

University of São Paulo
"Luiz de Queiroz" College of Agriculture

**Bovine milk microbiota: molecular characterization and evaluation of mastitis
pathogens detection methodologies**

Priscila Anchieta Trevisoli

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

Piracicaba
2022

Priscila Anchieta Trevisoli
Biotechnologist

Bovine milk microbiota: molecular characterization and evaluation of mastitis pathogens detection methodologies

versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:

Prof. Dr. **LUIZ LEHMANN COUTINHO**

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

Piracicaba
2022

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Trevisoli, Priscila Anchieta

Bovine milk microbiota: molecular characterization and evaluation of mastitis pathogens detection methodologies / Priscila Anchieta Trevisoli. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2022.

47 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

1. Leite bovino 2. Microbioma 3. Sequenciamento 16S 4. Patógenos de mastite I. Título

DEDICATION

I dedicate this work to my lovely and supporting parents, sisters, life partner and daughter. You means everything to me.

ACKNOWLEDGMENTS

First, undoubtedly, my family! Mom and Dad, you believe in me like nobody else! Thank you for your endless support, love and dedication! My sisters, you girls are amazing, thanks for all procrastination times shared, family moments, and laughs. My boyfriend and life partner, thank you for all the love, caring, patience, incredible moments, and dreams shared. And Pipoca, the reason I'm dog mom, thank you for all unimaginable ways of showing love and friendship.

My advisor Dr Luiz Lehmann Coutinho. Thank you for all the opportunities, challenges, guidance, and support for the last years.

Centro de Genômica or still, LBA team: Aline, Bruna, Gabriel, Nirlei, Jorge, Ricardo, Giovanna, Carol (Fernandes), Marcela, Pilar, Mirele, Barbara, Polyana, Horacio, Letícia, Marcelo, Priscilla, Shilton, Vinicius, Luan, and Graciela. Thank you for all support during this work. And also, the COVID-19 Lab team: Aurea, Carol, Raquel, Simone, Talita, Bia, Clovis, Gustavo, Francisco, Marianne and Fernanda. We shared distressing moments wearing NB2 outfits, lunches and coffees in unsteady times, and, reunions about the experiments and next steps. Thank you for the laughs and friendship in difficult pandemic times.

Professor Dr. Marcos Veiga, Juliano Gonçalves, and Professor Dr. Paulo Machado, thank you for the support and partnership, your collaboration was fundamental to the development of this work.

My incredible and supporting friends: Gabriel, May Salvian, Bruna, Elis, Rhay, Cintia, May Pinheiro, Oli, Mari, Lou, Apoca, Kceta, Balela, Lola, Kipora, ACM, Taty, Kani, Marcis, Vicente, Naira, Fasci, Isa, Zeca, Gio, Pepe, Juguri, Bia, Marcela, Marcha, Silvia, Fran, Vitor and Andressa, for always being with me, having a coffee, drinking a beer or wine, talking by audio podcasts, or just sending good vibes, even geographically distant. Thank you!

Finally, thanks all the institutions for the opportunity and support for the development and conclusion of my Doctorate: University of São Paulo – “Luiz de Queiroz” College of Agriculture (USP/ESALQ), NGS Soluções Genômicas, Clínica do Leite, University of São Paulo – School of Veterinary Medicine and Animal Science (USP/FMVZ), and Qualilete Laboratory. A special acknowledgment to Coordination for the Improvement of Higher Education Personnel (CAPES) for the financial support with a scholarship, and to São Paulo Research Foundation (FAPESP) - grant (18/00995-6) for the financial support of the analysis, making possible the development of my thesis.

EPIGRAPH

*“A scientist in **her** laboratory is not a mere technician: **she** is also a child confronting a natural phenomena that impress **her** as though they were fairy tales”*

Marie Curie (adapted)

(Physicist, Chemist and first woman to win a Nobel Prize)

SUMMARY

RESUMO	8
ABSTRACT	9
LIST OF FIGURES	10
LIST OF TABLES	11
LIST OF ABBREVIATIONS	12
1. INTRODUCTION	13
References	15
2. CHARACTERIZATION OF CORE MICROBIOME IN BULK TANK MILK AND ITS CORRELATION WITH SOMATIC CELL COUNT AND TOTAL BACTERIAL COUNT	19
Abstract	19
2.1. Introduction	19
2.2. Materials and Methods	20
2.2.1 Samples	20
2.2.2 Somatic cell count and total bacterial count	20
2.2.3. DNA extraction, library generation, and sequencing	21
2.2.4. Sequencing data analysis, database dereplication, and taxonomy assignment	21
2.2.5. Core microbiome and diversity indices	21
2.2.6. Microbiome profile and correlation with SCS and SPS	22
2.3. Results	22
2.3.1. Sample size, somatic cell count, total bacterial count	22
2.3.2. Sequencing and core microbiome	22
2.3.3. Diversity indices	23
2.3.4. Microbiome profile of each dairy plant	24
2.3.5. Microbiome correlation with SCC	24
2.3.6. Microbiome correlation with SPC	25
2.4. Discussion	26
2.4.1. Core microbiome	26
2.4.2. Descriptive analysis of SCC, SPC, and diversity indices	27
2.4.3. Microbiome profile correlation with SCC	27
2.4.4. Microbiome profile correlation with SPC	28
2.5. Conclusion	28
References	29

3. COMPARIOSION OF STANDARD MEDIA CULTURE, CHROMOGENIC MEDIA CULTURE, MALDI-TOF, 16S SEQUENCING WITH QPCR MULTIPLEX FOR MASTITIS PATHOGEN DETECTION.....	33
Abstract.....	33
3.1. Introduction	33
3.2. Materials and methods.....	35
3.2.1. Samples.....	35
3.2.2. Standard culture identification.....	35
3.2.3. Chromogenic culture media identification.....	35
3.2.4. MALDI-TOF MS identification.....	36
3.2.5. Multiplex qPCR identification.....	36
3.2.6. V4-16S rRNA sequencing identification.....	36
3.2.7. Statistical analysis.....	37
3.3. Results	37
3.3.1. Pathogen diagnosis.....	37
3.3.2. Methodologies comparison with qPCR as gold standard.....	38
3.4. Discussion.....	42
3.5. Conclusion.....	44
References	44

RESUMO

Microbiota do leite bovino: caracterização molecular e avaliação de metodologias de detecção de patógenos de mastite

O leite bovino possui valores nutricionais elevados e é um alimento importante para a dieta humana. A sua composição rica em água, gorduras, proteínas, carboidratos, vitaminas e minerais proporcionam um ambiente favorável para o crescimento e proliferação de microrganismos. Microrganismos classificados como psicrotóxicos possuem a habilidade de proliferar e produzir enzimas proteolíticas e lipolíticas em temperaturas baixas. Essas enzimas são termo resistentes e por isso, mesmo com procedimentos térmicos para a eliminação do microrganismo, estas enzimas continuam ativas degradando proteínas e gorduras prejudicando a qualidade final do produto lácteo. Outros microrganismos patogênicos são veiculados pelo leite cru causando doenças em humanos, como por exemplo a brucelose, listeriose e tuberculose. A presença de certos microrganismos na glândula mamária bovina pode causar inflamações, doença mais conhecida como mastite. Devido ao seu grande impacto financeiro, a detecção correta e rápida do patógeno causador é muito importante. Atualmente, os métodos mais utilizados, como cultura convencional, cultura com meios cromogênicos e por espectrometria de massa (MALDI-TOF). Esses métodos são dependentes da cultura bacteriana e por isso são suscetíveis as suas limitações, como tempo de cultivo, altas taxas de falsos negativos, e baixa repetibilidade. Com os avanços das técnicas moleculares, métodos como PCR quantitativo (qPCR) e sequenciamento de parte do gene 16S vem estabelecendo espaço como metodologias alternativas para a detecção desses patógenos. Neste trabalho, o perfil microbiano do leite bovino cru produzido no sudeste brasileiro foi caracterizado utilizando sequenciamento da região v4 do gene 16S. O perfil microbiano também foi correlacionado com indicadores de qualidade do leite, como contagem de célula somática (CCS) e contagem bacteriana total (CBT). Como resultado, foi observada correlação positiva significativa entre a abundância de *Streptococcus agalactiae* com CCS e CBT; *Streptococcus dysgalactiae* foi correlacionado positivamente com CCS, *Lactococcus lactis* e *Staphylococcus aureus* com CBT. Além de estabelecer o perfil microbiano do leite, cinco metodologias, sendo elas cultura convencional, cultura com meio cromogênico, MALDI-TOF MS, qPCR multiplex e sequenciamento, foram avaliadas e então discutidas suas vantagens e limitações para uma detecção sensível, rápida e acurada de patógenos relacionadas a mastite.

Palavras-chave: Leite bovino, Microbioma, Sequenciamento 16S, Patógenos de mastite

ABSTRACT

**Bovine milk microbiota: molecular characterization and evaluation of mastitis pathogens
detection methodologies**

Bovine milk has high nutritional values and is an important food for the human diet. Its composition rich in water, fats, proteins, carbohydrates, vitamins and minerals provides a favorable environment for the growth and proliferation of microorganisms. Microorganisms classified as psychrotrophic have the ability to proliferate and produce proteolytic and lipolytic enzymes at low temperatures. These enzymes are heat resistant and therefore, even with thermal procedures for the elimination of the microorganism, these enzymes remain active, degrading proteins and fats, spoiling the final quality of the dairy product. Others pathogenic microorganisms are carried by milk causing diseases in humans, such as brucellosis, listeriosis and tuberculosis. The presence of certain microorganisms in the mammary gland can cause inflammation, a disease commonly known as mastitis. Due to its great financial impact, the correct and rapid detection of the causative pathogen is very important. Currently, the most used methods, such as conventional culture, culture with chromogenic media and by mass spectrometry (MALDI-TOF), are dependent on bacterial culture and therefore have a low efficiency. With the advances in molecular techniques, methods such as quantitative PCR (qPCR) and sequencing of part of the 16S gene have been establishing space as alternative methodologies for the detection of these pathogens. In this work, the bovine raw milk microbial profile produced in southeastern Brazil was characterized using sequencing of the v4 region of the 16S gene. The microbial profile was also correlated with milk quality indicators such as somatic cell count (SCC) and total bacterial count (SPC). As results, abundances of *Streptococcus agalactiae* was correlated with SCC and SPC; *Streptococcus dysgalactiae* was correlated with SCC, *Lactococcus lactis* and *Staphylococcus aureus* with SPC. In addition, we comparatively tested five methodologies, namely conventional culture, chromogenic medium culture, MALDI-TOF MS, multiplex qPCR and 16S sequencing, and discussed their advantages and limitations for a sensitive, rapid and accurate detection of mastitis-related pathogens.

Keywords: Bovine milk, Microbiome, Sequencing 16S, Mastitis pathogen

LIST OF FIGURES

- Figure 1. Venn diagram demonstrating the number of species identified in all 575 samples, and separated by each dairy plant (a and b)23
- Figure 2. Box-plot for chao1 richness index (a) and shannon diversity index (b) for plant a and plant b. *: significant *p-value* for chao1 index (0.007) and, for shannon index (1.293e-12).24
- Figure 3. Venn diagram for the species for each dairy plant that were used for correlation analysis.24
- Figure 4. Correlation plot between microbiome profile and somatic cell count (a), and microbiome profile and standard plate count (b). Different colors are assigned according to the dairy plants (a and b). Adjusted *p-values* were calculated by bonferroni multiple-comparison correction.....25
- Figure 5. Venn diagram of identified species by diagnosis methodologies. Multiplex qpcr (red), chromogenic media culture (green), maldi-tof ms (yellow), standard culture (brown), and v4-16s rrna sequencing (blue).38

LIST OF TABLES

Table 1. Number of farms, samples, and descriptive analysis of somatic cell score and standard plate score according to the dairy plant (a and b).	22
Table 2. Abundance and presence of the core microbiome in bulk tank milk samples.	23
Table 3. Diagnostic parameters as sensitivity (Se), specificity (Sp) accuracy (Ac), positive predictive values (PPV), and negative predictive value (NVP) of standard culture methodology.	39
Table 4. Diagnostic parameters as sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive value (NVP) of chromogenic media culture methodology.	40
Table 5. Diagnostic parameters as sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive value (NVP) of MALDI-TOF MS method.	41
Table 6. Diagnostic parameters as (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive value (NVP) of v4-16S rRNA sequencing methodology.	42

LIST OF ABBREVIATIONS

ASV	Amplicon sequencing variants
BMT	Bulk tank milk
CFU	Colony-forming unit
LAB	Lactic acid bacteria
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass
Max	Maximum
Min	Minimum
NGS	Next generation sequencing
QMP	Quantitative microbiome profile
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal DNA
RPM	rotações por minute
rRNA	Ribosomal RNA
SCC	Somatic cell count
SCS	Somatic cell score
SD	Standard Deviation
SPC	Standard plate count
SPS	Standard plate score
Staph.	Staphylococcus
Strep.	Streptococcus
Se	Sensitivity
Sp	Specificity
Ac	Accuracy
PPV	Positive predictive values
NPV	Negative predictive values
GP	Gram-positive
GN	Gram-negative
TP	True positives
TN	True negatives
FP	False positives
FN	False negatives

1. INTRODUCTION

Dairy products are consumed worldwide and present in the diet of most Brazilians. The bovine milk composition is divided into water and total dry extract, with 87% and 13% respectively. The total dry extract is composed of 4.2% fat; 3.4% protein (serum and casein); 4.6% carbohydrates (mainly lactose); and 0.9% of vitamins and minerals, such as calcium, phosphorus, sodium and potassium, vitamins B2, B12, B6 and A (LIND-MARK MÅNSSON, 2008). This composition can be affected by several factors such as: breed, year, season, diet, disease, lactation stage, animal age and others (WALSTRA, 1999).

Due to the rich composition in nutrients and water, combined with the neutral pH (6.2 to 6.8), milk becomes an ideal environment for the proliferation of microorganisms (QUIGLEY et al., 2013; VITHANAGE et al., 2016). The specific microbiota of milk composition directly impacts the profitability of farms, the technological process of production of dairy products and the final quality of the product (LE MARÉCHAL et al., 2011; QUIGLEY et al., 2013). Microorganisms with psychrotrophic behavior are capable of producing and releasing thermoresistant proteases and lipases, which can compromise the dairy product quality even after the elimination of the microorganisms in vegetative state by heat treatments (RIBEIRO JÚNIOR et al., 2017).

Raw milk is also a vehicle for certain microorganisms that cause disease in humans, such as brucellosis (*Brucella* spp.), listeriosis (*Listeria monocytogenes*) and tuberculosis (*Mycobacterium tuberculosis*). Genera such as *Bacillus* and *Clostridium* can form spores and thus become resistant to heat treatments, leading to late deterioration of dairy products and also causing gastrointestinal infections in consumers.

The microbiological profile of raw milk impacts the technological processes adopted by the dairy industries, the production yield, and the dairy product quality throughout the shelf life, making it important to characterize the bacterial community in raw bovine milk. The microbial profile of milk is closely related to the health of the animal, especially the health of the mammary gland. The most important disease of the dairy industry is mastitis, an infectious disease caused by microorganisms (bacteria and fungi) that affect the mammary glands (WATTS, 1988). Mastitis is considered the most expensive disease in the dairy industry worldwide and is associated with economic losses of up to 26 billion dollars annually (www.dairy.ahdb.org.uk). Among the economic losses resulting from this disease, we can highlight the milk production reduction, changes in milk quality, higher labor, diagnosis, and treatment costs, as well as reduced longevity of animals (OLIVEIRA; HULLAND; RUEGG, 2013).

Clinical mastitis can be detected through inspection of changes in the appearance of the milk, local signs in the mammary gland (edema, pain, redness) or systemic signs in the animal (fever, apathy, anorexia, and dehydration). On the other hand, subclinical mastitis, responsible for 90-95% of mastitis cases in the herd, does not show obvious signs of infection and requires specific methods for somatic cell detection. The most used technique for the determination of this type of cells is the somatic cell count (SCC).

Traditionally, microbiological methods have been used to enumerate and diagnose the microbial population based on enzymatic and immunological reactions, bacterial growth, and changes in milk composition. However, even though that cultivation studies have contributed to the understanding of microorganisms, and may guided the treatments and management decisions, the limitations of these methodologies have led to an inaccurate and incomplete knowledge, wherein a large majority of microorganisms remain unknown (DEUSCH et al., 2015). The inconsistency between in situ and cultivable diversity has stimulated molecular approaches independent of the use of cultures (HUGENHOLTZ; GOEBEL; PACE, 1998; ZOETENDAL et al., 2004).

The identification of the causative agent of mastitis can be performed with methods based on culture medium, however, approximately 25% of samples from clinical mastitis are culture-negative or do not present significant pathogens (BRADLEY et al., 2007). Likewise, more than 30% of samples from cows or udders with high CCS (subclinical mastitis) were reported to be culture negative (BRADLEY et al., 2007). In addition, traditional culture and microbiological identification by biochemical tests presents some other limitations, such as analysis time, differences in reliability between tests from different laboratories, large number of erroneously identified mastitis bacteria and the impossibility of identifying microorganisms at the strain level.

Several on-farm culture systems have been developed to facilitate and accelerate pathogens identification in milk and thus contribute to rapid decision-making for the treatment of cows with clinical mastitis. The first tests, based on MacConkey agar plates and blood, allowed a low-cost categorization of microorganisms into Gram-positive, Gram-negative and no growth (GANDA et al., 2016). Currently, new culture media have been developed, such as chromogenic media. In these medium, chromogenic substrates are incorporated into the culture medium which, when degraded by specific bacterial enzymes, produce colored substrates that can be differentiated by naked eye. The Accumast plate, developed by FERA Animal Health LCC (Ithaca, NY), uses chromogenic culture medium for the on-farm identification of specific mastitis-related pathogens such as: *Staphylococcus aureus*, *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Lactococcus* spp., *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., in a single plate with three selective chromogenic media. A study developed by Ferreira et al (2018) compared four on-farm culture methodologies commercially available in the United States, Minnesota Easy System Tri-Plate, Accumast, Mastitis SSGN Quad plate and Mastitis SSGNC Quad Plate. The study concluded that Accumast was the methodology that obtained the most accurate results among the evaluated parameters.

Still culture-dependent, another method that has been used to identify microorganisms is matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). The technique is very sensitive and requires a small amount of sample (from 104 to 106 CFU, for bacteria). In this method, samples from culture are co-crystallized and ionized by laser, thus the ions are accelerated and the time of flight in vacuum is measured. The laser energy vaporizes the microorganism along with the matrix, causing the ionization of the ribosomal proteins. The equipment generates a mass spectrometry graph and, in comparison with databases, the identification of the microorganism is performed (WIESER et al., 2012). Several studies have already identified pathogens related to subclinical mastitis using the MALDI-TOF MS methodology in Brazil and in other countries (BARREIRO et al., 2010; BJÖRK et al., 2014; GONÇALVES et al., 2014; WERNER et al., 2012).

Rapid and reliable identification of the microorganisms that cause mastitis is important for disease management and treatment. Recent developments in molecular methods allowed faster and more sensitive analyzes than classical microbiology procedures (SOHIER et al., 2014). PCR detection of microorganisms present in foods is recognized by ISO and standardized through several guidelines (ISO, 2005a, b, 2006a, b, 2011a, b). PCR is also used to confirm characteristic colonies on agar plates as specified by ISO (ISO, 2007). In the last decade, the amplification technique has boosted towards quantitative PCR (qPCR) (LE DRÉAN et al., 2010; MALORNY et al., 2008; MASCO et al., 2007) and ISO 2012 and 2013 guidelines describe the use of qPCR for the detection of microorganisms in food. In this context, molecular identification of microorganisms should replace conventional culture-based characterization, providing a more precise, sensitive, and less laborious genomic definition.

To reduce time and facilitate the identification of mastitis-related pathogens, two qPCR multiplexes were developed and are available on the market. The VetMAX™ MastiType Multi Kit (Applied biosystems, ThermoFisher Scientific, USA) identifies 15 mastitis-causing pathogens and one antibiotic resistance gene, in 4 separate qPCR

multiplex reactions. The identified pathogens are *Staphylococcus aureus*, *Staphylococcus* spp. (including *Staph. aureus* and all collagenase negative staphylococci), *Enterococcus* spp. (including *Enterococcus faecalis* and *Enterococcus faecium*), *Corynebacterium bovis*, *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Trueperella pyogenes* and/or *Peptoniphilus indolicus*, *Klebsiella oxytoca* and/or *Klebsiella pneumoniae*, *Serratia marcescens*, *Mycoplasma bovis*, *Mycoplasma* spp., *Yeast*, *Prototheca* spp. and Staphylococcal β -lactamase gene (penicillin-resistance gene). The commercial Mastit4 qPCR kit (DNA Diagnostics A/S, Risskov, Denmark) has almost the same targets with the exchange of *Serratia marcescens* for *Lactococcus lactis*. Academic works have already been published with both kits, with samples from quarters and tanks, with clinical and subclinical mastitis (HOLMØY et al., 2018; KATHOLM et al., 2012; KEANE et al., 2013; KOSKINEN et al., 2010; SOLTAU et al., 2017; TAPONEN et al., 2009).

The progress of next generation sequencing (NGS) technologies and the consequent reduction of sequencing costs has revolutionized human medicine and can add value to agribusiness, contributing to the solution of several problems. These technologies allow the study of highly complex biological samples, enabling the taxonomic and functional characterization of microbial communities that practically colonize all ecological niches. The characterization of raw milk microbiological profile can contribute to the identification of possible problems associated with subclinical mastitis and hygienic procedures, guiding adequate protocols on farm and dairy industries to eliminate or minimize its consequences. The use of large-scale DNA sequencing to identify mastitis-causing pathogens is restricted to a few reports in the academic literature comparing healthy and infected udders. Thus, the use of sequencing data from the V4 hypervariable region of the 16S gene for the characterization of the microbiota can be implemented as a disease diagnostic tool for clinical and subclinical mastitis. Compared to the multiplex qPCR molecular methodology, it is not as fast, but sequencing methodology is not limited to the number of previously selected pathogens.

Here, chapter one considers the core microbiome profile characterization of bulk tank raw milk at the species taxonomic level, and its association with somatic cell and total bacteria count. In this chapter, the relationship between certain bacteria species and disease pathogens, and hygienic conditions are discussed. The second chapter, compares different methodologies for mastitis pathogen diagnosis. Five methodologies were considered, standard culture, chromogenic media culture, MALDI-TOF, qPCR multiplex and 16S rRNA sequencing. Using qPCR multiplex as the gold standard, sensibility, specificity, and accuracy were evaluated and discussed.

References

- Alessandria, V., Dolci, P., Rantsiou, K., Pattono, D., Dalmaso, A., Civera, T., & Cocolin, L. (2010). Microbiota of the Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Cape Verde Islands. *World Journal of Microbiology and Biotechnology* 26:12, 26(12), 2211–2221. <https://doi.org/10.1007/S11274-010-0406-7>
- Anderson, B. E., Greene, C. E., Jones, D. C., & Dawson, J. E. (1992). Ehrlichia ewingii sp. nov., the Etiologic Agent of Canine Granulocytic Ehrlichiosis. In *INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY* (Vol. 42, Issue 2).
- Berlau, J., Aucken, H. M., Houang, E., & Pitt, T. L. (1999). Isolation of Acinetobacterspp including A.baumannii from vegetables: implications for hospital-acquired infections. *Journal of Hospital Infection*, 42, 201–204.

- Burton, K. J., Rosikiewicz, M., Pimentel, G., Bütikofer, U., Ah, U. von, Voirol, M.-J., Croxatto, A., Aeby, S., Drai, J., McTernan, P. G., Greub, G., Pralong, F. P., Vergères, G., & Vionnet, N. (2017). Probiotic yogurt and acidified milk similarly reduce postprandial inflammation and both alter the gut microbiota of healthy, young men. *British Journal of Nutrition*, *117*(9), 1312–1322. <https://doi.org/10.1017/S0007114517000885>
- Cleto, S., Matos, S., Kluskens, L., & Vieira, M. J. (2012). Characterization of Contaminants from a Sanitized Milk Processing Plant. *PLoS ONE*, *7*(6), 40189. <https://doi.org/10.1371/journal.pone.0040189>
- Collins, C., Almuzara, M., Saigo, M., Montaña, S., Chiem, K., Traglia, G., Mussi, M. A., Tolmasky, M., Iriarte, A., Carlos Vay, , & Ramirez, M. S. (2018). Whole-Genome Analysis of an Extensively Drug-Resistance *Empedobacter falsenii* Strain Reveals Distinct Features and the Presence of a Novel Metallo- β -Lactamase (EBR-2). *Current Microbiology*, *75*, 1084–1089. <https://doi.org/10.1007/s00284-018-1498-9>
- de Haas, Y., Barkema, H. W., & Veerkamp, R. F. (2002). The effect of pathogen-specific clinical mastitis on the lactation curve for somatic cell count. *Journal of Dairy Science*, *85*(5), 1314–1323. [https://doi.org/10.3168/JDS.S0022-0302\(02\)74196-9](https://doi.org/10.3168/JDS.S0022-0302(02)74196-9)
- Delbè, C., Ali-Mandjee, L., & Montel, M.-C. (2007). Monitoring Bacterial Communities in Raw Milk and Cheese by Culture-Dependent and-Independent 16S rRNA Gene-Based Analyses. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *73*(6), 1882–1891. <https://doi.org/10.1128/AEM.01716-06>
- Ganda, E. K., Bisinotto, R. S., Lima, S. F., Kronauer, K., Decter, D. H., Oikonomou, G., Schukken, Y. H., & Bicalho, R. C. (2016). *Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin*. <https://doi.org/10.1038/srep37565>
- Harmon, R. J. (1994). Physiology of Mastitis and Factors Affecting Somatic Cell Counts. *Journal of Dairy Science*, *77*(7), 2103–2112. [https://doi.org/10.3168/JDS.S0022-0302\(94\)77153-8](https://doi.org/10.3168/JDS.S0022-0302(94)77153-8)
- Heyrman, J., Vanparrys, B., Logan, N. A., Balcaen, A., Rodríguez-Díaz, M., Felske, A., & Vos, P. De. (2004). *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. *International Journal of Systematic and Evolutionary Microbiology*, *54*(1), 47–57. <https://doi.org/10.1099/IJS.0.02723-0>
- Holmøy, I. H., Toftaker, I., Kirkeby, C., Østerås, O., Jørgensen, H. J., & Nødtvedt, A. (2019). A cohort study of the effect of *Streptococcus agalactiae* on milk yield and somatic cell count in Norwegian dairy cows. *Journal of Dairy Science*, *102*(9), 8385–8399. <https://doi.org/10.3168/JDS.2018-16155>
- Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1). <https://doi.org/10.1038/s41467-019-13036-1>
- Kampfer, P., Avesani, V., Janssens, M., Charlier, J., De Baere, T., & Vanechoutte, M. (2006). Description of *Wautersiella falsenii* gen. nov., sp.nov., to accommodate clinical isolates phenotypically resembling members of the genera *Chryseobacterium* and *Empedobacter*. *International Journal of Systematic and Evolutionary Microbiology*, *56*, 2323–2329. <https://doi.org/10.1099/ijms.0.64393-0>
- Kano, R., Kobayashi, Y., Nishikawa, A., Murata, R., Itou, T., Ito, T., Suzuki, K., & Kamata, H. (2018). Next-generation Sequencing Analysis of Bacterial Flora in Bovine Protothecal Mastitic Milk. *Medical Mycology Journal*, *59*(3), E41–E46. <https://doi.org/10.3314/mmj.18-00004>

- Katholm, J., Bennedsgaard, T. W., Koskinen, M. T., & Rattenborg, E. (2012). 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*, *95*(10), 5702–5708. <https://doi.org/10.3168/JDS.2011-5307>
- Keefe, G. P., Dohoo, I. R., & Spangler, E. (1997). Herd Prevalence and Incidence of *Streptococcus agalactiae* in the Dairy Industry of Prince Edward Island. *Journal of Dairy Science*, *80*(3), 464–470. [https://doi.org/10.3168/JDS.S0022-0302\(97\)75958-7](https://doi.org/10.3168/JDS.S0022-0302(97)75958-7)
- Lopes, J. E. F., Langei, C. C., Brito, M. A. V. P., Santos, F. R., Silva, M. A. S., de Moraes, L. C. D., & de Souza, G. N. (2012). Relationship between total bacteria counts and somatic cell counts from mammary quarters infected by mastitis pathogens. *Ciência Rural*, *42*(4), 691–696. <https://doi.org/10.1590/S0103-84782012000400019>
- Masoud, W., Vogensen, F., Lillevang, S., Abu Al-Soud, W., Sørensen, S., & Jakobsen, M. (2012). The fate of indigenous microbiota, starter cultures, *Escherichia coli*, *Listeria innocua* and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. *International Journal of Food Microbiology*, *153*(1–2), 192–202. <https://doi.org/10.1016/J.IJFOODMICRO.2011.11.014>
- Matías Traglia, G., Dixon, C., Chiem, K., Almuzara, M., Barberis, C., Montaña, S., Merino, C., Mussi, M. A., Tolmasky, M. E., Iriarte, A., Vay, C., & Soledad Ramírez, M. (2015). *Draft Genome Sequence of Empedobacter (Formerly Wautersiella) falsenii comb. nov. Wf282, a Strain Isolated from a Cervical Neck Abscess.* <https://doi.org/10.1128/genomeA.00235-15>
- Metzger, S. A., Hernandez, L. L., Skarlupka, J. H., Walker, T. M., Suen, G., & Ruegg, P. L. (2018). A Cohort Study of the Milk Microbiota of Healthy and Inflamed Bovine Mammary Glands From Dryoff Through 150 Days in Milk. *Frontiers in Veterinary Science*, *0*(OCT), 247. <https://doi.org/10.3389/FVETS.2018.00247>
- Miura, A., Kurumisawa, T., Kano, R., Ito, T., Suzuki, K., & Kamata, H. (2019). Next-generation sequencing analysis of bacterial flora in bovine protothecal mastitic milk and feces. *J Vet Med Sci.* <https://doi.org/10.1292/jvms.18-0649>
- Nemec, A., Musílek, M., Šedo, O., Baere, T. De, Maixnerová, M., Reijden, T. J. K. van der, Zdráhal, Z., Vanechoutte, M., & Dijkshoorn, L. (2010). *Acinetobacter bereziniae* sp. nov. and *Acinetobacter guillouiae* sp. nov., to accommodate *Acinetobacter* genomic species 10 and 11, respectively. *International Journal of Systematic and Evolutionary Microbiology*, *60*(4), 896–903. <https://doi.org/10.1099/IJS.0.013656-0>
- Ohta, H., Hattori, R., Ushiba, Y., Mitsui, H., Ito, M., Watanabe, H., Tonosaki, A., & Hattori, T. (2004). *Sphingomonas oligophenolica* sp. nov., a halo- and organo-sensitive oligotrophic bacterium from paddy soil that degrades phenolic acids at low concentrations. *International Journal of Systematic and Evolutionary Microbiology*, *54*(6), 2185–2190. <https://doi.org/10.1099/IJS.0.02959-0>
- Petzer, I. M., Karzis, J., Donkin, E. F., Webb, E. C., & Etter, E. M. C. (2017). Validity of somatic cell count as indicator of pathogen-specific intramammary infections. *Journal of the South African Veterinary Association*, *88*(0), 1019–9128. <https://doi.org/10.4102/JSAVA.V88I0.1465>
- Phuektes, P., Browning, G. F., Anderson, G., & Mansell, P. D. (2021). *Multiplex polymerase chain reaction as a mastitis screening test for Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis in bulk milk samples.* <https://doi.org/10.1017/S0022029903006010>

- Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. In *FEMS Microbiology Reviews* (Vol. 37, Issue 5, pp. 664–698). <https://doi.org/10.1111/1574-6976.12030>
- Ramos, G. L. de P. A., Nascimento, J. dos S., Ramos, G. L. de P. A., & Nascimento, J. dos S. (2019). Characterization of *Acinetobacter* spp. from raw goat Milk. *Ciência Rural*, *49*(10). <https://doi.org/10.1590/0103-8478CR20190404>
- Ribeiro Júnior, J. C., de Oliveira, A. M., Silva, F. de G., Tamanini, R., de Oliveira, A. L. M., & Beloti, V. (2017). The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. *Journal of Dairy Science*, *101*(1), 75–83. <https://doi.org/10.3168/jds.2017-13069>
- RJ, E., RJ, E., LJ, H., & SB, S. (1987). Herd management and prevalence of mastitis in dairy herds with high and low somatic cell counts. *Journal of the American Veterinary Medical Association*, *190*(11), 1411–1416.
- Rodrigues, M. X., Lima, S. F., Canniatti-Brazaca, S. G., & Bicalho, R. C. (2017). The microbiome of bulk tank milk: Characterization and associations with somatic cell count and bacterial count. *Journal of Dairy Science*, *100*(4), 2536–2552. <https://doi.org/10.3168/jds.2016-11540>
- Sharma, M., & Anand, S. K. (2002). Characterization of constitutive microflora of biofilms in dairy processing lines. *Food Microbiology*, *19*(6), 627–636. <https://doi.org/10.1006/FMIC.2002.0472>
- Shkreta, L., Talbot, B. G., Diarra, M. S., & Lacasse, P. (2004). Immune responses to a DNA/protein vaccination strategy against *Staphylococcus aureus* induced mastitis in dairy cows. *Vaccine*, *23*(1), 114–126. <https://doi.org/10.1016/J.VACCINE.2004.05.002>
- Speirs, L. B. M., Rice, D. T. F., Petrovski, S., & Seviour, R. J. (2019). *The Phylogeny, Biodiversity, and Ecology of the Chloroflexi in Activated Sludge*. <https://doi.org/10.3389/fmicb.2019.02015>
- Turton, J. F., Shah, J., Ozongwu, C., & Pike, R. (2010). Incidence of *Acinetobacter* Species Other than *A. baumannii* among Clinical Isolates of *Acinetobacter*: Evidence for Emerging Species †. *JOURNAL OF CLINICAL MICROBIOLOGY*, *48*(4), 1445–1449. <https://doi.org/10.1128/JCM.02467-09>
- Vacheyrou, M., Normand, A. C., Guyot, P., Cassagne, C., Piarroux, R., & Bouton, Y. (2011). Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. *International Journal of Food Microbiology*, *146*(3), 253–262. <https://doi.org/10.1016/j.ijfoodmicro.2011.02.033>
- Vithanage, N. R., Dissanayake, M., Bolge, G., Palombo, E. A., Yeager, T. R., & Datta, N. (2017). Microbiological quality of raw milk attributable to prolonged refrigeration conditions. *Journal of Dairy Research*, *84*(1), 92–101. <https://doi.org/10.1017/S0022029916000728>
- Watts, J. L. (1988). Etiological agents of bovine mastitis. *Veterinary Microbiology*, *16*(1), 41–66. [https://doi.org/10.1016/0378-1135\(88\)90126-5](https://doi.org/10.1016/0378-1135(88)90126-5)
- Whist, A. C., Østerås, O., & Sølvørød, L. (2007). *Streptococcus dysgalactiae* Isolates at Calving and Lactation Performance Within the Same Lactation. *Journal of Dairy Science*, *90*(2), 766–778. [https://doi.org/10.3168/JDS.S0022-0302\(07\)71561-8](https://doi.org/10.3168/JDS.S0022-0302(07)71561-8)
- XM, F., HJ, D., JL, B., WN, H., J, L., H, W., J, S., HY, L., YQ, Z., & LY, Y. (2020). *Ornithinimicrobium cerasi* sp. nov., isolated from the fruit of *Cerasus pseudocerasus* and emended description of the genus *Ornithinimicrobium*. *International Journal of Systematic and Evolutionary Microbiology*, *70*(3), 1691–1697. <https://doi.org/10.1099/IJSEM.0.003957>

2. CHARACTERIZATION OF CORE MICROBIOME IN BULK TANK MILK AND ITS CORRELATION WITH SOMATIC CELL COUNT AND TOTAL BACTERIAL COUNT

Abstract

Milk's nutrient-rich composition contributes to microorganism proliferation, therefore the two main quality indicators frequently used are somatic cell count (SCC) and standard plant count (SPC). Raw milk contamination at the farm has several sources and the microbiological profile can contribute to the identification of problems related to mammary gland diseases and hygienic deficiencies. This study aimed to identify the core microbiome of two dairy plants, and its correlation with quality parameters. A total of 575 bulk tank milk (BTM) samples were evaluated by somatic cell count, standard plate count, and microbiome profile by 16S gene rRNA sequencing of the V4 region. The core microbiome was determined by 14 bacterial species. *Staphylococcus aureus* was the most common, present in 96% of the samples, and the most abundant was *Streptococcus agalactiae*. Alpha diversity indices demonstrated significant differences between the dairy plants. Different species were found to be correlated with SCC and SPC for both dairy plants. Abundance of *Streptococcus agalactiae* was correlated with SCC in Plant A, and SPC in Plant B; *Streptococcus dysgalactiae* was correlated with SCC in Plant A, *Lactococcus lactis* and *Staphylococcus aureus* with SPC in Plant B. We also report here, for the first time, the presence of *Sphingomonas oligophenolica*, *Christensenellaceae* R-7 group, *Thermomicrobia JG30-KF-CM45* Species, *Bacillus dretensis*, and *Intertimbacter barlettii* in BTM of dairies. This study demonstrates that different tank milk microbiome profiles and their correlation with SPC, and SCC can contribute to the understanding of the sources of bacterial contamination and disease, and consequently improve milk quality in dairy farms.

2.1. Introduction

Milk is a nutrient-rich food with considerable amounts of protein, fat, carbohydrates, vitamins, and minerals. Thus, milk provides an optimal environment for microorganism proliferation, and the most common genera found in bovine raw milk are *Streptococcus*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, and *Pseudomonas* (QUIGLEY et al., 2013). The main indicators of bulk tank milk quality are the somatic cell count (SCC) and the standard plate count (SPC) (RODRIGUES et al., 2017). Somatic cells are naturally found in milk, and SCC is used as an udder health indicator, consequently higher SCCs in bulk milk tank (BMT) indicate the herd with mammary gland health disorders. SPC is considered a hygienic parameter.

Bulk milk contamination has several sources, such as teat, feces, bedding, water, feed, housing, milking equipment, and personnel hygiene (QUIGLEY et al., 2013). Bacteria's presence in raw milk has a direct and negative impact on dairy farms and dairy plants. On farms, bacteria are the main agents responsible for subclinical and clinical mastitis, an inflammation of the mammary gland. Mastitis is the most expensive disease in the dairy industry because of the time it takes for correct pathogen identification and cure, high treatment costs, reduction in milk production, and milk and animal discard (BONSAGLIA et al., 2017). Among the major mastitis pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Corynebacterium bovis*, *Bacillus* spp., *Pseudomonas* spp., *Escherichia coli*, and *Mycoplasma bovis* (WATTS, 1988).

Bacteria can also reduce the quality and shelf life of milk and dairy products. Lactic acid bacteria (LAB) ferment lactose to lactate, and thus influence the quality of the final dairy product, changing texture, flavor, and organoleptic properties. Some common LAB genera found in milk are *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus* (QUIGLEY et al., 2013). Psychrotrophic bacteria, which can proliferate under refrigeration temperature, are the main bacteria responsible for spoilage of dairy products because of their production and release of thermoresistant enzymes (CHAMPAGNE et al., 1994a; WOUTERS et al., 2002). These heat-stable lipases and proteases maintain their hydrolytic activities even after different thermic treatments (pasteurization and ultra-high tem-

perature) that are designed to eliminate the microorganisms (CHAMPAGNE et al., 1994b). *Pseudomonas*, *Acinetobacter* spp., and *Bacillus* spp. are psychrotrophic genera frequently found in raw milk (QUIGLEY et al., 2013; VITHANAGE et al., 2016). Another important characteristic of bacteria present in raw milk is the production of spores and biofilm. These bacteria with biofilm production ability can attach to the milking equipment, forming a protective barrel called biofilm against sanitizers and cleaning products. Some bacteria also produce spores and consequently become a constant source of contamination of pathogenic and spoilage bacteria, affecting raw milk quality, and shelf-life time.

The microbiological profile of raw milk tank can indicate potential problems associated with subclinical mastitis and hygienic procedures, guiding adequate protocols for farm and dairy industries to eliminate or minimize its consequences. The characterization of the milk microbiome profile has become more accessible because of the cost reduction of new DNA sequencing technologies. In particular, 16S rRNA gene sequencing method has been frequently used because of its capacity to identify most bacteria present, sometimes at species taxonomic level, being culturable or not, and with lower cost compared to whole-genome metagenomics methods.

The characterization and the establishment of BMT core microbiome and its association with SCC and SPC were previously done by Rodrigues and collaborators (RODRIGUES et al., 2017), however, their study was conducted with 19 samples and achieved the taxonomy classification until genera. In this study, we expand their findings by substantially increasing the number of samples (567 BMT) and, when possible, improving the classification at the species level, allowing the identification of species related to mastitis, cold storage, and hygienic conditions.

2.2. Materials and Methods

2.2.1. Samples

A total of 576 BTM samples were obtained from 174 dairy herds located in São Paulo State, Brazil. From these farms, 39 delivered their milk to dairy Plant A and 135 to dairy Plant B. Samples were collected during April, October, November, December, and January of 2018/19.

Samples were collected from the tank, after homogenization and before transportation of the milk to the plants. Samples for somatic cell count and v4 region of 16S rRNA gene (v4-16S) sequencing were collected and stored in tubes, and preserved with Bronopol® with a final concentration between 0.02% and 0.05%. Samples for total bacterial count were collected and stored in tubes, and preserved with Azidiol® with a final concentration between 0.004% - 0.005% of chloramphenicol and 0.10% and 0.12% of sodium azide. Both tubes were stored refrigerated for no more than five days until the analysis of somatic cell count, total bacterial count, and DNA extraction.

2.2.2. Somatic cell count and total bacterial count

Somatic cell count and total bacterial count were performed by the specialized laboratory Clínica do Leite (Piracicaba, São Paulo, Brazil). Both analyses were performed based on flow cytometry according to normative ISO 13366-2:2006/ IDF 148-2:2006 (for somatic cell count) and ISO 16297:2013/ IDF 161:2013 (for the total bacterial count). The bacterial count results were converted to colony-forming units and were designated as Standard Plate. Both measures, somatic cell and standard plate, were transformed into scores, being somatic cell score (SCS) and

standard plate score (SPS) using the respective equations, ($\text{score} = \log_2(\text{SCC} / 100,000) + 3$) and ($\text{score} = \log_2(\text{SPC} / 100,000) + 3$) (ALI; SHOOK, 1980).

2.2.3. DNA extraction, library generation, and sequencing

After SCC analysis, 2 mL of milk were transferred to a 2 mL microcentrifuge tube and centrifuged for 5 minutes at 14,000 rpm. After centrifugation, fat and supernatant were discarded, and the pellet was stored at -20°C for further DNA extraction. Genomic DNA was obtained using MagMAX™ CORE combined with MagMAX™ CORE Mechanical Lysis Module (ThermoFisher™) according to the manufacturer's instructions. Extracted DNA was quantified by spectrophotometry and quality evaluated in agarose gel.

Library construction was performed according to 16S Metagenomic Sequencing Library Preparation Guidelines (Illumina Inc., San Diego, CA). The primers 515F (5' GTGYCAGCMGCCGCGGTAA 3') and 806BR (5' GGACTACNVGGGTWTCTAAT 3') were used to amplify the V4 hypervariable region from the 16S rRNA gene by PCR. Equimolar quantities of each library were pooled and sequencing was performed using MiniSeq High Output reagent kit (300 cycles) on the MiniSeq platform (Illumina Inc., San Diego, CA).

2.2.4. Sequencing data analysis, database dereplication, and taxonomy assignment

Raw sequencing data were analyzed using the DADA2 program (CALLAHAN et al., 2016) in R software (R CORE TEAM, 2013). Raw reads were quality-filtered after primers removal and error rates learn. Error rates were estimated separately for each sequencing run. Chimeras were identified and removed using the remove Bimera Denovo function. Finally, an ASV (Amplicon Sequencing Variants) table was constructed. ASVs were used for taxonomy assignment against dereplicated DAIRYdb (MEOLA et al., 2019) database. Dereplication of the database was performed with homemade script to allow multiple species classification of the same read. In summary, sequences in DAIRYdb database with 100% of identity in the V4 hypervariable region were joint and the taxonomy information were concatenated. The sequences with less than 100% of identity were kept without adjustments.

After taxonomy assignment using the dereplicated DAIRYdb, data transformation was performed. For multiple gene copies correction, we used the database rrnDB (STODDARD et al., 2015). The total number of reads for each species was divided by the gene copy value of the lowest taxonomic level. When no information of gene copy number in the rrnDB was available, the average gene copy number of the taxonomic level above, was used. As presented by (VANDEPUTTE et al., 2017), a quantitative microbiome profile (QMP) was obtained with the construction of a relative count matrix, dividing the total count for each species by the total count of the sample. Next, the absolute count matrix was built by multiplying the species relative count by the sample bacterial total count (SPC) value, resulting in the values of QMP species abundance ($\times 10^3$ UFC/cell).

2.2.5. Core microbiome and diversity indices

To characterize the milk tank core microbiome at the genera and species levels, we used the species that were presented in at least 70% of the samples. Shannon diversity index and Chao1 richness index were calculated in

R with the phyloseq package (v. 1.30) (MCMURDIE; HOLMES, 2013). To evaluate the differences in diversity indices between the dairy plants, we performed a Wilcoxon test in R software with stats package (v. 3.6.1) (R CORE TEAM, 2013) for each index.

2.2.6. Microbiome profile and correlation with SCS and SPS

For the establishment of microbiome profile, we used only ASVs with classification at the species level and present in at least 20% of the samples for each dairy plant individually. For correlation of the microbiome profile with continuous variables, as SCS and SPS, we performed nonparametric Spearman tests with *pspearman* (SAVICKY, 2015) and *stats* (v. 3.6.1) packages in R software (R CORE TEAM, 2013). We used Bonferroni for multiple test corrections.

2.3. Results

2.3.1. Sample size, somatic cell count, total bacterial count

We obtained 108 and 467 BTM samples from dairy plant A and B, respectively. Descriptive statistics, as minimum, maximum, mean, and standard deviation values for SCS and SPS analyses, can be observed in Table 1. For Plant B, we observed higher means and standard deviations values of SCS and SPC than Plant A.

Table 1. Number of farms, samples, and descriptive analysis of Somatic Cell Score and Standard Plate Score according to the dairy Plant (A and B).

Plant	Farms	Samples	Analysis	Min	Max	Mean	SD
A	39	108	SCS ¹	-0.32	7.70	4.46	1.41
			SPS ²	-2.64	6.74	0.05	1.59
B	137	467	SCS	2.98	9.53	5.77	1.08
			SPS	-2,64	9.64	1.46	2.27

¹SCS: Somatic Cell Score; ²SPS: Standard Plate Score; Min: Minimum; Max: Maximum; SD: Standard Deviation.

2.3.2. Sequencing and core microbiome

After quality-filtering, primers removal, error rates learn, and ASV construction resulted in a mean of 49,870 ASVs per sample, with a max of 235,987 ASVs, min of 6,563 ASVs and standard deviation of 29,364.21 ASVs.

To characterize the bulk tank milk core microbiome in our samples, the first step was to evaluate all the species identified (Supplemental Table 1). With no filter, we identified 1019 species among the 575 samples, from that, 893 species were found in both dairy plants, 12 were exclusives for Plant A and 114 for Plant B (Figure 1). We identified 14 species that were present in at least 70% of the samples and defined these as the core microbiome. It is important to note that no single species was found in 100% of the samples analyzed. (Table 2). Among the 14 species of the core microbiome, some are frequently found in raw milk: *Streptococcus agalactiae*, *Lactococcus lactis*, *Empedobac-*

ter falsenii, *Acinetobacter berezinia*, *Staphylococcus aureus*, *Bacteroides plebeius*, *Sporobacter termitidis*, and *Moraxella osloensis*. In Table 2, we present the number of samples and the absolute counts for each species determined in the core microbiome. The bacteria species found in most samples were *Staphylococcus aureus* (554 samples, 96,3%) whereas *Streptococcus agalactiae* was the species with the highest sum of absolute count, accounting for 55.30 QMP species abundance ($\times 10^3$ UFC/ml)² present in 74.09% of the samples.



Figure 1. Venn diagram demonstrating the number of species identified in all 575 samples, and separated by each dairy plant (A and B).

Table 2. Abundance and presence of the core microbiome in bulk tank milk samples.

Species	Percentage of samples ¹	Mean of QMP specie abundance ($\times 10^3$ UFC/ml) ²
<i>Staphylococcus aureus</i>	96.35%	0.99
<i>Sporobacter termitidis</i>	90.43%	0.27
<i>Thermomicrobia JG30-KF-CM45 Species</i>	84.52%	0.68
<i>Lactococcus lactis</i>	83.83%	4.71
<i>Christensenellaceae R-7 group Species</i>	81.22%	0.22
<i>Empedobacter falsenii</i>	77.39%	1.80
<i>Acinetobacter berezinia</i>	77.22%	4.35
<i>Moraxella osloensis</i>	76.00%	3.36
<i>Intestinibacter bartlettii</i>	75.30%	0.08
<i>Streptococcus agalactiae</i>	74.09%	55.30
<i>Bacteroides plebeius</i>	73.57%	0.10
<i>Bacillus drentensis</i>	73.39%	0.04
<i>Sphingomonas oligophenolica</i>	71.65%	2.19
<i>Acinetobacter ursingii</i>	71.65%	4.67

¹% of samples containing the species. ²QMP: Quantitative microbiome profiling.

2.3.3. Diversity indices

To evaluate species richness and evenness between the two dairy plants, Shannon diversity index and Chao1 richness index were performed. Based on the results of Wilcoxon statistical test, the Shannon and Chao1 indexes were significantly different (p -value < 0.05) between the two dairy plants (A and B) (Figure 2). Hence, the correlation analysis between the microbiome profile, SCS, and SPS were performed separately for each plant.

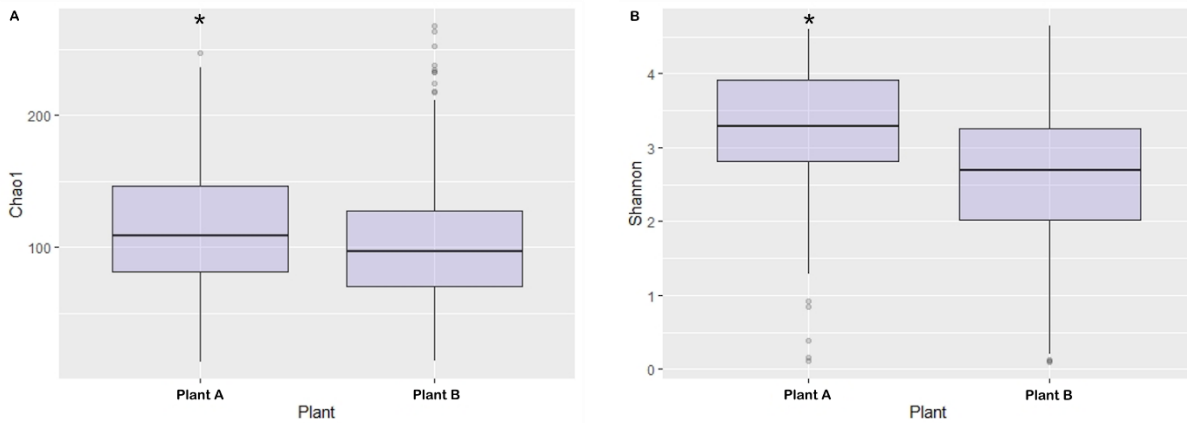


Figure 2. Box-plot for Chao1 richness index (A) and Shannon diversity index (B) for Plant A and Plant B. *: Significant p -value for Chao1 index (0.007) and, for Shannon index (1.293e-12).

2.3.4. Microbiome profile of each dairy plant

For microbiome correlation analysis, we used only the ASVs present in at least 20% of the samples. Because of this filter, Plant A had 184 species and Plant B 154 species. We construct a Venn diagram to evaluate the differences in the microbiome profile between the two dairy plants (Figure 3). As illustrated in Figure 3, 137 species were identified in both plants, 47 were exclusive for plant A and 17 for plant B. The list of species present in each group is available in Supplemental Table 2.

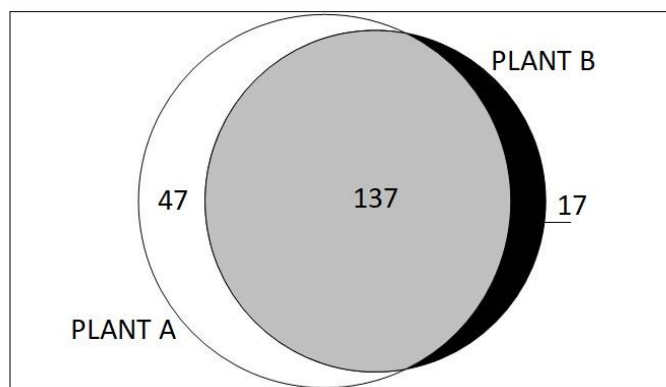


Figure 3. Venn diagram for the species for each dairy plant that were used for correlation analysis.

2.3.5. Microbiome correlation with SCC

To identify species associated with SCC BTM we performed correlation analyses for each dairy plant separately. For Plant A, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* abundancies had a significant correlation (adjusted p -value <0.05) (Figure 4a). For Plant B, *Streptococcus agalactiae*, *Ehrlichia ewingii*, *Bacillus drentensis*, and *Ornithinimicrobium spp.* were significantly correlated (adjusted p -value <0.05) with SCC (Figure 4a). *Ornithinimicrobium spp.* was the only species negatively correlated with SCC, and *Streptococcus agalactiae* was the only species positively correlated in both dairy plants.

2.3.6. Microbiome correlation with SPC

We also performed correlation analysis between SPC and microbiome profile for each plant separately to identify associated species. As result, for plant A, we identified *Acinetobacter ursingii* and *Bacillus drentensis* significantly correlated with SPC (adjusted p -value <0.05) (Figure 4b). For plant B, 21 species were significantly correlated (adjusted p -value <0.05) with SPC, they were: *Moraxella osloensis*, *Acinetobacter bereziniae*, *Streptococcus agalactiae*, *Bacillus drentensis*, *Lactococcus lactis*, *Staphylococcus aureus*, *Acinetobacter ursingii*, *Aeromonas spp.*, *Sporobacter termitidis*, *Kocuria spp.*, *Enterococcus italicus*, *Thermomicrobia JG30-KF-CM45 Species*, *Stenotrophomonas maltophilia*, *Empedobacter falsenii*, *Ornithinimicrobium Species*, *Anaerobacterium chartisolvens*, *Acinetobacter sp.*, *Chryseobacterium spp.*, *Pseudomonas spp.*, *Acetitomaculum spp.*, and *Caulobacter vibrioides* (Figure 4b). We observed a negative correlation with SPC between *Ornithinimicrobium spp.* and *Anaerobacterium chartisolvens* on plant B and, *Acinetobacter ursingii* and *Bacillus drentensis* in both dairy plants.

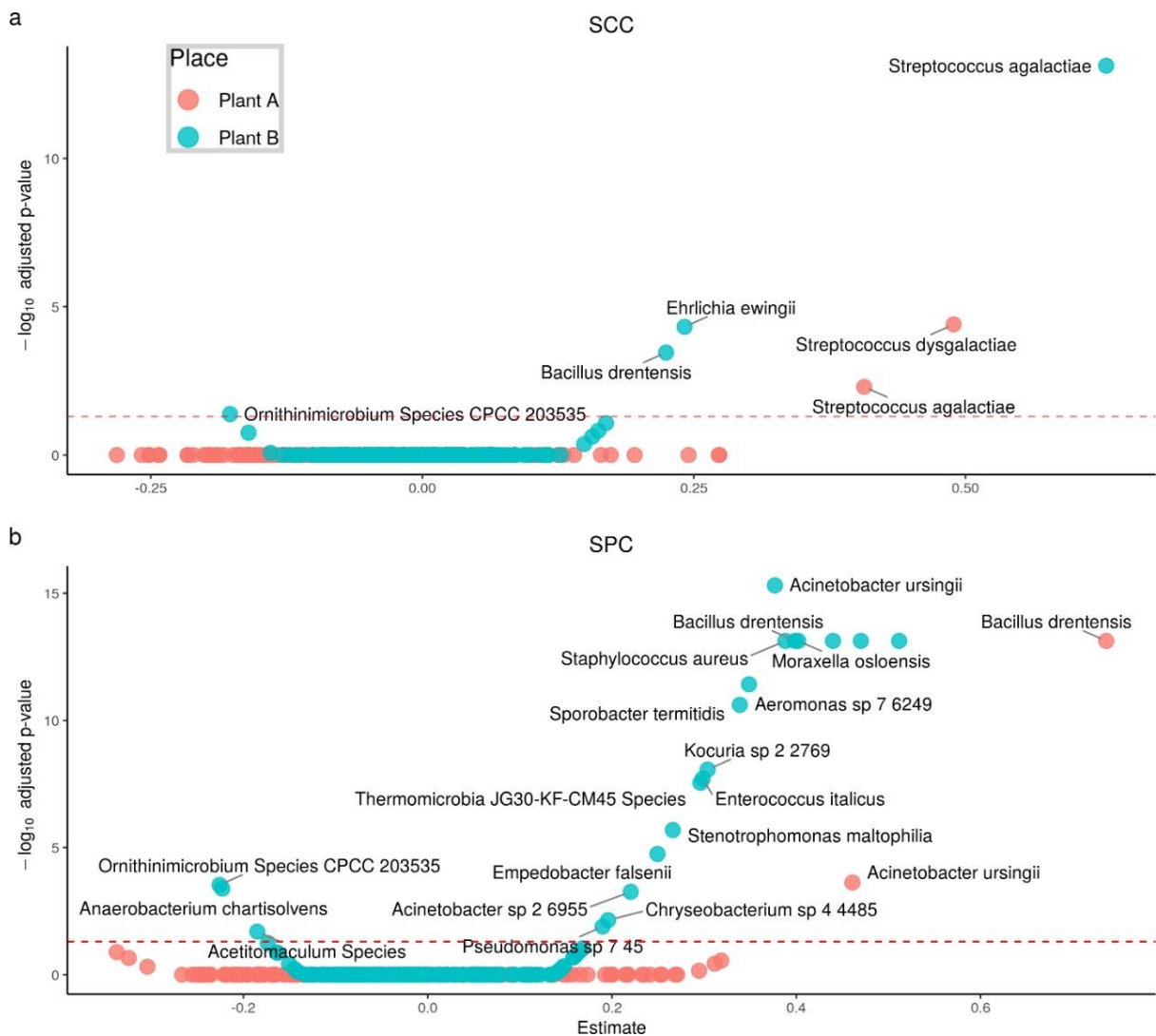


Figure 4. Correlation plot between microbiome profile and somatic cell count (a), and microbiome profile and standard plate count (b). Different colors are assigned according to the dairy plants (A and B). Adjusted p -values were calculated by Bonferroni multiple-comparison correction.

2.4. Discussion

In the current study, we used V4 region of 16S rRNA high-throughput sequencing technology to characterize the core microbiome profile of bulk tank milk in Brazil from 576 samples across 174 farms during the summer months. Also, the microbiome profile correlation with bulk tank milk quality parameters, as somatic cell count and standard plate count, were investigated. To our knowledge, a similar study was performed, although, using only 19 samples (Rodrigues et al., 2017).

2.4.1. Core microbiome

The core microbiome profile identified 14 bacteria species that were present in at least 70% of the bulk tank milk samples analyzed. We identified species frequently found in raw milk as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Lactococcus lactis*. *S. aureus* is a Gram-positive and catalase-positive coccus, and the most frequently isolated microorganisms from bovine mammary glands and, also considered a major mastitis pathogen. *S. aureus* is established both in the epithelial cells and alveolar function, and as a consequence, reduces milk yield and quality (Shkreta et al., 2004). *S. agalactiae* is an established bovine mastitis pathogen, and it is considered an obligate udder parasite in cows. Being one of the most widespread gram-positive contagious pathogens on dairy farms, its transmission between the cows occurs mainly during the milking process (Watts, 1988). *L. lactis* is also considered a mammary gland pathogen and, because of its lactate fermentation ability, *L. lactis* is known for its role in starter cultures in dairy industries and its probiotic properties (Quigley et al., 2013). Another study identified *L. lactis* by 16S rDNA sequencing and this species was one of the dominant species, representing 22.30% of the reads obtained (Masoud et al., 2012), and this finding is in agreement with our results as *L. lactis* was the second most abundant species, with a mean of 4,71 QMP species abundance ($\times 10^3$ UFC/ml).

We also identified some species that were already mentioned in dairies but are not common, such as *Empedobacter falsenii*, *Acinetobacter berezjinae*, *Bacteroides plebeius*, *Sporobacter termitidis*, *Acinetobacter ursingii* and *Moraxella osloensis*. *Empedobacter falsenii* (formerly *Wautersiella falsenii*) is a non-fermenting gram-negative bacillus and a nosocomial human pathogen with high levels of antibiotic resistance for several antibiotics (Collins et al., 2018; Kampf et al., 2006; Matías Traglia et al., 2015). *E. falsenii* was identified as one of the most prevalent species in protothecal mastitis cow milk samples and not in fecal samples (Miura et al., 2019). Another study also identified *E. falsenii* in a single mastitic milk sample with 4.27% of the reads (Kano et al., 2018). *Acinetobacter berezjinae* (formerly *Acinetobacter genospecies 10*) is an emerging pathogen already found in vegetables, several human specimens and also has resistance to at least 8 antibiotics (Berlau et al., 1999; Nemeč et al., 2010; Turton et al., 2010). Recent studies found *A. berezjinae* in goat and healthy cow milk. This species produces lipase and protease but does not form a biofilm (Kano et al., 2018; Ramos et al., 2019). So possibly, its presence in BTM could have a negative influence on the milk fat and protein fractions, because of the lipase and protease enzyme activity. *Bacteroides plebeius* (gram-negative non spore-forming bacteria) and *Sporobacter termitidis* (gram-positive spore-forming bacteria) were found in milk samples derived from healthy and mastitic cows (Kano et al., 2018; Miura et al., 2019) (Miura 2019, e Kano 2018). *Moraxella osloensis* is a gram-negative bacillus and was identified in cheese and cow's milk (Alessandria et al., 2010; Delbè et al., 2007). *Acinetobacter ursingii* is gram-negative bacteria that produce proteases and lipases but not biofilm and it was identified in goat's milk (Ramos et al., 2019).

Here we report, for the first time, the presence of the species *Sphingomonas oligophenolica*, *Christensenellaceae* R-7 group, *Thermomicrobia JG30-KF-CM45 Species*, *Bacillus dretensis* and *Intertinibacter barlettii* in dairies. However, these bacteria were found elsewhere, *Bacillus dretensis* in soil (Heyrman et al., 2004), *Intertinibacter barlettii* in human gut (Burton et al., 2017), *Thermomicrobia* genus in sludge (SPEIRS et al., 2019), and *Sphingomonas oligophenolica* in paddy soil (Ohta et al., 2004). Therefore, the identification of these bacteria in raw BTM might indicate dust, soil, or fecal contamination because of unsuccessful hygienic and cleaning protocols.

2.4.2. Descriptive analysis of SCC, SPC, and diversity indices

According to the Brazilian legislation for quality parameters of raw milk (Brazilian Normative Instruction MAPA 76, 2018), the refrigerated raw milk from tanks must present quarterly geometric averages of Standard Plate Count <300,000 CFU/mL and Somatic Cell Count of <500,000 cells/ ml. Plant A presented fewer samples above the legal limit for SPC (3 samples, 2.7%) and SCC (42 samples, 38.88%) than plant B. Dairy plant B had 49 samples (10,49%) above SPC legal limit and 292 samples (62,52%) above SCC legal limit.

The alpha diversity indices (Shannon and Chao1 index) were performed to evaluate species richness and evenness from the two dairy plants. We detected a significant difference in the Shannon and Chao1 index between the two dairy plants. Shannon index is a nonparametric diversity index that integrates the estimates of richness and evenness, which is the total number of ASVs and the relative abundance of ASVs, respectively. Hence, a low index indicates one dominant species and a higher index indicates a community with a more even distribution (Metzger et al., 2018). Ganda et al., (Ganda et al., 2016) reported that mastitic milk had a higher bacterial load and lower microbial diversity compared to healthy milk. So, combining our results of diversity indices and descriptive analysis of SPC and SCC, we demonstrate that milk from plant A, which presented better quality parameters, had higher microbial diversity compared to plant B. With that evidence, we decide to evaluate the correlation of microbiome profile with SCC and SPC of the two dairy plants separately.

2.4.3. Microbiome profile correlation with SCC

SCC has been used as a measure of inflammation, the severity of intramammary infection, and an indicator of economic losses (Petzer et al., 2017). Therefore, BTM SCC is an indicative of herd health, and positive correlations of major mastitis causing pathogen such as *Strep. agalactiae* and *Strep. dysgalactiae* were expected. In our study, *Strep. agalactiae* was present in 45% (49) of the samples in plant A and 72% (337) of the samples in plant B. As for *Strep. dysgalactiae*, it was present in 61% (66) of samples in plant A and 53% (247) of the samples in plant B. The significant correlation between *S. agalactiae* and SCC was also found by other studies (Katholm et al., 2012; Keefe et al., 1997; Phuektes et al., 2021; RJ et al., 1987). Holmøy et al., (2019) demonstrated that infected cows with *S. agalactiae* presented high SCC and milk production losses. Two other studies evaluated SCC values comparatively for *S. agalactiae*, *S. aureus*, other streptococci, and coagulase-negative staphylococcus. Both studies found high values of SCC in mammary quarter milk with *S. agalactiae* infection (Harmon, 1994; Lopes et al., 2012). Regarding *Strep. dysgalactiae*, other studies also found high values of SCC in milk samples with the presence of this pathogen (de Haas et al., 2002; Whist et al., 2007). It was interesting to observe that in Plant B, where the average SCS was higher than in Plant A

(5.77 and 4.46, respectively), there was no significant correlation of *S. dysgalactiae* and SCC. The presence of other bacteria associated with the SCC in Plant B could be responsible for the difference noted in SCS average.

In Plant B, *Bacillus drentensis* and *Ehrlichia ewingii* had a positive correlation with BTM SCC, and on the other hand, *Ornithinimicrobium* species presented a negative correlation. This is the first report of their presence in dairy products. *B. drentensis* and *Ornithinimicrobium* species are commonly isolated from soil (Heyman et al., 2004; XM et al., 2020) and *E. ewingii* is a canine pathogen that causes granulocytic ehrlichiosis (ANDERSON et al., 1992).

2.4.4. Microbiome profile correlation with SPC

The correlation analysis between SPC and the microbiome resulted in 2 species identified for plant A and 21 for plant B. High values of SPC can indicate hygienic deficiencies, inadequate cold chain temperature, or even the presence of psychrotrophic bacteria. Common psychrotrophic bacteria genera found in milk that also had a positive significant correlation with SPC were *Pseudomonas*, *Acinetobacter*, *Bacillus*, and *Staphylococcus* (RIBEIRO JÚNIOR et al., 2017; VITHANAGE et al., 2017). Vacheyrou et al. (VACHEYROU et al., 2011) evaluated the presence of several bacteria in different environments of dairy farms, and as a result, they identified *Streptococcus* presence in milk and hay, *Enterococcus* and *Lactococcus* in milk, *Stenotrophomonas* in air and hay, *Chryseobacterium* in dust, *Pseudomonas*, *Kocuria*, *Bacillus*, *Staphylococcus*, and *Acinetobacter* were found in several samples as milk, air, hay, dust, and teat surface. Hence, although some genera are common in milk, their presence could indicate problems in cleaning protocols and guide the attention for the primary sources of milk contamination in a dairy farm. Also, it is important to notice the presence of spore and biofilm-forming bacteria, as *S. maltophilia*, *Pseudomonas spp.*, *Bacillus spp.*, and *S. aureus* (CLETO et al., 2012; SHARMA; ANAND, 2002; WATTS, 1988). Because of its capability of adhering to surfaces and heat resistance, those bacteria are a potential source of constant contamination in the dairy industry.

2.5. Conclusion

Here, we report the bulk tank milk microbiome profile at the species level identified by 16S rDNA sequencing. Hence, we identified species frequently found in milk and reported new ones establishing the core microbiome for bulk tank milk for Brazilian dairy cattle. Among the bacteria on the core microbiome, some important and commonly observed in milk were *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Lactococcus lactis*. Also, other species were found, some of them being reported for the first time in BTM, as *B. drentensis* and *I. barlettii*. Relevant bacteria abundance was correlated with bulk tank SCC, such as *Strep. dysgalactiae* and *Strep. agalactiae*, and with bulk tank SPC, as *Pseudomonas*, *Bacillus*, *Streptococcus* and *Staphylococcus*. Identification of bacteria present in bulk tank milk and its correlations with SCC and SPC can provide an important first evaluation of the microbial profile of dairy herds. The microbiome information can help identify pathogenic bacteria, contaminants, and sources of contamination, and thus improve milk quality and shelf life of milk products.

References

- ALESSANDRIA, V.; DOLCI, P.; RANTSIOU, K.; PATTONO, D.; DALMASSO, A.; CIVERA, T.; COCOLIN, L. Microbiota of the Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Cape Verde Islands. **World Journal of Microbiology and Biotechnology** 2010, 26:12, v. 26, n. 12, p. 2211–2221, 10 abr. 2010.
- ALI, A. K. A.; SHOOK, G. E. An Optimum Transformation for Somatic Cell Concentration in Milk. **Journal of Dairy Science**, v. 63, n. 3, p. 487–490, 1 mar. 1980.
- ANDERSON, B. E.; GREENE, C. E.; JONES, D. C.; DAWSON², J. E. Ehrlichia ewingii sp. nov., the Etiologic Agent of Canine Granulocytic Ehrlichiosis. **International Journal of Systematic Bacteriology**. [s.l: s.n.].
- BERLAU, J.; AUCKEN, H. M.; HOUANG, E.; PITT, T. L. Isolation of Acinetobacterspp including A.baumannii from vegetables: implications for hospital-acquired infections. **Journal of Hospital Infection**, v. 42, p. 201–204, 1999.
- BONSAGLIA, E. C. R.; GOMES, M. S.; CANISSO, I. F.; ZHOU, Z.; LIMA, S. F.; RALL, V. L. M.; OIKONOMOU, G.; BICALHO, R. C.; LIMA, F. S. Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland. **Scientific Reports**, v. 7, n. 1, p. 1–10, 1 dez. 2017.
- BURTON, K. J.; ROSIKIEWICZ, M.; PIMENTEL, G.; BÜTIKOFER, U.; AH, U. von; VOIROL, M.-J.; CROXATTO, A.; AEBY, S.; DRAI, J.; MCTERNAN, P. G.; GREUB, G.; PRALONG, F. P.; VERGÈRES, G.; VIONNET, N. Probiotic yogurt and acidified milk similarly reduce postprandial inflammation and both alter the gut microbiota of healthy, young men. **British Journal of Nutrition**, v. 117, n. 9, p. 1312–1322, 14 may 2017.
- CALLAHAN, B. J.; MCMURDIE, P. J.; ROSEN, M. J.; HAN, A. W.; JOHNSON, A. J. A.; HOLMES, S. P. DA2: High-resolution sample inference from Illumina amplicon data. **Nature Methods**, v. 13, n. 7, p. 581–583, 29 jun. 2016.
- CHAMPAGNE, C.; LAING, R.; ROY, D.; MAFU, A.; GRIFFITHS, M. Psychrotrophs in dairy products: their effects and their control. **Critical reviews in food science and nutrition**, v. 34, n. 1, p. 1–30, 1 jan. 1994a.
- CHAMPAGNE, C. P.; LAING, R. R.; ROY, D.; MAFU, A. A.; GRIFFITHS, M. I. W. Psychrotrophs in dairy products: their effects and their control. **Critical reviews in food science and nutrition**, v. 34, n. 1, p. 1–30, 1 jan. 1994b.
- CLETO, S.; MATOS, S.; KLUSKENS, L.; VIEIRA, M. J. Characterization of Contaminants from a Sanitized Milk Processing Plant. **PLoS ONE**, v. 7, n. 6, p. 40189, 2012.
- COLLINS, C.; ALMUZARA, M.; SAIGO, M.; MONTAÑA, S.; CHIEM, K.; TRAGLIA, G.; MUSSI, M. A.; TOLMASKY, M.; IRIARTE, A.; CARLOS VAY, ; RAMIREZ, M. S. Whole-Genome Analysis of an Extensively Drug-Resistance Empedobacter falsenii Strain Reveals Distinct Features and the Presence of a Novel Metallo-β-Lactamase (EBR-2). **Current Microbiology**, v. 75, p. 1084–1089, 2018.
- DE HAAS, Y.; BARKEMA, H. W.; VEERKAMP, R. F. The effect of pathogen-specific clinical mastitis on the lactation curve for somatic cell count. **Journal of dairy science**, v. 85, n. 5, p. 1314–1323, 2002.
- DELBÈ, 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**, v. 73, n. 6, p. 1882–1891, 2007.

- GANDA, E. K.; BISINOTTO, R. S.; LIMA, S. F.; KRONAUER, K.; DECTER, D. H.; OIKONOMOU, G.; SCHUKKEN, Y. H.; BICALHO, R. C. Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. 2016.
- HARMON, R. J. Physiology of Mastitis and Factors Affecting Somatic Cell Counts. **Journal of Dairy Science**, v. 77, n. 7, p. 2103–2112, 1 jul. 1994.
- HEYRMAN, J.; VANPARYS, B.; LOGAN, N. A.; BALCAEN, A.; RODRÍGUEZ-DÍAZ, M.; FELSKÉ, A.; VOS, P. De. *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. **International Journal of Systematic and Evolutionary Microbiology**, v. 54, n. 1, p. 47–57, 1 jan. 2004.
- HOLMØY, I. H.; TOFTAKER, I.; KIRKEBY, C.; ØSTERÅS, O.; JØRGENSEN, H. J.; NØDTVEDT, A. A cohort study of the effect of *Streptococcus agalactiae* on milk yield and somatic cell count in Norwegian dairy cows. **Journal of Dairy Science**, v. 102, n. 9, p. 8385–8399, 1 set. 2019.
- KAMPFER, P.; AVESANI, V.; JANSSENS, M.; CHARLIER, J.; DE BAERE, T.; VANEECHOUTTE, M. Description of *Wautersiella falsenii* gen. nov., sp. nov., to accommodate clinical isolates phenotypically resembling members of the genera *Chryseobacterium* and *Empedobacter*. **International journal of systematic and evolutionary microbiology**, n. 56, p. 2323–2329, 2006.
- KANO, R.; KOBAYASHI, Y.; NISHIKAWA, A.; MURATA, R.; ITOU, T.; ITO, T.; SUZUKI, K.; KAMATA, H. Next-generation Sequencing Analysis of Bacterial Flora in Bovine Protothecal Mastitic Milk. **Medical Mycology Journal**, v. 59, n. 3, p. E41–E46, 2018.
- 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**, v. 95, n. 10, p. 5702–5708, 1 out. 2012.
- KEEFE, G. P.; DOHOO, I. R.; SPANGLER, E. Herd Prevalence and Incidence of *Streptococcus agalactiae* in the Dairy Industry of Prince Edward Island. **Journal of Dairy Science**, v. 80, n. 3, p. 464–470, 1 mar. 1997.
- LOPES, J. E. F.; LANGEI, C. C.; BRITO, M. A. V. P.; SANTOS, F. R.; SILVA, M. A. S.; DE MORAES, L. C. D.; DE SOUZA, G. N. Relationship between total bacteria counts and somatic cell counts from mammary quarters infected by mastitis pathogens. **Ciência Rural**, v. 42, n. 4, p. 691–696, abr. 2012.
- MASOUD, W.; VOGENSEN, F.; LILLEVANG, S.; ABU AL-SOUD, W.; SØRENSEN, S.; JAKOBSEN, M. The fate of indigenous microbiota, starter cultures, *Escherichia coli*, *Listeria innocua* and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. **International journal of food microbiology**, v. 153, n. 1–2, p. 192–202, 1 fev. 2012.
- MATÍAS TRAGLIA, G.; DIXON, C.; CHIEM, K.; ALMUZARA, M.; BARBERIS, C.; MONTAÑA, S.; MERINO, C.; MUSSI, M. A.; TOLMASKY, M. E.; IRIARTE, A.; VAY, C.; SOLEDAD RAMÍREZ, M. Draft Genome Sequence of *Empedobacter* (Formerly *Wautersiella*) *falsenii* comb. nov. Wf282, a Strain Isolated from a Cervical Neck Abscess. 2015.
- MCMURDIE, P. J.; HOLMES, S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. **PLOS ONE**, v. 8, n. 4, 2013.
- MEOLA, M.; RIFA, E.; SHANI, N.; DELBÈS, C.; BERTHOUD, H.; CHASSARD, C. DAIRYdb: A manually curated reference database for improved taxonomy annotation of 16S rRNA gene sequences from dairy products. **BMC Genomics**, v. 20, n. 1, 8 jul. 2019.

- METZGER, S. A.; HERNANDEZ, L. L.; SKARLUPKA, J. H.; WALKER, T. M.; SUEN, G.; RUEGG, P. L. A Cohort Study of the Milk Microbiota of Healthy and Inflamed Bovine Mammary Glands From Dryoff Through 150 Days in Milk. **Frontiers in Veterinary Science**, v. 0, n. OCT, p. 247, 9 out. 2018.
- MIURA, A.; KURUMISAWA, T.; KANO, R.; ITO, T.; SUZUKI, K.; KAMATA, H. Next-generation sequencing analysis of bacterial flora in bovine protothecal mastitic milk and feces. **J Vet Med Sci.**, 2019.
- NEMEC, A.; MUSÍLEK, M.; ŠEDO, O.; BAERE, T. De; MAIXNEROVÁ, M.; REIJDEN, T. J. K. van der; ZDRÁHAL, Z.; VANECHOUTTE, M.; DIJKSHOORN, L. *Acinetobacter bereziniae* sp. nov. and *Acinetobacter guillouiae* sp. nov., to accommodate *Acinetobacter* genomic species 10 and 11, respectively. **International Journal of Systematic and Evolutionary Microbiology**, v. 60, n. 4, p. 896–903, 1 abr. 2010.
- OHTA, H.; HATTORI, R.; USHIBA, Y.; MITSUI, H.; ITO, M.; WATANABE, H.; TONOSAKI, A.; HATTORI, T. *Sphingomonas oligophenolica* sp. nov., a halo- and organo-sensitive oligotrophic bacterium from paddy soil that degrades phenolic acids at low concentrations. **International Journal of Systematic and Evolutionary Microbiology**, v. 54, n. 6, p. 2185–2190, 1 nov. 2004.
- PETZER, I. M.; KARZIS, J.; DONKIN, E. F.; WEBB, E. C.; ETTER, E. M. C. Validity of somatic cell count as indicator of pathogen-specific intramammary infections. **Journal of the South African Veterinary Association**, v. 88, n. 0, p. 1019–9128, 2017.
- PHUEKTES, P.; BROWNING, G. F.; ANDERSON, G.; MANSELL, P. D. Multiplex polymerase chain reaction as a mastitis screening test for *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* in bulk milk samples. 2021.
- QUIGLEY, L.; O'SULLIVAN, O.; STANTON, C.; BERESFORD, T. P.; ROSS, R. P.; FITZGERALD, G. F.; COTTER, P. D. The complex microbiota of raw milk. **FEMS Microbiology Reviews**, set. 2013.
- R CORE TEAM. **R: A Language and Environment for Statistical Computing**. [s.l.: s.n.]. Disponível em: <<https://www.R-project.org>>. Access in: 2 jun. 2019.
- RAMOS, G. L. de P. A.; NASCIMENTO, J. dos S.; RAMOS, G. L. de P. A.; NASCIMENTO, J. dos S. Characterization of *Acinetobacter* spp. from raw goat Milk. **Ciência Rural**, v. 49, n. 10, 23 set. 2019.
- RIBEIRO JÚNIOR, J. C.; DE OLIVEIRA, A. M.; SILVA, F. de G.; TAMANINI, R.; DE OLIVEIRA, A. L. M.; BELOTTI, V. The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. **Journal of Dairy Science**, v. 101, n. 1, p. 75–83, 2017.
- RJ, E.; RJ, E.; LJ, H.; SB, S. Herd management and prevalence of mastitis in dairy herds with high and low somatic cell counts. **Journal of the American Veterinary Medical Association**, v. 190, n. 11, p. 1411–1416, 1 jun. 1987.
- RODRIGUES, M. X.; LIMA, S. F.; CANNIATTI-BRAZACA, S. G.; BICALHO, R. C. The microbiome of bulk tank milk: Characterization and associations with somatic cell count and bacterial count. **Journal of dairy science**, v. 100, n. 4, p. 2536–2552, 1 abr. 2017.
- SAVICKY, P. **Spearman's rank correlation test**, 2015.
- SHARMA, M.; ANAND, S. K. Characterization of constitutive microflora of biofilms in dairy processing lines. **Food Microbiology**, v. 19, n. 6, p. 627–636, 1 dez. 2002.
- SHKRETA, L.; TALBOT, B. G.; DIARRA, M. S.; LACASSE, P. Immune responses to a DNA/protein vaccination strategy against *Staphylococcus aureus* induced mastitis in dairy cows. **Vaccine**, v. 23, n. 1, p. 114–126, 15 nov. 2004.

- SPEIRS, L. B. M.; RICE, D. T. F.; PETROVSKI, S.; SEVIOUR, R. J. The Phylogeny, Biodiversity, and Ecology of the Chloroflexi in Activated Sludge. 2019.
- STODDARD, S. F.; SMITH, B. J.; HEIN, R.; ROLLER, B. R. K.; SCHMIDT, T. M. rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. **Nucleic Acids Research**, v. 43, n. D1, p. D593–D598, 28 jan. 2015.
- TURTON, J. F.; SHAH, J.; OZONGWU, C.; PIKE, R. Incidence of Acinetobacter Species Other than *A. baumannii* among Clinical Isolates of Acinetobacter: Evidence for Emerging Species †. **JOURNAL OF CLINICAL MICROBIOLOGY**, v. 48, n. 4, p. 1445–1449, 2010.
- VACHEYROU, M.; NORMAND, A. C.; GUYOT, P.; CASSAGNE, C.; PIARROUX, R.; BOUTON, Y. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. **International Journal of Food Microbiology**, v. 146, n. 3, p. 253–262, 29 abr. 2011.
- VANDEPUTTE, D.; KATHAGEN, G.; VIEIRA-SILVA, S.; VALLES-COLOMER, M.; SABINO, J.; WANG, J.; TITO, raul Y.; DE COMMER, L.; DARZI, Y.; VERMEIRE, S.; FALONY, G.; RAES, J. Quantitative microbiome profiling links gut community variation to microbial load. **Nature**, v. 551, 2017.
- VITHANAGE, N. R.; DISSANAYAKE, M.; BOLGE, G.; PALOMBO, E. A.; YEAGER, T. R.; DATTA, N. Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential. **International Dairy Journal**, v. 57, p. 80–90, 2016.
- VITHANAGE, N. R.; DISSANAYAKE, M.; BOLGE, G.; PALOMBO, E. A.; YEAGER, T. R.; DATTA, N. Microbiological quality of raw milk attributable to prolonged refrigeration conditions. **Journal of Dairy Research**, v. 84, n. 1, p. 92–101, 2017.
- WATTS, J. L. Etiological agents of bovine mastitis. **Veterinary Microbiology**, v. 16, n. 1, p. 41–66, 1 jan. 1988.
- WHIST, A. C.; ØSTERÅS, O.; SØLVERØD, L. Streptococcus dysgalactiae Isolates at Calving and Lactation Performance Within the Same Lactation. **Journal of Dairy Science**, v. 90, n. 2, p. 766–778, 1 fev. 2007.
- WOUTERS, J. T. M.; AYAD, E. H. E.; HUGENHOLTZ, J.; SMIT, G. Microbes from raw milk for fermented dairy products. **International Dairy Journal**, v. 12, n. 2–3, p. 91–109, 1 jan. 2002.
- XM, F.; HJ, D.; JL, B.; WN, H.; J, L.; H, W.; J, S.; HY, L.; YQ, Z.; LY, Y. *Ornithinimicrobium cerasi* sp. nov., isolated from the fruit of *Cerasus pseudocerasus* and emended description of the genus *Ornithinimicrobium*. **International journal of systematic and evolutionary microbiology**, v. 70, n. 3, p. 1691–1697, 2020.

3. COMPARISON OF STANDARD MEDIA CULTURE, CHROMOGENIC MEDIA CULTURE, MALDI-TOF, 16S SEQUENCING WITH qPCR MULTIPLEX FOR MASTITIS PATHOGEN DETECTION

Abstract

The precise and quickly detection of mastitis pathogens is necessary to guide the better choice of treatment protocol in dairy cattle. Nowadays, the methods available for pathogen detection are divided in two, culture-based and molecular methods. From culture-based, the mainly used is the standard microbiological culture with biochemical and physiological tests. The chromogenic media culture, knowing as on-farm kits, have different media for different bacterial groups (gram-positive and negative) or genera/species, and the detection is evaluated by the colony color produced. Other culture-based method is Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), the ribosomal proteins of an isolate is prepared and analyzed by mass spectrometry, and then, contrasted against a reference database for taxonomy classification. About the molecular-based method, the multiplex quantitative polymerase chain reaction (qPCR) has been used and, several commercial kits are available, such as VetMAX MastiType Multi Kit (by ThermoFisher Scientific). Here, we evaluated the performance of standard culture, chromogenic media culture, MALDI-TOF MS, multiplex qPCR, and, for the first time, the v4-16S rRNA sequencing methods for mastitis pathogen detection in bovine milk with subclinical mastitis. Multiplex qPCR was used as gold standard method to calculate sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive values (NPV). As result, only three species *Streptococcus dysgalactiae*, *Escherichia coli*, and *Staphylococcus aureus* were identified by all techniques. Standard media culture resulted on overall Se = 25.77%, Sp = 99.28%, Ac = 88.16%, PPV = 90.91%, and NPV = 82.65%. Chromogenic media culture methodology resulted on Se = 23.71%, Sp = 95.80%, Ac = 80.00%, PPV = 61.33%, and NPV = 81.73%. MALDI-TOF methodology resulted on Se = 31.96%, Sp = 99.28 %, Ac = 84.52%, PPV = 92.54%, and NPV = 83.86%. And, v4-16S rRNA sequencing methodology resulted on Se = 39.69%, Sp = 91.03%, Ac = 79.77%, PPV = 55.40%, and NPV = 84.32%. The higher Sp and Ac of MALDI-TOF MS, and the higher Se of v4-16S rRNA sequencing suggests that these methods, along with qPCR, are suitable for mastitis pathogen identification. Each method has its limitations; therefore, the establishment of the better method will be dependent of tested target pathogens, cost, time of result, and importance of specie determination for treatment choice.

3.1. Introduction

Mastitis is a disease of the mammary gland, caused mainly by bacterial, but also by fungal, algae, and viral infections. Bovine mastitis is the predominant disease in the dairy industry, causing severe economic losses by reducing animal welfare, milk production and quality and, increasing costs associated with treatment (Kitchen, 1981; Horstet and Seegers, 1998; Seegers et al., 2003). Mastitis can present in two ways, clinical and subclinical. Clinical mastitis is characterized by udder swelling, abnormal milk with clots, discoloration secretions, and flakes. On the other hand, subclinical mastitis does not present visual discrepancies of the milk but reduces milk production and quality and its common diagnosis is by laboratory analysis of somatic cell count (Adkins and Middleton, 2018).

The correct identification of mastitis causing pathogen is important for treatment and mastitis control in herd. Different pathogens require different approaches in treatments and management strategies to control the spread of the disease, such as systemic or intramammary antimicrobial, antibiotic spectrum, early animal disposal, or no antimicrobial treatment (Lago et al., 2011, 2016).

Traditionally, mastitis pathogen identification is based on standard microbiological culturing alongside with biochemical and physiological tests. This methodology is laborious, time consuming, and although misidentification of bacteria is not uncommon is still considered as standard for many diagnostic laboratories. However, several reports demonstrated that culturing of mastitis milk samples resulted a 27-50% of false negative results (Makovec and Ruegg, 2003; Olde Riekerink et al., 2006; Spain and Barrett, 2015).

On farm kits have been developed to accelerate pathogens diagnosis based on culturing. These kits are composed by chromogenic media, and are available for pathogens groups (e.g., Gram-negative/Gram-positive), genus (e.g., *Staphylococcus sp.* and *Streptococcus sp.*) or species (*Staphylococcus aureus*) (Ganda et al., 2016; Lago and Godden, 2018). Chromogenic media culturing tests is based on the cleavage of chromogens (substrate) incorporated in the culture media by specific bacterial enzymes, generating a visible change of the colony color, making the test simple to interpret, and accessible for low-trained individuals.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be used for pathogen detection. Extracted and treated ribosomal protein of an isolate is submitted to mass spectrum analysis where peptide mass fingerprint is generated and contrasted with databases for the microbe identification (Singhal et al., 2015). Several studies applied this technique for mastitis pathogen detection, such as group B and D streptococci, non-aureus staphylococci, *Enterobacter*, *Enterococcus sp.*, *Streptococcus sp.*, *Staphylococcus sp.* including *Staph. aureus*. (Barreiro et al., 2010; Werner et al., 2012; Cameron et al., 2017; Nonnemann et al., 2019).

The culturing prerequisite in the methods mentioned above have some limitations, such as high frequency of false-negatives, growth competition between bacteria, no detection of non-viable bacteria, and specific media composition and different growth time rate for different bacteria species/genera. In the last two decades, molecular methods become more available, and PCR turn into the gold standard for bacterial, fungal, and viral pathogen detections due to its higher sensitivity and specificity (Yang and Rothman, 2004) and successful application for mastitis pathogen detection (Phuektes et al., 2001; Riffon et al., 2001; Graber et al., 2007). PCR or qPCR tests are limited by the number of pathogens tested, which can increase the cost and time of diagnosis. Multiplex qPCR reduces time and costs, but as increased number of detectable pathogens is tested, higher are the challenges for multiplex qPCR optimization, such as primer dimers formation, annealing conditions, reduce sensitivity or specificity, and preferential amplification targets. Commercially available kits such as, VetMAX MastiType Multi Kit (ThermoFisher Scientific), and Multiple mastitis pathogens Real-Time PCR Kit (Noak Group, Barentz), enables the accurate detection of 15 major mastitis-causing pathogens.

Recent advances in high throughput next-generation sequencing combined with cost reduction opens the possibility for new strategies for mastitis pathogen detection. Amplification of one or more variable regions of 16S ribosomal RNA gene (16S rRNA) combined with next-generation sequencing, enables the bacterial microbiome identification in specie level (Johnson et al., 2019). 16S rRNA sequencing have been used to comprehend the microbiome present in mastitic milk with culture-negative results (Kuehn et al., 2013), identify the predominant pathogens in subclinical mastitis milk (Pang et al., 2018), or even understand the microbiota diversity in healthy milk (Kuehn et al., 2013; Oikonomou et al., 2014)

The pros and cons of each method for mastitis pathogen diagnosis is extensive, therefore a comparative study using five methods, three culture-dependent (standard culture, chromogenic media culture, and MALDI-TOF MS) and two molecular based (multiplex qPCR and v4-16S rRNA sequencing) is reported here for the first time. We determined specificity, sensitivity, accuracy, predicted positive value and predictive negative value for standard culture, chromogenic media culture, MALDI-TOF MS, and v4-16S rRNA sequencing adopting the multiplex qPCR as the gold standard methodology for mastitis pathogen detection.

3.2. Materials and Methods

3.2.1. Samples

The procedures for milk sample collection were guided by NMC (2017). A total of 60 milk samples were aseptically collected (teat cleaned and disinfected with 70% iodized alcohol (70% alcohol + 2% iodine) and discarding of the first milk) in sterile tube from 60 cows with subclinical mastitis (SCC > 4 x 10⁵ cells per ml and no clinical symptoms) from one farm in São Paulo, Brazil. 15 ml of milk per sample were collected, and to reduce bacterial growth, the samples were transported in coolers and frozen until the analysis. Also, to obtain only comparable samples between all methods, we used only culture-positive samples.

3.2.2. Standard culture identification

For bacteria diagnostic by standard culture methodology, milk samples were inoculated in blood agar media enriched with 5% bovine blood. After inoculation the plates were incubated at 37°C for 24 h. After, standard procedures of conventional microbiological identification were realized, as Gram staining, colony morphology and hemolytic patterns on blood agar (Adkins et al., 2017).

3.2.3. Chromogenic culture media identification

To identify the bacteria in selective chromogenic culture media, we used a Gram-positive (GP), Gram-negative (GN), Streptococcus and Staphylococcus (CHROMagar™, Paris, France) differentiation media. Using a platinum loop, 0.01 mL of milk samples were inoculated in the three chromogenic culture media. After, the plates were submitted to an aerobically incubation at 37°C for 24h followed by visual inspection to evaluate microbiological growth.

For the bacteria identification, the evaluation was carried out with a white background for better color differentiation. The presumptive results were interpreted according to the manufacturer's recommendation, therefore, for GP culture media, the colonies were interpreted according to the colors: turquoise blue - *Streptococcus agalactiae/dysgalactiae*; dark blue/metallic blue - *Streptococcus uberis/Enterococcus spp.*; pink/mauve - *Staphylococcus aureus*. For GN culture media, the colonies were interpreted according to the colors: blue – *Enterobacter spp./Klebsiella spp./Citrobacter spp.*; colorless – *Pseudomonas spp.*; purple - *Escherichia coli*. For Staph. culture media, the colonies were interpreted according to the colors: pink/mauve - *Staphylococcus aureus*; colorless/pinkish - *Staphylococcus epidermidis*; turquoise blue - *Staphylococcus saprophyticus*. And, for Strep. culture media, the colonies were interpreted according to the colors: blue - *Streptococcus agalactiae*; mauve/purple – *Enterococcus*; blue/metallic blue - *Streptococcus uberis*. For all chromogenic culture media, growth colonies with different colors were considered as “other microorganisms”.

3.2.4. MALDI-TOF MS identification

After bacterial growth in chromogenic culture media, all the isolates were submitted to microbiological identification by Matrix Associated Laser Desorption-Ionization—Time of Flight (MALDI-TOF MS). Each isolate of a pure bacterial colony was submitted to a ribosomal protein extraction followed by preparation in the steel plate for analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Daltonics, Inc. Billerica, MA, USA). For internal controls, in each plate was used a positive control (*Escherichia coli*) and a negative control (formic acid and matrix) were analyzed. For protein extraction identification the plate reading was performed according to the specifications (Bruker Daltonik, Bremen, Germany), and for microorganism identification the spectral data processing was done using the MALDI Biotyper 4.1.70 (Bruker Daltonik, Bremen, Germany) computer software (MBT version 7311 MPS library). More protocol details were described by Barcelos et al., (2019).

3.2.5. Multiplex qPCR identification

For DNA extraction, was centrifuged for 5 minutes at 14,000 rpm 2 ml of milk, and then only the pellet was used for DNA extraction protocol. The genomic DNA was obtained using MagMAX™ CORE combined with MagMAX™ CORE Mechanical Lysis Module (ThermoFisher™) according to the manufacturer's instructions. The obtained DNA were used for bacterial identification for both DNA based methodologies, qPCR and sequencing. For qPCR bacterial identification, was used the VetMAX™ MastiType Multi Kit (Applied Biosystems™, CA, USA) according to the manufacturer's instructions. The VetMAX MastiType Multi assay detects 15 pathogens and one resistance gene in four separate PCR reactions: *Staphylococcus aureus*; *Staphylococcus spp.* (including all major coagulase-negative staphylococci); *Streptococcus agalactiae*; *Streptococcus dysgalactiae*; *Streptococcus uberis*; *Escherichia coli*; *Enterococcus spp.* (including *E. faecalis* and *E. faecium*); *Klebsiella oxytoca* (and/or *K. pneumoniae*); *Serratia marcescens*; *Corynebacterium bovis*; *Trueperella pyogenes* and/or *Peptoniphilus indolicus*; Staphylococcal β -lactamase gene (penicillin-resistance gene); *Mycoplasma bovis*; *Mycoplasma spp.*; yeasts; and *Prototheca spp.*. Determination of pathogen presence were according of Ct values established by manufactures instruction for each pathogen.

3.2.6. V4-16S rRNA sequencing identification

For pathogen identification by sequencing, we used the V4 hypervariable region from the 16S rRNA gene. Extracted genomic DNA was used for library construction that was performed according to 16S Metagenomic Sequencing Library Preparation Guidelines (Illumina Inc., San Diego, CA). The primers 515F (5' GTGYCAGCMGCCGCGTAA 3') and 806BR (5' GGACTACN VGGGTWTCTAAT 3') were used to amplify the V4 hypervariable region by PCR. Libraries were pooled together with equimolar quantities, quantified using KAPA Library Quantification Kit (KAPA Biosystems), and sequencing was performed using MiniSeq High Output reagent kit (300 cycles) on the MiniSeq platform (Illumina Inc., San Diego, CA). The v4-16S rRNA sequencing data analysis were conducted in R software (R Core Team, 2013) using DADA2 program (Callahan et al., 2016). Raw reads were submitted to primers removal, error rates learn, quality-filtering, and chimeras identification and removing. After, an ASV (Amplicon Sequencing Variants) table was constructed, and taxonomy assignment against dereplicated DAIRYdb v1.2.5 (Meola et al., 2019) database. Homemade script was used to dereplicate the database to allow

multiple species classification of the same read. As result, sequences in DAIRYdb database with 100% of identity in the V4 hypervariable region were joint and the taxonomy information were concatenated. The sequences with less than 100% of identity were kept without adjustments.

3.2.7. Statistical analysis

Diagnostic tests as specificity (Sp), sensitivity (Se), accuracy (Ac), positive (PPV) and negative predictive values (NPV) were calculated based in true positives (TP) and negatives (TN), false positives (FP) and negatives (FN) as described by Dohoo et al., (2009), and considering the multiplex qPCR as the gold standard methodology. The following formulas were used to calculate the specificity (TN divide by FP and TN); sensitivity (TP divide by TP and FN), accuracy (TP and TN divide by total number of tests); positive predictive value (TP divide by TP and FP); and negative predictive value (TN divide by TN and FN).

3.3. Results

3.3.1. Pathogen diagnosis

From the 60 milk samples analyzed, the standard culture methodology identified 21 species and one genus. The species with the respective number of samples are: *Acinetobacter towneri* (2), *Aerococcus viridans* (2), *Corynebacterium bovis* (3), *Cronobacter sakazakii* (1), *Enterobacter aerogenes*, *Enterobacter asburiae* (1), *Enterobacter cloacae* (2), *Escherichia coli* (5), *Lactococcus lactis* (1), *Prototheca sp.* (1), *Pseudomonas aeruginosa* (1), *Pseudomonas fulva* (2), *Serratia marcescens* (8), *Staphylococcus aureus* (4), *Staphylococcus chromogenes* (11), *Staphylococcus haemolyticus* (2), *Staphylococcus saprophyticus* (1), *Staphylococcus sciuri* (1), *Staphylococcus simulans* (1), *Streptococcus dysgalactiae* (2), *Streptococcus lutetiensis* (1), *Streptococcus uberis* (12).

The chromogenic culture identified five genera being *Citrobacter sp.* (8), *Enterobacter sp.* (8), *Enterococcus sp.* (8), *Klebsiella sp.* (8), *Pseudomonas sp.* (5), and seven species: *Escherichia coli* (8), *Staphylococcus aureus* (7), *Staphylococcus epidermidis* (1), *Staphylococcus saprophyticus* (11), *Streptococcus agalactiae* (8), *Streptococcus dysgalactiae* (8), *Streptococcus uberis* (11).

With MALDI-TOF MS methodology, 25 species were identified *Aerococcus viridans* (1), *Bacillus pumilus* (4), *Enterobacter asburiae* (1), *Enterobacter cloacae* (1), *Enterobacter kobei* (1), *Enterococcus mundtii* (1), *Escherichia coli* (7), *Kosakonia cowanii* (1), *Lactococcus lactis* (1), *Macrocococcus caseolyticus* (1), *Pantoea agglomerans* (1), *Pseudomonas aeruginosa* (1), *Serratia marcescens* (9), *Staphylococcus aureus* (6), *Staphylococcus chromogenes* (21), *Staphylococcus haemolyticus* (7), *Staphylococcus hyicus* (1), *Staphylococcus saprophyticus* (1), *Staphylococcus sciuri* (5), *Staphylococcus simulans* (1), *Streptococcus alactolyticus* (1), *Streptococcus dysgalactiae* (2), *Streptococcus uberis* (10), *Vagococcus fluvialis* (1), *Vagococcus lutrae* (1).

From 15 species/genera and one resistance gene that the multiplex qPCR test can identify, only *Mycoplasma bovis*, *Mycoplasma spp.*, and *Klebsiella spp.* were not detected. The species/genera identified were (with the following quantity of samples) *Corynebacterium bovis* (24), *Enterococcus spp.*, (11), *Escherichia coli* (27), *Prototheca spp.*, (3), *Serratia marcescens* (24), *Staphylococcus aureus* (6), *Staphylococcus spp.* (51), *Streptococcus agalactiae* (5), *Streptococcus dysgalactiae* (7), *Streptococcus uberis* (19), *Trueperella pyogenes/ Peptoniphilus indolicus* (10) and Yeast (7).

The v4-16S rRNA sequencing analysis of the 60 samples resulted on the identification of 587 genera and 927 species. *Fusobacterium nucleatum* was identified in 59 samples. Some important mastitis pathogens were identified

such as *Staphylococcus aureus* (22), *Pseudomonas* sp. (56), *Escherichia coli* (18), *Stenotrophomonas maltophilia* (17), *Pseudomonas aeruginosa* (1), *Streptococcus dysgalactiae* (3), *Streptococcus agalactiae* (2), and *Mycoplasma bovis* (1).

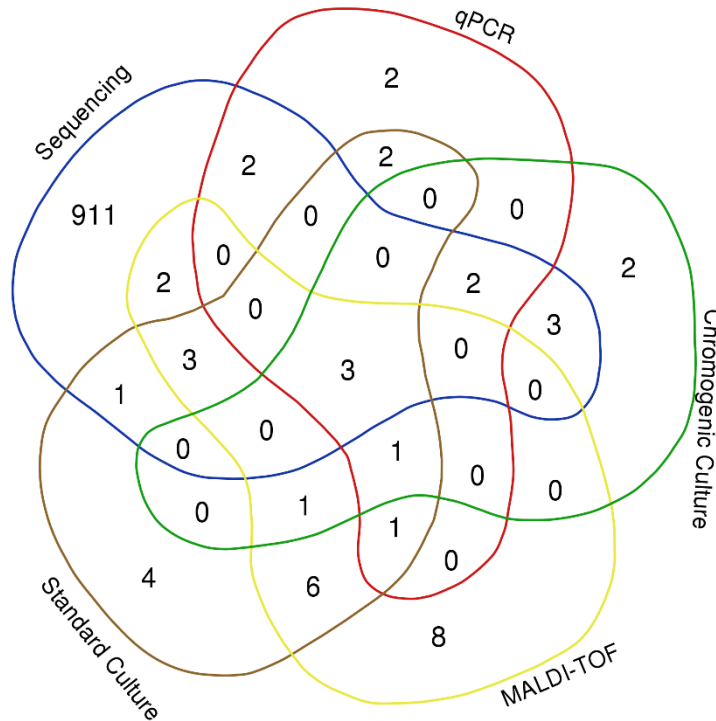


Figure 1. Venn diagram of identified species by diagnosis methodologies. Multiplex qPCR (red), Chromogenic media culture (green), MALDI-TOF MS (yellow), standard culture (brown), and v4-16S rRNA sequencing (blue).

On figure 1, a Venn Diagram was constructed to visualize the distribution of the species identified in the five methodologies. Only *Streptococcus dysgalactiae*, *Escherichia coli*, and *Staphylococcus aureus* were identified by all techniques. The higher merge between the methodologies was with Standard Culture and MALDI-TOF MS with 15 species in common.

3.3.2. Methodologies comparison with qPCR as gold standard

To evaluate the different methods, we used multiplex qPCR as gold standard and calculated sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV) and negative predictive values (NPV), for standard culture, chromogenic media culture, MALDI-TOF MS and v4-16S rRNA sequencing. For those estimations, we used only the 15 species/genera identified in the qPCR multiplex. Standard media culture resulted on overall Se = 25.77%, Sp = 99.28%, Ac = 88.16%, PPV = 90.91%, and NPV = 82.65%. The specie with higher Se was *Streptococcus uberis* with 63.16%. *Enterococcus spp.*, *Streptococcus agalactiae*, *Trueperella pyogenes/ Peptoniphilus indolicus*, and *Yeast* were not identified by standard culture methodology (Table 3).

Table 3. Diagnostic parameters as sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive value (NPV) of standard culture (SC) methodology.

Specie	N qPCR ¹	N SC ²	Se	Sp	Ac	PPV	NPV
<i>Corynebacterium bovis</i>	24	3	8.33%	97.14%	61.02%	66.67%	60.71%
<i>Enterococcus spp.</i>	11	0	0.00%	100.00%	81.36%		81.36%
<i>Escherichia coli</i>	27	5	14.81%	96.88%	59.32%	80.00%	57.41%
<i>Klebsiella spp.</i>	0	0		100.00%	100.00%		100.00%
<i>Mycoplasma bovis</i>	0	0		100.00%	100.00%		100.00%
<i>Mycoplasma spp.</i>	0	0		100.00%	100.00%		100.00%
<i>Prototheca spp.</i>	3	1	33.33%	100.00%	96.61%	100.00%	96.55%
<i>Serratia marcescens</i>	24	8	29.17%	97.14%	69.49%	87.50%	66.67%
<i>Staphylococcus aureus</i>	6	4	50.00%	98.11%	93.22%	75.00%	94.55%
<i>Staphylococcus spp.</i>	51	20	37.25%	87.50%	44.07%	95.00%	17.95%
<i>Streptococcus agalactiae</i>	5	0	0.00%	100.00%	91.53%		91.53%
<i>Streptococcus dysgalactiae</i>	7	2	28.57%	100.00%	91.53%	100.00%	91.23%
<i>Streptococcus uberis</i>	19	12	63.16%	100.00%	88.14%	100.00%	85.11%
<i>Trueperella pyogenes/ Peptoniphilus indolicus</i>	10	0	0.00%	100.00%	83.05%		83.05%
<i>Yeast</i>	7	0	0.00%	100.00%	88.14%		88.14%
Total			25.77%	99.28%	83.16%	90.91%	82.65%

¹Number of samples detected by multiplex qPCR;

²Number of samples detected by standard culture;

Chromogenic media culture methodology resulted on Se = 23.71%, Sp = 95.80%, Ac = 80.00%, PPV = 61.33%, and NPV = 81.73% (Table 2). The specie with higher sensitivity was *Staphylococcus aureus* with 83.33%. *Corynebacterium bovis*, *Mycoplasma bovis*, *Mycoplasma spp.*, *Prototheca spp.*, *Serratia marcescens*, *Trueperella pyogenes/ Peptoniphilus indolicus*, and *Yeast* were not identified by chromogenic media culture methodology. Chromogenic media culture identified 8 samples positive for *Klebsiella spp.* that multiplex qPCR resulted negative (Table 4).

Table 4. Diagnostic parameters as sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive value (NPV) of chromogenic media culture methodology.

Specie	N qPCR ¹	N SC ²	Se	Sp	Ac	PPV	NPV
<i>Corynebacterium bovis</i>	24	0	0.00%	100.00%	59.32%		59.32%
<i>Enterococcus spp.</i>	11	8	27.27%	89.58%	77.97%	37.50%	84.31%
<i>Escherichia coli</i>	27	8	25.93%	96.88%	64.41%	87.50%	60.78%
<i>Klebsiella spp.</i>	0	8		86.44%	86.44%	0.00%	100.00%
<i>Mycoplasma bovis</i>	0	0		100.00%	100.00%		100.00%
<i>Mycoplasma spp.</i>	0	0		100.00%	100.00%		100.00%
<i>Prototheca spp.</i>	3	0	0.00%	100.00%	94.92%		94.92%
<i>Serratia marcescens</i>	24	0	0.00%	100.00%	59.32%		59.32%
<i>Staphylococcus aureus</i>	6	7	83.33%	96.23%	94.92%	71.43%	98.08%
<i>Staphylococcus spp.</i>	51	17	33.33%	100.00%	42.37%	100.00%	19.05%
<i>Streptococcus agalactiae</i>	5	8	20.00%	87.04%	81.36%	12.50%	92.16%
<i>Streptococcus dysgalactiae</i>	7	8	42.86%	90.38%	84.75%	37.50%	92.16%
<i>Streptococcus uberis</i>	19	11	52.63%	97.50%	83.05%	90.91%	81.25%
<i>Trueperella pyogenes/ Peptoniphilus indolicus</i>	10	0	0.00%	100.00%	83.05%		83.05%
<i>Yeast</i>	7	0	0.00%	100.00%	88.14%		88.14%
Total			23.71%	95.80%	80.00%	61.33%	81.73%

¹Number of samples detected by multiplex qPCR;

²Number of samples detected by chromogenic media culture;

MALDI-TOF methodology resulted on Se = 31.96%, Sp = 99.28 %, Ac = 84,52%, PPV = 92.54%, and NPV = 83.86% (Table 5). The specie with higher sensitivity was *Staphylococcus aureus* with 83.33%. *Corynebacterium bovis*, *Klebsiella spp.*, *Mycoplasma bovis*, *Mycoplasma spp.*, *Prototheca spp.*, *Streptococcus agalactiae*, *Trueperella pyogenes/ Peptoniphilus indolicus*, and *Yeast* were not identified by MALDI-TOF methodology.

Table 5. Diagnostic parameters as sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive value (NPV) of MALDI-TOF MS method.

Specie	N qPCR ¹	N SC ²	Se	Sp	Ac	PPV	NPV
<i>Corynebacterium bovis</i>	24	0	0.00%	100.00%	59.32%		59.32%
<i>Enterococcus spp.</i>	11	1	9.09%	100.00%	83.05%	100.00%	82.76%
<i>Escherichia coli</i>	27	7	25.93%	100.00%	66.10%	100.00%	61.54%
<i>Klebsiella spp.</i>	0	0		100.00%	100.00%		100.00%
<i>Mycoplasma bovis</i>	0	0		100.00%	100.00%		100.00%
<i>Mycoplasma spp.</i>	0	0		100.00%	100.00%		100.00%
<i>Prototheca spp.</i>	3	0	0.00%	100.00%	94.92%		94.92%
<i>Serratia marcescens</i>	24	9	29.17%	94.29%	67.80%	77.78%	66.00%
<i>Staphylococcus aureus</i>	6	6	83.33%	98.11%	96.61%	83.33%	98.11%
<i>Staphylococcus spp.</i>	51	32	58.82%	75.00%	61.02%	93.75%	22.22%
<i>Streptococcus agalactiae</i>	5	0	0.00%	100.00%	91.53%		91.53%
<i>Streptococcus dysgalactiae</i>	7	2	28.57%	100.00%	91.53%	100.00%	91.23%
<i>Streptococcus uberis</i>	19	10	52.63%	100.00%	84.75%	100.00%	81.63%
<i>Trueperella pyogenes/Peptoniphilus indolicus</i>	10	0	0.00%	100.00%	83.05%		83.05%
<i>Yeast</i>	7	0	0.00%	100.00%	88.14%		88.14%
Total			31.96%	99.28%	84.52%	92.54%	83.86%

¹Number of samples detected by multiplex qPCR;

²Number of samples detected by MALDI-TOF MS

The v4-16S rRNA sequencing methodology resulted on Se = 39.69%, Sp = 91.03%, Ac = 79.77%, PPV = 55.40%, and NPV = 84.32% (Table 4). The specie with higher sensitivity was *Staphylococcus aureus* with 100%. *Corynebacterium bovis*, *Klebsiella spp.*, *Prototheca spp.*, *Serratia marcescens*, *Streptococcus uberis*, and *Yeast* were not identified by v4-16S sequencing methodology. Although multiplex qPCR resulted negative in all samples for *Mycoplasma bovis* and *Mycoplasma spp.*, 16S sequencing methodology was able to identify in one and three samples, respectively (Table 6). Even *Streptococcus agalactiae* identified in two samples by v4-16S rRNA sequencing, Se and PPV were 0% because the samples were not the same ones that multiplex qPCR detected.

Table 6. Diagnostic parameters as (Se), specificity (Sp), accuracy (Ac), positive predictive values (PPV), and negative predictive value (NPV) of v4-16S rRNA sequencing methodology.

Specie	N qPCR ¹	N SC ²	Se	Sp	Ac	PPV	NPV
<i>Corynebacterium bovis</i>	24	0	0.00%	100.00%	59.32%		59.32%
<i>Enterococcus spp.</i>	11	13	27.27%	79.17%	69.49%	23.08%	82.61%
<i>Escherichia coli</i>	27	19	59.26%	90.63%	76.27%	84.21%	72.50%
<i>Klebsiella spp.</i>	0	0		100.00%	100.00%		100.00%
<i>Mycoplasma bovis</i>	0	1		98.31%	98.31%	0.00%	100.00%
<i>Mycoplasma spp.</i>	0	3		94.92%	94.92%	0.00%	100.00%
<i>Prototheca spp.</i>	3	0	0.00%	100.00%	94.92%		94.92%
<i>Serratia marcescens</i>	24	0	0.00%	100.00%	59.32%		59.32%
<i>Staphylococcus aureus</i>	6	47	100.00%	22.64%	30.51%	12.77%	100.00%
<i>Staphylococcus spp.</i>	51	48	92.16%	87.50%	91.53%	97.92%	63.64%
<i>Streptococcus agalactiae</i>	5	2	0.00%	96.30%	88.14%	0.00%	91.23%
<i>Streptococcus dysgalactiae</i>	7	3	42.86%	100.00%	93.22%	100.00%	92.86%
<i>Streptococcus uberis</i>	19	0	0.00%	100.00%	67.80%		67.80%
<i>Trueperella pyogenes/ Peptoniphilus indolicus</i>	10	3	20.00%	97.96%	84.75%	66.67%	85.71%
Yeast	7	0	0.00%	100.00%	88.14%		88.14%
Total			39.69%	91.03%	79.77%	55.40%	84.32%

¹Number of samples detected by multiplex qPCR;

²Number of samples detected by v4-16S rRNA sequencing;

3.4. Discussion

Defining the best approach for mastitis pathogen diagnosis can be challenging. Each methodology has its limitations, costs, processing time, advantages, and disadvantages. Here, we discuss the performance of standard media culture, chromogenic media culture, MALDI-TOF MS, v4-16S rRNA sequencing and multiplex qPCR.

An overall agreement of the different detection methods can be observed by the diagram (Figure 1). Only three species (*Strep. dysgalactiae*, *Escherichia coli*, and *Staph. aureus*) were identified by the five methods. V4-16S rRNA sequencing method detected more unique species (911), as expected, because of the premises of identification of all bacteria present. The identification of unique by the other methods and not from 16S sequencing (27) could be due to the failure of achieve the taxonomic classification on genus level for 412 ASV (10.3%), and for specie level for 606 ASV (15.2%).

Our standard culture showed a lower value of Se, and a similar value of Sp compared with the findings of Chamchoy et al., (2022) [Se = 88.4 and Sp = 99.8 for *Staph. aureus*, and Se = 76.5 and Sp = 99.6 for *Streptococcus spp.*], Cederlöf et al., (2012) [Se = 83% and Sp = 97% for *Staph. aureus*], and Mahmmmod et al., (2013) [Se = 52.8% and Sp = 89.4 for major mastitis pathogens]. Several factors may influence on culture-based tests estimates, such as procedure of sample collection, duration of infections, fresh or frozen samples, culturing procedures, and contamination definition.

Chromogenic media culture results of low Se = 23.71% and PPV = 61.33% may be affected by the lack of chromogenic media for *Corynebacterium bovis*, *Mycoplasma sp.*, *Prototheca spp.*, and *Trueperella pyogenes/Peptoniphilus indolicus*. A study that evaluated the CHROMAgar *Staph. aureus* media for identification of *Staph. aureus*, resulted on a Se = 69%, Sp = 79.65, PPV = 28.13%, and NPV = 95.74% in comparison with isolates sequencing (Bautista-Trujillo et

al., 2013). Similar values were observed here for *Staph. aureus* with a Se = 83%, Sp = 96%, PPV = 71%, and NVP = 98%.

MALDI-TOF MS have been evaluated comparatively for mastitis pathogen identification with several methodologies. Gene sequencing of *rpoB*, *hsp60*, and *tuf* resulted an Ac = 99.94% (Cameron et al., 2018); and, comparison with 16S rDNA sequencing resulted an agreement of 93% at genus level and 74% at specie level (Jahan et al., 2021). An important fact to understand and consider with the sequencing studies is that the sequencing (gene and 16S methodologies) was performed with DNA extracted from the isolates and instead of whole milk samples. Here, were considered the multiplex qPCR as gold standard and we obtained high values for Ac = 84.52%, Sp = 99.28%, PPV = 92.54%, and NPV = 83.86%, and for sensitivity we obtained a value of 31.96%. MALDI-TOF MS mainly limitation is the requirement of a pure culture specimen, which can be challenging to obtain due to all already cited issues regarding to culturing method.

Neither *Mycoplasma sp.* nor *Mycoplasma bovis* were identified by any of the culture-dependent methods. Mycoplasma genus requires a long period for growth, which may be suppressed by other species (Ashraf et al., 2018). Considering the molecular methods, *Mycoplasma bovis* and *Mycoplasma spp.* were identified only by v4-16S rRNA sequencing, a low amount of DNA obtained could be the reason for the identification by v4-16S rRNA sequencing but not the multiplex qPCR.

The results obtained for multiplex qPCR and v4-16S rRNA sequencing in some unexpected findings. Multiplex qPCR detected *Corynebacterium bovis*, *Prototheca spp.*, *Serratia marcescens*, *Streptococcus uberis* and Yeast, that were not identified by v4-16S rRNA sequencing. Multiplex qPCR detected *Corynebacterium bovis* in 24 samples, on the other hand, v4-16S rRNA sequencing only identified *Corynebacterium* genera in those samples. Concerning *Streptococcus uberis*, multiplex qPCR detected in 19 samples, witch in 17 (89.5%) samples the v4-16S rRNA sequencing identified *Streptococcus* genera. Therefore, the non-detection of *Corynebacterium bovis* and *Streptococcus uberis* by v4-16S rRNA sequencing might due to the incapacity of the database to classify at specie level. Regarding *Serratia marcescens*, v4-16S rRNA sequencing detected one ASV for *Serratia* genera that was classified as *Serratia liquefaciens* specie. In contrast, Yeast and *Prototheca spp.* were expected the non-identification by v4-16S rRNA sequencing. Yeast is a Fungi, which do not have 16S ribosomal DNA gene; and, the DAIRYdb reference database does not have sequences of *Prototheca spp.* genus.

Escherichia coli was detected in 27 samples by qPCR and 19 samples by v4-16S rRNA sequencing, but in only 16 with agreement for both methods. *Enterococcus spp.* had only three samples identified by both methods, in contrast of the 11 samples detected by multiplex qPCR and 13 by v4-16S rRNA sequencing, these explains the low results for Se = 27.27% and PPV = 23.08%. For *Strep. agalactiae*, multiplex qPCR was able to detect five positive samples, in agreement with v4-16S rRNA sequencing results at genera level (*Streptococcus*) unlikely at specie level where none of the samples had agreement. *Strep. dysgalactiae*, was detected in three samples only by multiplex qPCR, other three samples by both methods (multiplex qPCR and v4-16S rRNA sequencing at specie level), and one sample by multiplex qPCR and v4-16S rRNA sequencing at genera level.

Staph. aureus was identified in 47 samples by v4-16S rRNA sequencing conversely with six detected by the multiplex qPCR. The Se = 100% of *Staph. aureus* demonstrate the agreement between the six samples positive by multiplex qPCR and v4-16S rRNA sequencing. Additionally, the number of samples in agreement for *Staph. aureus* detection between the v4-16S rRNA sequencing and the other methods were: six samples with chromogenic media culture, one with MALDI-TOF MS, and four with standard culture. Therefore, 39 samples were positive for *Staph. aureus* only by v4-16S rRNA sequencing method. In addition, from these 39 samples, 38 were positive for *Staphylococ-*

aus spp. genus, these findings might indicate a misidentification at specie level of *Staphylococcus aureus* by v4-16S rRNA sequencing using DAIRYdb database.

Some explanations related to the disagreements between qPCR and sequencing methods results, might be: i) although the multiplex qPCR and the 16S rRNA sequencing protocol is well established, we cannot discard the possibilities of poor efficiency during PCR amplification, such as temperatures for annealing and extension, DNA amount near the limits up and down, inhibitor in sample, and primers specificity; ii) the choose of 16S v4 hypervariable region could not be the best, considering the not classification at level specie for some important pathogens; iii) the incapacity of reliable classification at specie level by our methodology using DAIRYdb, as presented above for *Staph. aureus*. Additionally, other concern about the detection of live and dead bacteria by molecular methods was minimized here by the procedures on DNA extraction. The steps of milk samples centrifugation before the lysis procedure, guided by manufacture instruction, ensures that we obtained only bacteria with intact membrane and not free DNA, with a small portion might be from dead cells (Svennesen et al., 2018).

3.5. Conclusion

Researchers from different fields are in intense work to establish a simple, accurate, sensitive, and cheap diagnosis test for mastitis pathogen. The higher Sp and Ac of MALDI-TOF MS, and the higher Se of v4-16S rRNA sequencing suggests that these methods, along with qPCR are suitable for mastitis pathogen identification. Both methods classify the bacteria based on a reference database with constant updates. Regarding the limitations, MALDI-TOF MS requires a pure culture specimen, which can be challenging to obtain due to all already cited issues regarding to culturing method. Moreover, the correct identification at specie level by 16S rRNA sequencing is crucial and need constant improvement. Each method has its advantages and disadvantages; therefore, the establishment of the better method will be dependent of tested target pathogens, cost, time of result, and importance of specie determination for treatment choice.

References

- Adkins, P.R.F., and J.R. Middleton. 2018. Methods for Diagnosing Mastitis. *Veterinary Clinics of North America: Food Animal Practice* 34:479–491. doi:10.1016/J.CVFA.2018.07.003.
- Adkins, P.R.F., J.R. Middleton, L.K. Fox, G. Pighetti, and C. Petersson-Wolfe. 2017. *Laboratory Handbook on Bovine Mastitis*. Third edition. National Mastitis Council, New Prague.
- Ashraf, A., M. Imran, T. Yaqub, M. Tayyab, W. Shehzad, C.N. Mingala, and Y.-F. Chang. 2018. Development and validation of a loop-mediated isothermal amplification assay for the detection of *Mycoplasma bovis* in mastitic milk. *Folia Microbiologica* 63:373–380. doi:10.1007/s12223-017-0576-x.
- Barcelos, M.M., & L. Martins, R.C. Grenfell, & L. Juliano, & K.L. Anderson, M. v dos Santos, and J.L. Gonçalves. 2019. Comparison of standard and on-plate extraction protocols for identification of mastitis-causing bacteria by MALDI-TOF MS. *Brazilian Journal of Microbiology* 50:849–857. doi:10.1007/s42770-019-00110-5.
- Barreiro, J.R., C.R. Ferreira, G.B. Sanvido, M. Kostrzewa, T. Maier, B. Wegemann, V. Böttcher, M.N. Eberlin, and M.V. dos Santos. 2010. Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Dairy Science* 93:5661–5667. doi:10.3168/jds.2010-3614.

- Bautista-Trujillo, G.U., J.L. Solorio-Rivera, I. Rentería-Solórzano, S.I. Carranza-Germán, J.A. Bustos-Martínez, R.I. Arteaga-Garibay, V.M. Baizabal-Aguirre, M. Cajero-Juárez, A. Bravo-Patiño, and J.J. Valdez-Alarcón. 2013. Performance of culture media for the isolation and identification of *Staphylococcus aureus* from bovine mastitis. *Journal of Medical Microbiology* 62:369–376. doi:10.1099/JMM.0.046284-0/CITE/REFWORKS.
- Callahan, B.J., P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, and S.P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13:581–583. doi:10.1038/nmeth.3869.
- Cameron, M., H.W. Barkema, J. de Buck, S. de Vliegher, M. Chaffer, J. Lewis, and G.P. Keefe. 2017. Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. *Journal of Dairy Science* 100:2137–2147. doi:10.3168/JDS.2016-12020.
- Cameron, M., J. Perry, J.R. Middleton, M. Chaffer, J. Lewis, and G.P. Keefe. 2018. Short communication: Evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. *Journal of Dairy Science* 101:590–595. doi:10.3168/JDS.2017-13226.
- Cederlöf, S.E., N. Toft, B. Aalback, and I.C. Klaas. 2012. Latent class analysis of the diagnostic characteristics of PCR and conventional bacteriological culture in diagnosing intramammary infections caused by *Staphylococcus aureus* in dairy cows at dry off. *Acta Vet Scand* 54:65. doi:10.1186/1751-0147-54-65/TABLES/3.
- Chamchoy, T., E. Okello, D.R. Williams, K. Tonooka, K. Glenn, K. Maehana, I.A. Gardner, and S.S. Aly. 2022. Bayesian estimation of sensitivity and specificity of a rapid mastitis test kit, bacterial culture, and PCR for detection of *Staphylococcus aureus*, *Streptococcus* species, and coliforms in bovine milk samples. *Journal of Dairy Science* 105:6240–6250. doi:10.3168/JDS.2021-20940.
- Dohoo, I.Robert, S.W. Martin, and H. Stryhn. 2009. Screening and Diagnostic Tests. In: *Veterinary Epidemiologic Research*. VER, Inc, Charlottetown.
- Ganda, E.K., R.S. Bisinotto, D.H. Decker, and R.C. Bicalho. 2016. Evaluation of an On-Farm Culture System (Accumast) for Fast Identification of Milk Pathogens Associated with Clinical Mastitis in Dairy Cows. *PLoS One* 11:e0155314. doi:10.1371/journal.pone.0155314.
- Graber, H.U., M.G. Casey, J. Naskova, A. Stelner, and W. Schaeren. 2007. Development of a Highly Sensitive and Specific Assay to Detect *Staphylococcus aureus* in Bovine Mastitic Milk. *Journal of Dairy Science* 90:4661–4669. doi:10.3168/JDS.2006-902.
- Hortet, P., and H. Seegers. 1998. Calculated milk production losses associated with elevated somatic cell counts in dairy cows: review and critical discussion - PubMed. *Veterinary Research* 29:497–510.
- Jahan, N.A., S.M. Godden, E. Royster, T.C. Schoenfuss, C. Gebhart, J. Timmerman, and R.C. Fink. 2021. Evaluation of the matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) system in the detection of mastitis pathogens from bovine milk samples. *Journal of Microbiological Methods* 182:106168. doi:10.1016/J.MIMET.2021.106168.
- Johnson, J.S., D.J. Spakowicz, B.Y. Hong, L.M. Petersen, P. Demkowicz, L. Chen, S.R. Leopold, B.M. Hanson, H.O. Agresta, M. Gerstein, E. Sodergren, and G.M. Weinstock. 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications* 10. doi:10.1038/s41467-019-13036-1.
- Kitchen, B.J. 1981. Bovine mastitis: milk compositional changes and related diagnostic tests. *Journal of Dairy Research* 48:167–188. doi:10.1017/S0022029900021580.

- Kuehn, J.S., P.J. Gorden, D. Munro, R. Rong, Q. Dong, P.J. Plummer, C. Wang, and G.J. Phillips. 2013. Bacterial Community Profiling of Milk Samples as a Means to Understand Culture-Negative Bovine Clinical Mastitis. *PLOS ONE* 8:e61959. doi:10.1371/JOURNAL.PONE.0061959.
- Lago, A., and S.M. Godden. 2018. Use of Rapid Culture Systems to Guide Clinical Mastitis Treatment Decisions. *Veterinary Clinics of North America: Food Animal Practice* 34:389–412. doi:10.1016/J.CVFA.2018.06.001.
- Lago, A., S.M. Godden, R. Bey, P.L. Ruegg, and K. Leslie. 2011. The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. *Journal of Dairy Science* 94:4441–4456. doi:10.3168/JDS.2010-4046.
- Lago, A., C. Tovar, J. Zaragoza, D. Luiz, and D. Pearce. 2016. 0155 The treatment of only environmental Streptococci clinical mastitis cases reduced antibiotic use, days out of the tank, recurrence of clinical mastitis and a tendency to reduce culling. *Journal of Animal Science* 94:75–75. doi:10.2527/JAM2016-0155.
- Mahmmod, Y.S., N. Toft, J. Katholm, C. Grønbaek, and I.C. Klaas. 2013. Bayesian estimation of test characteristics of real-time PCR, bacteriological culture and California mastitis test for diagnosis of intramammary infections with *Staphylococcus aureus* in dairy cattle at routine milk recordings. *Preventive Veterinary Medicine* 112:309–317. doi:10.1016/J.PREVETMED.2013.07.021.
- Makovec, J.A., and P.L. Ruegg. 2003. Results of Milk Samples Submitted for Microbiological Examination in Wisconsin from 1994 to 2001. *Journal of Dairy Science* 86:3466–3472. doi:10.3168/JDS.S0022-0302(03)73951-4.
- Meola, M., E. Rifa, N. Shani, C. Delbès, H. Berthoud, and C. Chassard. 2019. DAIRYdb: A manually curated reference database for improved taxonomy annotation of 16S rRNA gene sequences from dairy products. *BMC Genomics* 20. doi:10.1186/s12864-019-5914-8.
- Nonnemann, B., U. Lyhs, L. Svennesen, K.A. Kristensen, I.C. Klaas, and K. Pedersen. 2019. Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. *Journal of Dairy Science* 102:2515–2524. doi:10.3168/JDS.2018-15424.
- Oikonomou, G., M.L. Bicalho, E. Meira, R.E. Rossi, C. Foditsch, V.S. Machado, A.G.V. Teixeira, C. Santisteban, Y.H. Schukken, and R.C. Bicalho. 2014. Microbiota of Cow's Milk; Distinguishing Healthy, Sub-Clinically and Clinically Diseased Quarters. *PLOS ONE* 9:e85904. doi:10.1371/JOURNAL.PONE.0085904.
- Olde Riekerink, R.G.M., H.W. Barkema, S. Veenstra, D.E. Poole, R.T. Dingwell, and G.P. Keefe. 2006. Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island. *The Canadian Veterinary Journal* 47:567.
- Pang, M., X. Xie, H. Bao, L. Sun, T. He, H. Zhao, Y. Zhou, L. Zhang, H. Zhang, R. Wei, K. Xie, and R. Wang. 2018. Insights into the bovine milk microbiota in dairy farms with different incidence rates of subclinical mastitis. *Frontiers in Microbiology* 9:2379. doi:10.3389/FMICB.2018.02379/BIBTEX.
- Phuektes, P., P.D. Mansell, and G.F. Browning. 2001. Multiplex Polymerase Chain Reaction Assay for Simultaneous Detection of *Staphylococcus aureus* and Streptococcal Causes of Bovine Mastitis. *Journal of Dairy Science* 84:1140–1148. doi:10.3168/JDS.S0022-0302(01)74574-2.
- R Core Team. 2013. *R: A Language and Environment for Statistical Computing*. Vienna.
- Riffon, R., K. Sayasith, H. Khalil, P. Dubreuil, M. Drolet, and J. Lagacé. 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *Journal of Clinical Microbiology* 39:2584–2589. doi:10.1128/JCM.39.7.2584-2589.2001/ASSET/4CDAA27F-A1BB-4E4E-A65E-874DA039BDBE/ASSETS/GRAPHIC/JM0711639003.JPEG.

- Seegers, H., C. Fourichon, and F. Beaudeau. 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary Research* 34:475–491. doi:10.1051/VETRES:2003027.
- Singhal, N., M. Kumar, P.K. Kanaujia, and J.S. Viridi. 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology* 6:791. doi:10.3389/fmicb.2015.00791.
- Spain, S.L., and J.C. Barrett. 2015. Strategies for fine-mapping complex traits. *Human Molecular Genetics* 24Spain, S:R111–R119. doi:10.1093/hmg/ddv260.
- Svennesen, L., Y.S. Mahmmod, N.K. Skjølstrup, L.R. Mathiasen, J. Katholm, K. Pedersen, I.C. Klaas, and S.S. Nielsen. 2018. Accuracy of qPCR and bacterial culture for the diagnosis of bovine intramammary infections and teat skin colonisation with *Streptococcus agalactiae* and *Staphylococcus aureus* using Bayesian analysis. *Preventive Veterinary Medicine* 161:69–74. doi:10.1016/J.PREVETMED.2018.10.013.
- Werner, G., C. Fleige, A.T. Feßler, M. Timke, M. Kostrzewa, M. Zischka, T. Peters, H. Kaspar, and S. Schwarz. 2012. Improved identification including MALDI-TOF mass spectrometry analysis of group D streptococci from bovine mastitis and subsequent molecular characterization of corresponding *Enterococcus faecalis* and *Enterococcus faecium* isolates. *Veterinary Microbiology* 160:162–169. doi:10.1016/J.VETMIC.2012.05.019.
- Yang, S., and R.E. Rothman. 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. *Lancet Infect Dis* 4:337–348. doi:10.1016/S1473-3099(04)01044-8.