., 2013). The species *Staphylococcus aureus* is the main etiological agent of mastitis in dairy cows (LUINI et al., 2015), a disease economically important to the dairy industry (HAYAKAWA et al., 2001; SHAHEEN; TANTARY; NABI, 2016). In addition, staphylococci from bovine milk, excluding *S. aureus*, also represent a heterogeneous group of microorganisms, namely coagulase-negative staphylococci (CNS), which are commonly associated with bovine mastitis (LANGE et al., 2015). In different studies, CNS has been highlighted in human (BECKER; HEILMANN; PETERS, 2014; JEAN-BAPTISTE et al., 2011) and animal infections (LANGE et al., 2015), and also in food poisoning cases (PODKOWIK et al., 2013).

Food poisoning is widely related to *S. aureus* and is frequently detected in milk and dairy products (CARFORA et al., 2015; JAMALI et al., 2015; XING et al.,

2016). Specifically in cheese production, André et al. (2008) and Arcuri et al. (2010) showed that the source of *S. aureus* contamination could be multifactorial, such as raw milk, processing environment, and handlers. In general, *S. aureus* is a major concern for the food processing industry due to its virulence factors (XING et al., 2016). Strains can produce coagulase, hemolysins, exfoliative toxins, toxic shock syndrome toxin-1, protein A, staphylococcal enterotoxins (SEs), in addition to others (HAYAKAWA et al., 2001).

Moreover, the presence of strains resistant to antibiotics has been reported and its transmission via food is a concern for public health (JAMALI et al., 2015). Resistance of *Staphylococcus aureus* isolated from raw milk and dairy products to tetracycline, kanamycin, gentamicin, streptomycin, methicillin, and other antibiotics was reported by Jamali et al. (2015). Other studies have described the antibiotic resistance of *Staphylococcus* spp. isolated from milk (ANDRÉ et al., 2008; ROLA et al., 2015). Additionally, *S. aureus* is a pathogen identified by World Health Organization (WHO) as an international concern due to its resistance to antibacterial drugs (WORLD HEALTH ORGANIZATION, 2014). Considering this, the aim of this study was to characterize coagulase-positive and negative staphylococci isolated from raw milk, *Minas* cheese (fresh cheese), and from production lines of *Minas* cheese processing. Virulence factor genes, antibiotic resistance genes, and antibiogram testing of eleven different antibiotics were all assessed for all isolates; molecular typing of *S. aureus* (*spa* and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) types) was also performed.

5.2 Material and methods

5.2.1 Origin of isolates and dairies descriptions

One hundred isolates from the bacterial collection of Hygiene and Dairy Laboratory, University of São Paulo, Brazil were used in this study. In a previous study performed by Hygiene and Dairy Laboratory's research group, staphylococci were isolated using standard methods (DOWNES; ITO, 2001) from samples collected from three different processing plants of traditional Brazilian fresh cheese called *Minas Frescal* cheese or *Minas* cheese. All processing plants were located in São Paulo State, Brazil. The isolates used were storage at -20 ºC and their origins are shown in Table 1.

Table 1 –Origin and number of isolates per cheese processing plant enrolled in this study

	Number of isolates			
Origin	Dairy A	Dairy B	Dairy C	Total
Raw milk	23	2	4	29
Pasteurized milk	0	0	1	1
Food handler	20	7	0	27
Table	0	1	2	3
Floor	0	1	0	1
Cheese before packing	6	2	0	8
Cheese after packing	15	8	1	24
Brine	2	0	0	2
Milk tank	1	0	2	3
Packer	1	0	0	1
Cheese mold	1	0	0	1

Dairy “A” produced approximately 600 kilograms (kg) of cheese per week and the pasteurization used was High Temperature Short Time (HTST). Dairy “B” processed 150 kg per week and slow pasteurization was used, 73 ºC/15 seconds. Dairy “C” produced its own milk, the pasteurization was rapid (HTST) and 400 kg of cheese was produced per week.

5.2.2 Molecular characterization

The strains were reactivated in Brain Heart Infusion broth (BHI, Oxoid™, Hampshire, UK). The cell pellet was harvested by centrifugation at 12.000 rpm/2 minutes. The cell pellet was used and DNA was extracted from samples using “AxyPrep™ Blood Genomic DNA Miniprep Kit” (Axygen Scientific Inc., California, USA) according to manufacturer's instructions. The genomic DNA was stored at −20 °C until further analysis.

Classification of isolates as negative or positive-coagulase was conducted using Polymerase Chain Reaction (PCR) of *coa* gene according to Aarestrup, Dangler and Sordillo (1995) with modifications. When confirmed as coagulase-positive, a multiplex PCR was performed to identify *S. intermedius*, *S. aureus*, and *S.*

hyicus (SASAKI et al., 2010). For identification of other species, amplification of *sod*A gene as described by Silva et al. (2014) was completed. DNA was purified using "Illustra^{TM}, GFX^{TM} PCR DNA and Gel Band Purification Kit" (GE Healthcare, Buckinghamshire, UK) and Sanger sequencing was performed. Posterior comparison of the FASTA sequence against sequences stored in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (BENSON et al., 2009; SAYERS et al., 2009) was completed to identify the species.

Genes encoding enterotoxin, hemolysin, exfoliative toxin, Panton-Valentine leukocidin, and toxic shock syndrome toxin were identify using oligonucleotide sequences previously described, the sequences corresponding to the genes *sea*, *seb*, *sec*, *sed* (JOHNSON et al., 1991), *see* (MEHROTRA; WANG; JOHNSON, 2000), *seg*, *seh*, *sei* (OMOE et al., 2002), *selj* (NASHEV et al., 2004), *selk* (OMOE et al., 2005), *sell* (CREMONESI et al., 2005), *selm*, *seln*, *selo* (OTE et al., 2011), *selp*, *selq*, *ser* (OMOE et al., 2005), *ses*, *set* (ONO et al., 2008), *selu* (FISCHER et al.,2009), *selv* (THOMAS et al., 2009), *selx* (WILSON et al., 2011), *hla*, *hlb*, *hld*, *hlg*, *hlg-v* (JARRAUD et al., 2002), *eta*, *etb* (JARRAUD et al., 2002), *etd* (YAMAGUCHI et al., 2002), *tst* (JARRAUD et al., 2002), and *pvl* (LINA et al., 1999). Multiplex or uniplex PCR's were completed as described by previously published studies, with modifications, which were described in another study conducted by our research group (data not published). The antibiotic resistant genes *tet*K, *tet*L, *tet*M (GÓMEZ-SANZ et al., 2010), *ant(4')-Ia* (van de KLUNDERT; VLIEGENTHART, 1993), *erm*A, *erm*B, *erm*C (GÓMEZ-SANZ et al., 2010), *mec*A (MOON et al., 2007), and *mec*A$_{LGA251}$ (CUNY et al., 2011) were identified as previously described. The antibiotic resistance genes and virulence factor genes were tested in all isolates. All PCR's performed included positive and negative controls, where the positive controls were provided by Hygiene and Dairy Laboratory, University of São Paulo.

Through molecular typing, the *spa* region was amplified by PCR according to the website www.ridom.com. DNA was purified using "Illustra^{TM}, GFX^{TM} PCR DNA and Gel Band Purification Kit" (GE Healthcare, Buckinghamshire, UK) and sequenced using the Sanger method. Repeats and *spa* types were identified using the databases accessed in the http://spa.ridom.de/repeats.shtml, and http://spa.ridom.de/spatypes.shtml, respectively. Multi Locus Sequence Type (MLST) was completed as described by Enright et al. (2000). SCC*mec* type (I to V) for *mec*A

positive strains was performed according to Zhang et al. (2005) and Kondo et al. (2007).

5.2.3 Phenotypic characterization of antibiotic resistance

Disk-diffusion method was carried out according to the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2015) to identify antibiotic resistance of the isolates. The antibiotics tested were: penicillin (PEN), cefoxitin (FOX), oxacillin (OXA), erythromycin (ERI), clindamycin (CLI), chloramphenicol (CHL), ciprofloxacin (CIP), vancomycin (VAN), tobramycin (TOB), tetracycline (TET), and gentamicin (GEN).

5.3 Results

Of the total, 92.0% of the isolates were identified as coagulase-positive, and 8.0% were coagulase-negative. The prevalence of *S. aureus* (88% of the isolates) was observed (Table 2), followed by *S. epidermidis* (6%), *S. hyicus* (4%), and *S. warneri* (2%).

Table 2 – Genes and phenotypic resistance to antibiotics identified in *Staphylococcus* isolated from dairies

(to be continued)

Dairy/ Specie (n)	Enterotoxin genes (n)	Hemolysin genes (n)	TSST-1 and PVL gene (n)	Antibiotic resistance (n)	Antibiotic resistance genes (n)
Dairy A					
S. aureus (61)	*sea* (4) *seb* (6) *sec* (1) *seg* (13) *seh* (36) *sei* (1) *sej* (4) *sek* (14) *sel* (20) *sem* (7) *sen* (10) *seo* (5) *sep* (9) *seq* (5) *ser* (26) *seu* (7) *selx* (38)	*hla* (33) *hlb* (36) *hld* (31) *hlg* (31) *hlg-v* (34)	*tst* (1) pvl (2)	CIP (3) ERI (10) OXA (9) FOX (13) CLI (8) VAN (5) CLO (1) TET (5) GEN (3)	*tetK* (2) *tetM* (5) *ermA* (7) *ermC* (6) *mec*A (6)

Table 2 – Genes and phenotypic resistance to antibiotics identified in *Staphylococcus* isolated from dairies

(to be continued)

Dairy/ Specie (n)	Enterotoxin genes (n)	Hemolysin genes (n)	TSST-1 and PVL gene (n)	Antibiotic resistance (n)	Antibiotic resistance genes (n)
Dairy A					
				TOB (5) PEN (30)	
S. hyicus (4)	*seh* (2) *sek* (1) *sel* (2) *sen*(1) *sep* (2) *ser*(1)	-	-	PEN (1)	*ermA* (1)
S. warneri (2)	*seb* (1) *sek* (1)	-	-	CLI (1) PEN (2)	
S. epidermidis (2)	-	-	-	ERI (1) OXA (1) PEN (2)	
Dairy B					
S. aureus (17)	*seg* (9) *seh*(15)*sel* (2) *sem* (9) *sen* (7) *seo* (8) *ser* (7) *seu* (11) *selx* (1)	*hla* (4) *hlb*(13) *hld* (10) *hlg* (6)	-	ERI (1) FOX (2) CLI (1) VAN (1) PEN (3) OXA(1)	
S. epidermidis (4)	*seg* (3) *seh* (2) *seu* (3)	*hlb* (1)	-	ERI (1) PEN (2)	
Dairy C					
S. aureus (10)	*seg* (10) *seh*(10) *sel* (6) *sem* (9) *sen* (9) *seo*(9) *sep* (1) *ser* (8) *seu* (10) *sel*x (6)	*hla* (1) *hlb* (8) *hld* (6) *hlg* (8) *hlg-v* (5)	-	ERI (1) OXA (5) FOX (6) CLI (5) VAN (2)	*tetM* (4)

Table 2 – Genes and phenotypic resistance to antibiotics identified in *Staphylococcus* isolated from dairies

(conclusion)

Dairy/ Specie (n)	Enterotoxin genes (n)	Hemolysin genes (n)	TSST-1 and PVL gene (n)	Antibiotic resistance (n)	Antibiotic resistance genes (n)
Dairy C					
S. aureus (10)				TET (4) GEN (2) TOB (1) PEN (8)	

The genes *sed*, *see*, *ses*, *set* and *selv* were not detected. The *seh* gene was the most identified across all isolates, it was detected in 76.6% of the raw milk samples, while it was detected in 42.8% of handler, packer, table, floor, brine, milk tank, and cheese mold isolates, and in 84.3% of the cheeses isolates. In total, fifty-five staphylococcal enterotoxin profiles were detected, two being the most frequent, *seg+seh+sel/+selm+seln+selo+ser+seu+selx,* and *seh+ser+selx*, with seven isolates for each one. Twelve coagulase-positive staphylococci (CPS) were negative for all genes encoding enterotoxins tested. Among CNS three were negative for all toxin genes assessed in this study i.e. two *S. epidermidis* and one *S. warneri*. However, four CNS isolates identified as *S. epidermidis* were detected as enterotoxins genes. The profiles identified were *seg, seg+seh+selu, seh+selu*, and *seg+selu*, and one *S. warneri* was also positive for enterotoxins genes, *seb+selk*.

For hemolysin genes, 14 hemolysin gene profiles were identified in total. The profile representing all hemolysin genes (*hla*, *hlb*, *hld*, *hlg*, and *hlg-v*) tested was the most prevalent (28 isolates). The most abundant hemolysin gene was *hlb* (58%), next *hld* (47%), *hlg* (45%), *hlg-v* (39%), and *hla* (38%). Exfoliative toxin genes were not found, the *tst* gene was detected in one isolate from raw milk (*S. aureus*, Table 2), the same isolate was carrying enterotoxin genes (*sec*, *seg*, *sei*, *selk*, *sell*, *selm*, *seln*, *selo*, *selp*, *selu*, and *selx*), while *pvl* gene was detected in two isolates (*S. aureus*, Table 2), one from raw milk, and another from food handler, their profiles are shown in the Table 3. Antibiotic resistance genes were identified (Table 2), however, *mec*LGA251, *tet*L, *ant(4')-Ia*, and *erm*B genes were not observed. The gene *mec*A

was found in isolates from the same dairy, and the SCC*mec* types were described as well as the profile of these strains, which are shown in the Table 3.Using the disk-diffusion method, antibiotic resistance was observed in 52% of the isolates, resistance to penicillin was the most frequent followed by cefoxitin, oxacillin, clindamycin, erythromycin, tetracycline, vancomycin, tobramycin, gentamicin, ciprofloxacin, and chloramphenicol (Table 2). In total, 20 antibiotic resistance profiles were detected in this study, the most abundant profile was PEN (23%) followed by OXA, FOX, ERI, CLI, VAN, PEN (3%). Antibiotic multiresistance was observed in 27% of the isolates, 45% of the isolates were susceptible to all antibiotics tested, and three isolates presented intermediate resistance.

Table 3 – Genotypic, phenotypic profiles of methicillin-resistant *Staphylococcus aureus* identified in this study

(to be continued)

Strain	Origin	Enterotoxin genes	Hemolysin genes	*pvl* gene	Antibiotic resistance genes	SCC*mec* type	Phenotypic resistance to antibiotic	*spa* type
A	Raw milk	*sea, seb, seh,* selj*, selk, sell, selq, ser, selx*	*hla, hlb, hld, hlg, hlg-v*	-	*mec*A, *erm*A, *erm*C	IVd	CIP, OXA, FOX, ERI, CLI, PEN, TET, GEN, TOB	t064
B	Food handler	*sea, seb, seh, selj, selk, sell, selq, ser, selx*	*hla, hlb, hld, hlg, hlg-v*	+	*mec*A, *erm*A, *erm*C	IVd	CIP, OXA, FOX, ERI, CLI, PEN, TET, GEN, TOB	t064

Table 3 – Genotypic, phenotypic profiles of methicillin-resistant *Staphylococcus aureus* identified in this study

(conclusion)

Strain	Origin	Enterotoxin genes	Hemolysin genes	*pvl* gene	Antibiotic resistance genes	SCC*mec* type	Phenotypic resistance to antibiotic	*spa* type
C	Packer	*sea, seb, selk, selq, selx*	*hla, hlb, hld, hlg-v*	-	*mec*A, *erm*C	-	CIP, OXA, FOX, ERI, CLI, PEN, TET, GEN, TOB	t064
D	Cheese mold	*seg, seh, sell, seln, selp, selu, selx*	*hlb*	-	*mec*A, *erm*A	-	OXA, FOX, ERI, CLI, VAN, PEN	t127
E	Raw milk	*seh, selk, sell, selp, selq, selx*	*hla, hlb, hld, hlg, hlg-v*	-	*mec*A, *tet*K, , *erm*C	III	OXA, FOX, ERI, CLI, VAN, CLO, PEN	t037
F	Raw milk	*selk, selq, selx*	*hla, hlb, hld, hlg, hlg-v*	+	*mec*A, *tet*K	IVa	FOX, ERI, TET	t008

The *spa* types detected were t002 (1.14%), t008 (5.68%), t021 (1.14%), t037 (3.41%), t064 (6.82%), t114 (1.14%), t127 (22.72%), t128 (2.27%), t177 (2.27%), t267 (1.14%), t318 (1.14%), t521 (4.54%), t605 (32.95%), t777 (1.14%), t922 (1.14%), t4158 (4.54%), t5605 (1.14%),t6367 (2.27%), and a new type, t14969 (3.41%). The three isolates with *spa* type t14969 were isolated from fresh cheese samples after packing, the repeats are 15-12-16-16-02-16-02-02-25-17-17-24, and its sequence type (ST) was identified as ST30 (Clonal Complex 30). Enterotoxin gene profile was *seg*+*seh*+*selm*+*seln*+*selo*+*ser*+*selu*, hemolysin gene profiles were

hld+hlg+hlg-v (one isolate) and *hld+hlg* (two isolates), and were sensitive to all antibiotics tested.

5.4 Discussion

This study showed that the distribution of *Staphylococcus* species is a real concern for Brazilian dairies. *Staphylococcus* is an important pathogen in dairy products and contributes to food deterioration. Thus, it is essential to control the presence of this pathogen in the food processing plant. Herein, isolates from dairies were assessed and *S. aureus* was widely identified followed by *S. epidermidis*, *S. hyicus*, and *S. warneri*, which were carrying several virulence factor genes and demonstrated antibiotic resistance. The enterotoxin gene most abundant was *seh*, representing SEH. Interestingly, genes encoding classical enterotoxins were identified either in low frequency or were completely absent. The data highlights the importance of detecting new enterotoxins in food as well as their screening in CNS. Hemolysin genes were detected across isolates, and *pvl* and *tst* were also detected. Although, *pvl* and *tst* have been detected in few isolates, their importance as virulence factors should be emphasized. The research on antibiotic resistance showed the diversity on resistance among the isolates, and reported the presence of methicillin-resistant *Staphylococcus aureus* (MRSA), where many virulence factor genes were detected. Using molecular typing (SCC*mec* and *spa* typing), we could observe different strains, which may suggest several sources of contamination. The t605 was detected in high frequency and in samples from all dairies indicating an endemic clone was present. Additionally, we described a new *spa* type, t14969, isolated from cheese.

The identification and abundance of *S. aureus* in dairy products is common (CARFORA et al., 2015; ROLA et al., 2015). *Minas Frescal* cheese is composed of 25% to 44.9% of fat and moisture ≥55% which is consumed fresh (BRAZIL, 2004) and due to its characteristics, is a good environment for bacterial growth. André et al. (2008) studied a dairy processing plant in Goiás State, Brazil, where they collected 140 samples (food, food handler, and fresh cheese). They obtained 73 *S. aureus* isolates, 75%, 66.7% and 70.8% from handler, milk, and cheese samples, respectively, showing the prevalence of this pathogen in processing plant as was observed in the present study. All species identified also have been reported in other

studies involving *Staphylococcus* in dairies and bovine milk (ANDRÉ et al., 2008; LANGE et al., 2015; OTE et al., 2011).

In our study 85% of isolates were enterotoxigenic, carrying one or more SE gene. Aydin, Sudagidan and Muratoglu (2011) observed that 62.6% of 147 isolates were enterotoxigenic and they performed PCR to investigate seventeen SE-encoding genes. Ote et al. (2011) investigated the presence of thirteen SE-encoding genes in *S. aureus*. The enterotoxin genes encode SEH, followed by SElX, and SER were predominantly identified. Ote et al. (2011) reported that only 1.7% of the isolates associated with bovine mastitis carried *seh*. Interestingly, *seh* was reported as rare or absent in mastitis associated isolates (OTE et al., 2011). However, Liu et al. (2014) described *seh* as the most prevalent gene among isolates from bovine mastitis; a disease that it is an important entry point for the pathogen in dairy products. In another study using isolates from food samples, *seh* was found in 16.3% of the isolates (AYDIN; SUDAGIDAN; MURATOGLU, 2011). Therefore, our findings suggest that *seh* is a common gene in staphylococci and is distributed in these specific cheese productions, predominantly in isolates from raw milk. André et al. (2008) reported that raw milk appears to be the potential source of cheese contamination. The second most identified SE gene was *selx*, which was recently discovered and was described as a unique core genome-encoded superantigen (SAg) (WILSON et al., 2011). It was acquired by an ancestor of *S. aureus* species, and has undergone functional and genetic diversification in clones that infect humans and animals (WILSON et al., 2011), which can explain the high frequency of the *selx* gene. To the best of our knowledge, there is no report identifying *selx* in isolates from cheeses, and samples from food processing plant. The gene *ser* was widely identified (42% of the isolates); however studies have demonstrated the lower frequency of this gene, e.g. 5.4% (isolates from food poisoning cases) (CHIANG et al., 2008), 4.3% (isolates from bovine mastitis) (LIU et al., 2014) and 28.6% (isolates from milk, and dairy products of different animals) (CARFORA et al., 2015). Both enterotoxins SEH, and SER have emetic activity and staphylococcal food poisoning (SFP) caused by SEH has been described (IKEDA et al., 2005; JØRGENSEN et al., 2005). SER has been identified in isolates from food poisoning cases (CHIANG et al., 2008); which is a concern because the screening procedures commonly performed in food typically involve detection of classical enterotoxins (LIS et al., 2012). The frequency of classical enterotoxins was low or absent and similar results have been

cited in previous studies (ARCURI et al., 2010; AYDIN; SUDAGIDAN; MURATOGLU, 2011; LIU et al., 2014). Moreover, isolates from cheese before and after packing boasted the enterotoxin gene in 100%, and 95.83%, respectively, this high prevalence of isolates from ready-to-eat food are a concern. In addition, fifty-five enterotoxin profiles were observed in one hundred isolates enrolled in this study, showing the high distribution of enterotoxin genes, e.g. in a previous study fifty-nine superantigenic toxin gene profiles in 229 isolates were described (OTE et al., 2011).

Regarding hemolysin genes, 63.4% of isolates carried one or more genes, and it is important to underline the significance of *hlb*, since beta-hemolytic *S. aureus* has been reported as being more virulent to cattle than beta-hemolytic negative strains. The *hlb* may be an active factor in development of bovine mastitis (LARSEN; AARESTRUP; JENSEN, 2002); which may indicate that the presence of the majority of the staphylococci in cheese production process was caused by use of raw milk contaminated by staphylococci from mastitic milk.

Herein, exfoliative toxin encoding genes were not detected and similar results were previously cited (AYDIN; SUDAGIDAN; MURATOGLU, 2011; OTE et al., 2011). The *tst* gene was identified only in one isolate from raw milk, which showed several enterotoxin genes, and resistance to antibiotics. Similarly, another study showed that 2.8% of the isolates from bulk tank milk, and *Minas* cheese were positive for *tst* (ARCURI et al., 2010). In isolates from mastitic milk, the presence of *tst* gene was reported to be 27.5% (OTE al., 2011). Regarding the Panton-Valentine leukocidin (*pvl* gene), Naimi et al. (2003) reported that the MRSA acquired or associated with the community (CA-MRSA) boasts *pvl*. In our study, it was observed that both isolates carrying *pvl* were MRSA, and these are SCC*mec* types associated with CA-MRSA.

Antibiotic resistance genes were identified and the *mec*A positive isolates (6.81% of the *S. aureus* isolates) detected were carrying different genes, and presenting different *spa* types. Meanwhile, Rola et al. (2015) studied isolates from raw milk and observed no positive isolate for the *mecA*, and *mecC* genes. MRSA containing *mec*C from human, and cattle were reported in the United Kingdom, and Denmark (CUNY et al., 2011). In high proportions, MRSA implies greater risk, and the need for more toxic drug treatments, thus increasing costs and side-effects, and ultimately driving resistance in this species and/or in other species (WORLD HEALTH ORGANIZATION, 2014). MRSA have evolved with increased pathogenic potential,

the strains are capable of causing persistent infections in hospitalized patients, and healthy individuals in the community (CHATTERJEE; OTTO, 2013). Moreover, MRSA harbor resistance to majority of the antibiotics (CHATTERJEE; OTTO, 2013). On SCC*mec* typing, the types III and IV were found, they are usually associated with HA-MRSA (hospital-associated MRSA), and CA-MRSA (community-associated MRSA) or LA-MRSA (livestock-associated MRSA), respectively (CATRY et al., 2010; DEURENBERG; STOBBERINGH, 2009). Currently, LA-MRSA infection has been cited in several species, including bovines (LUINI et al., 2015). Thus, it is important to know SCC*mec* elements because they work as a vehicle to transfer genetic markers, as genes mediating antibiotic resistance or virulence (ZHANG et al., 2005). Although, MRSA have been widely used to define *S. aureus* resistant against methicillin, the Centers for Disease Control and Prevention cited that this definition is not only regarding methicillin resistance, the MRSA also included common antibiotics, e.g. oxacillin (CHATTERJEE; OTTO, 2013). The *mec*A, in general, present resistance against many β-lactam antibiotics, and SCC*mec* elements may contain genes for resistance to antibiotics not β-lactam (CHATTERJEE; OTTO, 2013). Thus, the not detection of *mec*A in isolates positives for antibiotic used to screening MRSA may suggest the presence of other resistance mechanism, not classical MRSA, such as modified *S. aureus* (MODSA) which possess modification of existing penicillin-binding proteins (BHUTIA et al., 2012). Moreover, the absence of resistance to antibiotic considered markers, e.g. oxacillin and penicillin, in isolate *mec*A positive can be a result intrinsic to the method used. Oxacillin disk diffusion can fail to detected heterogeneous MRSA populations, which motivated also the use of cefoxitin disc test described as more efficient to methicillin resistance screening (MAALEJ et al., 2012).

Using phenotypic test of antibiotic resistance, we detected resistance to penicillin was the most frequent among the isolates. In addition, resistance for all antibiotics was identified as well as multiresistance. Rola et al. (2015) tested 10 antibiotics using the minimum inhibitory concentration method in isolates from milk, they detected that 43% of isolates were resistant to one or more antibiotic and all strains were susceptible to ciprofloxacin, erythromycin, gentamicin, cefoxitin, and streptomycin, they reported also that resistance to penicillin was the most frequent. André et al., (2008) did not detect resistant for ciprofloxacin, gentamicin, vancomycin, they observed that resistance to penicillin (69.9% of isolates) was most frequent than other antibiotics tested. The majority strains are resistant to penicillin or ampicillin

because of long-term of use of these antibiotics in agriculture and healthcare (MOON et al., 2007).

Regarding typing, we identified several *spa* types across *S. aureus* from different samples. Aydin, Sudagidan and Muratoglu (2011) reported that the high genetic diversity indicates that contamination of food products with *S. aureus* could originate from different sources, e.g. pre-processing environments, processing areas, and market place. Furthermore, the same *spa* type was detected in different samples, e.g. t127 was identified in isolates from handler, cheese, mold cheese, and raw milk, which can suggest cross contamination. The type t605 was widely identified; this type t605 was identified in 37.5% of isolates from mastitic milk in Brazil (SILVA et al., 2013). Another study using isolates from food handlers this type was not detected (HO; BOOST; O'DONOGHUE, 2015). However, in the present study, 10 isolates from handlers were identified with this *spa* type. Ho et al. (2015) indicated t127 as the most abundant among the types found in *S. aureus* from food handlers, and they reported that it was observed in persistent and transient carriers. In our study, the type t127 was the second type most detected and it had between two to nine enterotoxin genes and one to five hemolysin genes. Both, t605 and t127 were previously identified as abundant in isolates from mastitis milk in the same region sampled in Brazil (SILVA et al., 2013).

The new *spa* type t14969 had the first report in this study; it was ST30, and CC30. Contemporary *S. aureus* CC30 lineage is associated with infections, infections considered complicated (SHARMA-KUINKEL et al., 2015). The lineage diverged from the phage-type 80/81 *S. aureus*, clone that was responsible for the major bacterial epidemic in the 20th century (SHARMA-KUINKEL et al., 2015).

5.5 Conclusions

In this study, we identified and characterized staphylococci isolated from dairy processing plants, located in São Paulo State, Brazil. The majority of isolates from different samples, e.g. raw milk, table, and cheese, were identified as *S. aureus*. In addition, CNS were also detected and were carrying several genes as well as demonstrating antibiotic resistance. The enterotoxin genes were widely identified across isolates, the most abundant gene being *seh*, followed by *sel*x, and *ser*. The SEH represents an important staphylococcal enterotoxin, since it can cause SFP, thus highlighting the importance of screening for others staphylococcal enterotoxins

in food. Hemolysin, Panton-Valentine leukocidin, and toxic shock syndrome toxin genes were also detected among the isolates, illustrating the pathogenic potential of these. Resistance to all antibiotics tested was observed and many of the isolates were multiresistant. MRSA were identified, specifically SCCmec III, IVa, IVd, and two nontypeable, and carried hemolysin, enterotoxin, Panton-Valentine leukocidin, tetracycline, and erythromycin genes. Using spa typing, we detected several types distributed in the isolates from different samples; however, a dominant type was identified. The majority of *S. aureus* was identified as t605, and this was found in all dairies.

Most importantly, a new *spa* type was described, the *spa* type t14969 (ST30 and CC30) isolated from fresh cheese samples after packing. According to all findings reported in the present study, it can be suggested that antibiotic resistance and highly virulent strains from different sources can be found in a dairy processing environment in Brazil, which is a real concern for producers and consumers. Thus, improvements are still needed in Brazilians dairies to avoid contamination by staphylococci. Prevention and control actions in all dairy processing should be observed and improved to warrant food safety.

References

AARESTRUP, F.M.; DANGLER, C.A.; SORDILLO, L.M. Prevalence of coagulase gene polymorphism in *Staphylococcus aureus* isolates causing bovine mastitis. **Canadian Journal of Veterinary Research**, Ottawa, v. 59, n. 2, p. 124-128, Apr. 1995.

ANDRÉ, M.C.D.P.B.; CAMPOS, M.R.H; BORGES, L.J; KIPNIS, A. Comparison of *Staphylococcus aureus* isolates from food handlers, raw bovine milk and Minas Frescal cheese by antibiogram and pulsed-field gel electrophoresis following SmaI digestion. **Food Control**, Guildford, v. 19, n. 2, p. 200-207, Feb. 2008.

ARCURI, E.F.; ANGELO, F.F.; GUIMARÃES, M.F.; TALON, R.; BORGES, M.DE F.; LEROY, S.; LOISEAU, G.; LANGE, C.C.; ANDRADE, N.J.; MONTET, D. Toxigenic status of *Staphylococcus aureus* isolated from bovine raw milk and Minas Frescal cheese in Brazil. **Journal of Food Protection**, Ames, v. 73, n. 12, p. 2225-31, Dec. 2010.

AYDIN, A.; SUDAGIDAN, M.; MURATOGLU, K. Prevalence of staphylococcal enterotoxins, toxin genes and genetic-relatedness of foodborne *Staphylococcus aureus* strains isolated in the Marmara Region of Turkey. **International Journal of Food Microbiology**, Washington, v. 148, n. 2, p. 99-106, Aug. 2011.

BECKER, K.; HEILMANN, C.; PETERS, G. Coagulase-negative staphylococci. **Clinical Microbiology Reviews**, Washington, v. 27, n. 4, p. 870-926, Oct. 2014.

BENSON, D.A.; KARSCH-MIZRACHI, I.; LIPMAN, D.J.; OSTELL, J.; SAYERS, E.W. GenBank. **Nucleic Acids Research**, London, v. 37, D26-D31, Jan. 2009.

BHUTIA, K.O.; SINGH, T.S.; BISWAS, S.; ADHIKARI, L. Evaluation of phenotypic with genotypic methods for species identification and detection of methicillin resistant in *Staphylococcus aureus*. **International Journal of Applied and Basic Medical Research**, v. 2, n. 2, p. 84-91, July 2012.

BRAZIL. Ministério da Agricultura, Pecuária e Abastecimento. **Instrução normativa nº 4, de 01 de março de 2004**. Disponível em: <http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&id=5974>. Acesso em: 04 maio 2016.

CARFORA, V.; CAPRIOLI, A.; MARRI, N.; SAGRAFOLI D.; BOSELLI C.; GIACINTI, G.; GIANGOLINI, G.; SORBARA, L.; DOTTARELLI, S.; BATTISTI, A.; AMATISTE, S. Enterotoxin genes, enterotoxin production, and methicillin resistance in *Staphylococcus aureus* isolated from milk and dairy products in Central Italy. **International Dairy Journal**, Amsterdam, v. 42, p. 10-15, Mar. 2015.

CATRY, B., VAN DUIJKEREN, E., POMBA, M.C., GREKO, C., MORENO, M.A., PYÖRÄLÄ. S., RUZAUSKAS, M.; SANDERS, P.; THRELFALL, E.J.; UNGEMACH, F.; MUNOZ-MADERO, C.; TORREN-EDO, J. Reflection paper on MRSA in food-producing and companion animals: epidemiology and control options for human and animal health. **Epidemiology & Infection**, Cambridge, v. 138, n. 5, p. 626-644, May 2010.

CHATTERJEE, S.S.; OTTO, M. Improved understanding of factors driving methicillin-resistant *Staphylococcus aureus* epidemic waves. **Clinical Epidemiology**, Auckland, v. 5, p. 205-217, July 2013.

CHIANG, Y.C.; LIAO, W.W.; FAN, C.M.; PAI, W.Y.; CHIOU, C.S.; TSEN, H.Y. PCR detection of staphylococcal enterotoxins (SEs) N, O, P, Q, R, U, and survey of SE types in *Staphylococcus aureus* isolates from food-poisoning cases in Taiwan. **International Journal of Food Microbiology**, Washington, v. 121, n.1, p. 66-73, Jan. 2008.

CLINICAL LABORATORY STANDARDS INSTITUTE. Performance standards for antimicrobial susceptibility testing. In: _____. **National Committee for Clinical Laboratory standards**. Wayne, 2015. p. 146-156. (Twenty-fifth Informational Supplement, M100–S25).

CREMONESI, P.; LUZZANA, M.; BRASCA, M., MORANI, S.; LODI, R.; VIMERCATI, C.; AGNELLINI, D.; CARAMENTI, G.;MORONI, P.; CASTIGLIONI, B. Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. **Molecular and Cell Probes**, London, v. 19, n. 5, p. 299-305, Oct. 2005.

CUNY, C.; LAYER, F.; STROMMENGER, B.; WITTE, W. Rare occurrence of methicillin-resistant *Staphylococcus aureus* CC130 with a Novel *mec*A Homologue in Humans in Germany. **PLoS One**, San Francisco, v. 6, n. 9, p.1-5, Sep. 2011.

DEURENBERG, R.H.; STOBBERINGH, E.E. The molecular evolution of hospital- and community-associated methicillin-resistant *Staphylococcus aureus*. **Current Molecular Medicine**, Hilversum, v. 9, n.2, p. 100-115, Mar. 2009.

DOWNES, F.P.; ITO, K. **Compendium of methods for the microbiological examination of foods**. 4th ed. Washington: American Public Health Association, 2001. 676p.

ENRIGHT, M.C.; DAY, N.P.; DAVIES, C.E.; PEACOCK, S.J.; SPRATT, B.G. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. **Journal of Clinical Microbiology**, Washington, v. 38, n. 3, p. 1008-1015, Mar. 2000.

FISCHER, A.; FRANCOIS, P.; HOLTFRETER, S.;BROEKER, B.; SCHRENZEL, J. Development and evaluation of a rapid strategy to determine enterotoxin gene content in *Staphylococcus aureus*. **Journal of Microbiological Methods**, Amsterdam, v. 77, n. 2, p. 184-190, May 2009.

GÓMEZ-SANZ, E.; TORRES, C.; LOZANO, C.; FERNÁNDEZ-PÉREZ, R.; ASPIROZ, C.; RUIZ-LARREA, F.; ZARAZAGA, M. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. **Foodborne and Pathogens Disease**, Larchmont, v. 7, n. 10, p. 1269-1277, Oct. 2010.

HAYAKAWA,Y.; HASHIMOTO, N.; IMAIZUMI, K.; KAIDOH, T.; TAKEUCHI, S. Genetic analysis of exfoliative toxin A-producing *Staphylococcus aureus* isolated from mastitic cow's milk. **Veterinary Microbiology**, Amsterdam, v. 78, n. 5, p. 39–48, Jan. 2001.

HO, J.; BOOST, M.V.; O'DONOGHUE, M.M. Tracking sources of *Staphylococcus aureus* hand contamination in food handlers by *spa* typing. **American Journal of Infection Control**, New York, v. 43, n.7, p. 759–761, July 2015.

IKEDA, T.; TAMATE, N.; YAMAGUCHI, K.; MAKINO, S. Mass outbreak of food poisoning disease caused by small amounts of staphylococcal enterotoxins A and H. **Applied and Environmental Microbiology**, Washington, v. 71, n. 5, p. 2793-2795, May 2005.

JAMALI, M.; PAYDAR, M.; RADMEHR, B.; ISMAIL, S.; DADRASNIA, A. Prevalence and antibiotic resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. **Food Control**, Guildford, v. 54, p. 384-388, Aug. 2015.

JARRAUD, S.; MOUGEL, C.; THIOULOUSE, J.; LINA, G.; MEUGNIER, H.; FOREY, F.; NESME, X.; ETINNE, J.; VANDENESCH, F. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. **Infection and Immunity**, Washington, v. 70, n. 2, p.631-641, Feb. 2002.

JEAN-BAPTISTE, N.; BENJAMIN JR., D.; COHEN-WOLKOWIEZ, M.; FOWLER JR., V.G.; LAUGHON, M.; CLARK, R.H.; SMITH, P.B. Coagulase-negative staphylococcal infections in the neonatal intensive care unit. **Infection Control & Hospital Epidemiology**, Chicago, v. 37, n. 7, p. 679-686, July 2011.

JOHNSON, W.M.; TYLER, S.D.; EWAN, E.P.; ASHTON, F.E.; POLLARD, D.R.; ROZEE, K.R. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. **Journal of Clinical Microbiology**, Washington, v. 29, n. 3, p. 426–430, Mar. 1991.

JORGENSEN, H.J.; MATHISEN, T.; LOVSETH, A.; OMOE, K.; QVALE, K.S.; LONCAREVIC, S. An outbreak of *Staphylococcus* food poisoning cause by enterotoxin H in mashed potato made with raw milk. **FEMS Microbiology Letters**, Amsterdam, v. 252, n. 2, p. 267-272, Nov. 2005.

KONDO, Y.; ITO, T.; MA, X.X.; WATANABE, S.; KREISWIRTH, B.N.; ETIENNE, J.; HIRAMATSU, K. Combination of multiplex PCRs for Staphylococcal Cassette Chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. **Antimicrobial Agents and Chemotherapy**, Washington, v. 51, n. 1, p. 264–274, Jan. 2007.

LANGE, C.C.; BRITO, M.A.V.P.; REIS, D.R.L.; MACHADO, M.M; GUIMARÃES, A.S.; AZEVEDO, A.L.; SALLES, E.B.; ALVIM, M.C; SILVA, F.S.; MEURER, I.R. Species-level identification of staphylococci isolated from bovine mastitis in Brazil using partial 16S rRNA sequencing. **Veterinary Microbiology**, Amsterdam, v. 176, n. 3/4, p. 382-388, Apr. 2015.

LARSEN, H.D.; AARESTRUP, F.M.; JENSEN, N.E. Geographical variation in the presence of genes encoding superantigenic exotoxins and beta-hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. **Veterinary Microbiology**, Amsterdam, v. 26, n. 1, p. 61-67, Feb. 2002.

LINA, G.; PIÉMONT, Y.; GODAIL-GAMOT, F.; BES, N.M.; PETER, M.O.; GAUDUCHON, V.; VANDENESCH, F.; ETIENNE, J. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. **Clinical Infectious Diseases**, Chicago, v. 29, n. 5, p. 1128-1132, Nov. 1999.

LIS, E.; PODKOWIK, M.; SCHUBERT, J.; BYSTRON, K.; STEFANIAK, T.; BANIA, J. Production of staphylococcal enterotoxin R by *Staphylococcus aureus* strains. **Foodborne Pathogens and Disease**, Larchmont, v. 9, n. 8, p. 762-766, Aug. 2012.

LIU, Y.; CHEN, W.; ALI, T.; ALKASIR, R.; YIN, J.; LIU, G.; HAN, B. Staphylococcal enterotoxin H induced apoptosis of bovine mammary epithelial cells *in vitro*. **Toxins**, Basel, v. 6, n. 12, p. 3552-3567, Dec. 2014.

LUINI, M., CREMONESI, P., MAGRO, G., BIANCHINI, V., MINOZZI, G., CASTIGLIONI, B.; PICCININI, R. Methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: genotyping of isolates. **Veterinary Microbiology**, Amsterdam, v. 178, n. 3-4, p. 270-274, Aug. 2015.

MAALEJ, S.M.; RHIMI, F.M.; FINES, M.; MNIF, B.; LECLERCQ, R.; HAMMAMI, A. Analysis of borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) strains isolated in Tunisia. **Journal of Clinical Microbiology**, Washignton, v. 50, n. 10, p. 3345-3348, Oct. 2012.

MEHROTRA, M.; WANG, G.; JOHNSON, W.M. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. **Journal of Clinical Microbiology**, Washington, v. 38, n. 3, p.1032–1035, Mar. 2000.

MOON, J.S.; LEE, A.R.; KANG, H.M.; LEE, E.S.; KIM, M.N.; PAIK, Y.H.; PARK, Y.H.; JOO, Y.S.; KOO, H.C. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. **Journal of Dairy Science**, Champaign, v. 90, n. 3, p. 1176-1185, Mar. 2007.

NAIMI, T.S.; LEDELL, K.H.; COMO-SABETTI, K.; BORCHARDT, S.M., BOXRUD, D.J.; ETIENNE, J.; JOHNSON, S.K.; VANDENESCH, F.; FRIDKIN, S.; O'BOYLE, C.; DANILA, R.N.; LYNFIELD, R. Comparison of community- and health care–associated methicillin-resistant *Staphylococcus aureus* infection. **JAMA: the Journal of the American Medical Association**, Chicago, v. 290, n.22, p. 2976-2984, Dec. 2003.

NASHEV, D.; TOSHKOVA, K.; SALASIA, S.I.;HASSAN, A.A.; LAMMLER, C.; ZSCHÖCK, M. Distribution of virulence genes of *Staphylococcus aureus* isolated from stable nasal carriers. **FEMS Microbiology Letters**, Amsterdam, v. 233, n. 1, p. 45–52, Apr. 2004.

OMOE, K.; HU, D.L.; TAKAHASHI-OMOE, H.; NAKANE, A.; SHINAGAWA, K. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. **FEMS Microbiology Letters**, Amsterdam, v. 246, n. 2, p. 191-198, May 2005.

OMOE, K.; ISHIKAMA, M.; SHIMODA, Y.; HU, D.L.; UEDA, S.; SHINAGAWA, K. Detection of *seg, seh* and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolate harboring *seg*, *seh* or *sei* genes. **Journal of Clinical Microbiology**, Washington, v. 40, n. 3, p. 857–862, Mar. 2002.

ONO, H.K.; OMOE, K.; IMANISHI, K.; IWAKABE, K.; HU, D.L.; KATO, H.; SAITO, N.; NAKANE, A.; UCHIVAMA, T.; SHINAGAWA, K. Identification and characterization of two novel staphylococcal enterotoxins, types S and T. **Infection and Immunity**, Washington, v. 76, n. 11, p. 4999-5005, Nov. 2008.

OTE, I.; TAMINIAU, B.; DUPREZ, J.N.; DIZIER, I.; MAINIL, J.G. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. **Veterinary Microbiology**, Amsterdam, v.153, n. 3/4, p. 285-292, Dec. 2011.

PODKOWIK, M.; PARK, J.Y.; SEO, K.S.; BYSTROŃ, J.; BANIA, J. Enterotoxigenic potential of coagulase-negative staphylococci. **International Journal of Food Microbiology**, Washington, v. 163, n. 1, p. 34-40, Apr. 2013.

ROLA, J.G; KORPYSA-DZIRBA, W.; CZUBKOWSKA, A.; OSEK, J. Prevalence of enterotoxin genes and antibiotic resistance of coagulase-positive staphylococci recovered from raw cow milk. **Journal of Dairy Science**, Champaign, v. 98, n. 7, p. 4273-4378, July 2015.

SASAKI, T.; TSUBAKISHITA, S.; TANAKA, Y.; SAKUSABE, A.; OHTSUBA,M.; HIROTAKI, S.; KAWAKAMI, T.; FUKATA, T.; HIRAMATSU, K. Multiplex-PCR method species identification of coagulase-positive staphylococci. **Journal of Clinical Microbiology**, Washington, v. 48, n. 3, p. 765-769, Mar. 2010.

SAYERS, E. W.; BARRETT,T.; BENSON, D.A.; BRYANT,S.H.; CANESE,K.; CHETVERNIN,V.; CHURCH,D.M.; DICUCCIO, M.; EDGAR,R.; FEDERHEN,S.; FEOLO,M.; GEER, L.Y.; HELMBERG, W.; KAPUSTIN, Y.; LANDSMAN, D.; LIPMAN, D.J.; MADDEN, T.L.; MAGLOTT, D.R.; MILLER, V.; MIZRACHI,I.; OSTELL,J.; PRUITT, K.D.; SCHULER, G.D.; SEQUEIRA, E.; SHERRY, S.T.; SHUMWAY,M.; SIROTKIN, K.; SOUVOROV, A.; STARCHENKO,G.; TATUSOVA, T.A.; WAGNER,L.; YASCHENKO,E.; YE, J. Database resources of the National Center for Biotechnology Information. **Nucleic Acids Research**, London, v. 37, D5-D15, Jan. 2009.

SHAHEEN, M.; TANTARY, H.A.; NABI, S.U.A treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention and control strategy. **Advances in Dairy Research**, Las Vegas, v. 4, p. 1-10, Jan. 2016.

SHARMA-KUINKEL, B.K.; MONGODIN, E.F.; MYERS, J.R.; VORE, K.L.; CANFIELD, G.S.; FRASER, C.M.; RUDE, T.H.; FOWLER, V.G.; GILL, S.R. Potential influence of *Staphylococcus aureus* Clonal Complex 30 genotype and transcriptome on hematogenous infections. **Open Forum Infectious Diseases**, Oxford, v. 2, n.3, p.1-14, July 2015.

SILVA, N.C.C.; GUIMARÃES, F.F.; MANZI, M.P.; BUDRI, P.E.; GÓMEZ-SANZ, E.; BENITO, D.; LANGONI, H.; RALL, V.L.; TORRES, C. Molecular characterization and clonal diversity of methicillin susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil. **Journal of Dairy Science**, Champaign, v. 96, n. 11, p. 6856-6862, 2013.

SILVA, N.C.C.; GUIMARÃES, F.F.; MANZI, M.P.; GÓMEZ-SANZ, E.; GÓMEZ, P.; ARAÚJO-JÚNIOR, J.P.; LANGONI, H.; RALL, V.L.; TORRES, C. Characterization of methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. **Antonie van Leeuwenhoek**, Dordrecht, v. 106, n. 2, p. 227–223, Aug. 2014.

THOMAS, D.; DAUWALDER, O.; BRUN, V.; BADIOU, C.; FERRY, T.; ETIENNE, J.; VANDENESCH, F.; LINA, G. *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. **Infection and Immunity**, Washington, v. 77, n. 5, p. 2043–2050, May 2009.

van de KLUNDERT, J.A.M.; VLIEGENTHART, J.S. PCR detection of genes for aminoglycoside-modifying enzymes. In: PERSING, D.H.; SMITH, T.F.; TENOVER, F.C.; WHITE, T.J. **Diagnostic molecular microbiology**. Washington: American Society for Microbiology, 1993, p. 547–552.

WORLD HEALTH ORGANIZATION. **Antibiotic resistance**: global report of surveillance.2014. Disponível em: <http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1>. Acesso em: 05 maio 2016.

WILSON, G.J.; SEO, K.S., CARTWRIGHT, R.A.; CONNELLEY, T.; CHUANG-SMITH, O.N.; MERRIMAN, J.A.; GUINANE, C.M.; PARK, J.Y.; BOHACH, G.A.; SCHLIEVERT, P.M.; MORRISON, W.I.; FITZGERALD, J.R. A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. **PLoS Pathogens**, San Francisco, v. 7, p. 1-16, June 2011.

XING, X.; ZHANG, Y.; WU, Q.; WANG, X.; GE, W.; WU, C. Prevalence and characterization of *Staphylococcus aureus* isolated from goat milk powder processing plants. **Food Control**, Guildford, v. 59, p. 644-650, Jan. 2016.

YAMAGUCHI, T.; NISHIFUJI, K.; SASAKI, M.; FUDABA, Y.; AEPFELBACHER, M.; TAKATA, T.; OHARA, M.; KOMATSUZAWA, H.;AMAGAI, M.; SUGAI, M. Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. **Infection and Immunity**, Washington, v. 70, n. 10, p. 5835–5845, Oct. 2002.

ZHANG, K.; McCLURE, J.A.; ELSAYED, S.; LOUIE, T.; CONLY, J.M. Novel Multiplex PCR assay for characterization and concomitant subtyping of Staphylococcal Cassette Chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. **Journal of Clinical Microbiology**, Washington, v. 43, n. 10, p. 5026-5033, Oct. 2005.

6 GENERAL CONCLUSIONS

This study characterized and analyzed bacterial isolates and microbiome of raw materials from the dairies. The relevance of raw milk quality regarding dairy herd health and storage on dairy farms was discussed. Furthermore, contamination in the processing lines of dairy product was observed. Conventional microbiological methods were used as well as a molecular biology approach including PCR and next generation sequencing using the Illumina MiSeq platform.

Staphylococci isolates from mastitic milk were analyzed and several virulence factor genes were detected as well as antibiotic resistance. Methicillin-resistant *Staphylococcus aureus* (MRSA) were identified. Its presence is a concern, since these strains can be more virulent and boast increased resistance. *Staphylococcus aureus* was abundant and enterotoxin genes were widely identified, with *seh* and *sel*x genes as the most frequent. Genes encoding classical enterotoxins were not among the most detected, some of which were not detected. Few coagulase negative staphylococci (CSN) were identified; however, these showed virulence potential. Regarding *spa* typing, the t605 (*agr* type II) was detected in the majority of the *S. aureus*.

In this work, an emerging mastitis pathogen, associated with a mastitis outbreak was investigated. Herein, *Lactococcus* was investigated and was identified as abundant in mastitic milk samples from clinical mastitis cases. Furthermore, when compared with healthy milk a large difference in relative abundance of the genus was evident. However, further investigations should be conducted with a focus on *L. lactis* as mastitis pathogen.

An overview of the microbiome from bulk tank milk samples was generated and genera previously not reported in raw milk were identified (*Thermoanaerobacterium* and *5-7N15*). The core microbiome was determined presented, which included spoilage, pathogens, and spore forming bacteria. Several bacterial taxa were detected with higher relative abundance in samples classified as high SCC when compared to samples classified as low SCC, e.g. *Corynebacterium, Streptococcus, Lactobacillus,* and *Coxiella*. Similarly, samples classified as high SPC presented higher relative abundance for specific bacterial taxa when compared to low SPC, e.g. *Acinetobacter, Enterobacteriaceae, Corynebacterium,* and *Streptococcus*. Moreover, *Streptococcus* was a genus with high prevalence and significance following analysis of samples with high SCC and SPC. Interestingly, bacterial load of

bulk tank milk samples correlated with diversity (Shannon index) indicating that microbiome of high bacterial load samples are dominated by smaller groups of bacterial taxa.

Lastly, highly virulent and antibiotic resistant staphylococci were identified from raw milk and production lines of *Minas Frescal* cheese. This correlates to data outlined in Chapter 2; however, a new spa type was identified. *spa* type t14969 was discovered, which was identified as ST30 and CC30. Herein, staphylococci with potential to cause disease and presenting antibiotic resistance were detected in ready-to-eat cheese highlighting the need to implement improvements in cheese processing.

Thus, in this study was possible to highlight the importance of dairy herd health, raw milk quality, and quality control in the dairy products processing in order to provide safety food to consumers.