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"Luiz de Queiroz" College of Agriculture

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

Marjory Xavier Rodrigues

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Marjory Xavier Rodrigues
Food Technologist

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Advisor:
Prof. Dr. **SOLANGE GUIDOLIN CANNIATTI-BRAZACA**

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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.

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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.