ORIGINAL ARTICLE

https://doi.org/10.21708/avb.2023.17.2.11673

Bacterial diversity and detection of resistance genes to broad-spectrum betalactams in dairy family farm soils

Diversidade bacteriana e detecção de genes de resistência a betalactâmicos de amplo espectro em solos de propriedade familiar leiteira

Danilo Alves de França[1], Karen Caroline Ferreira Santaren[2], Gustavo Souza Lima Sant'Anna[3], Miliane Moreira Soares de Souza[3], Shana de Mattos de Oliveira Coelho[3], Irene da Silva Coelho[3]*

ABSTRACT: Bovine mastitis is a complex disease that brings great losses to the dairy producer. The microbial diversity of the soils, as well as the presence of resistance genes in the environment directly influence the maintenance of mastitis in the farm. The objective of this work was to analyze the bacterial diversity in pasture soils of a dairy family farm, detecting enterobacteria that may be involved in the etiology of bovine mastitis, and to detect genes that encode broad-spectrum betalactamases in these soils. Twelve soil samples, representative of different areas of the farm located in the municipality of Barra do Piraí, Rio de Janeiro, were collected at different times of the year. Total DNA was extracted from the samples, gene amplified by Nested-PCR and then the amplification products were separated by DGGE (Denaturing Gradient Gel Electrophoresis). With the DGGE it was possible to construct dendograms that effectively represented the bacterial diversity of these soils. Eight of the soil samples were used to amplify the genes encoding the betalactamase enzymes TEM (blaTEM gene), SHV (blaSHV gene) and CTX (blaCTXM gene). In three of the eight soil samples, the blaSHV gene was found to be present. The blaTEM and blaCTX-M genes were not detected in any of the samples. The detection of genes encoding broad-spectrum betalactamases in dairy cattle pasture soils is of concern, because the transfer of gene material between pathogenic and non-pathogenic bacteria in this environment is a reality.

KEYWORDS: Betalactamase; DGGE; enterobacteria; mastitis; resistome.

RESUMO: A mastite bovina é uma doença complexa que traz grandes prejuízos ao produtor de leite. A diversidade microbiana dos solos, bem como a presença de genes de resistência no ambiente, influencia diretamente a manutenção da mastite na fazenda. O objetivo deste trabalho foi analisar a diversidade bacteriana em solos de pastagem de uma propriedade familiar leiteira, detectando enterobactérias que possam estar envolvidas na etiologia da mastite bovina, e detectar genes que codificam betalactamases de amplo espectro nesses solos. Doze amostras de solo, representativas de diferentes áreas da fazenda localizada no município de Barra do Piraí, Rio de Janeiro, foram coletadas em diferentes épocas do ano. O DNA total foi extraído das amostras, o gene foi amplificado por Nested-PCR e, em seguida, os produtos de amplificação foram separados por DGGE (*Denaturing Gradient Gel Electrophoresis*). Com a DGGE, foi possível construir dendogramas que representavam efetivamente a diversidade bacteriana desses solos. Oito das amostras de solo foram usadas para amplificar os genes que codificam as enzimas betalactamase TEM (gene blaTEM), SHV (gene blaSHV) e CTX (gene blaCTXM). Em três das oito amostras de solo, foi constatada a presença do gene blaSHV. Os genes blaTEM e blaCTX-M não foram detectados em nenhuma das amostras. A detecção de genes que codificam betalactamases de amplo espectro em solos de pastagens de gado leiteiro é preocupante, pois a transferência de material genético entre bactérias patogênicas e não patogênicas nesse ambiente é uma realidade.

PALAVRAS-CHAVE: Betalactamase; DGGE; enterobactérias; mastite; resistoma.

INTRODUCTION

Environmental mastitis, whose infection occurs in the interval between milking processes, within the pickets, is considered to be one of the main problems encountered in the production environment with serious repercussions on the internal milk supply, and clinically, generating evident lesions on the animals' udders as well as the possibility of generalized infections. *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* e *Enterobacter cloacae* are enterobacteria commonly found in

[1]Universidade Estadual Paulista/SP, Brasil
[2]Universidade Federal do Rio de Janeiro/RJ, Brasil
[3]Universidade Federal Rural do Rio de Janeiro/RJ, Brasil
*Corresponding author: irenecoelho@ufrrj.br
Received: 01/23/2023. Accepted: 05/15/2023

manure, feces, urine and organic bedding contaminated by animal excretions, important in the onset of mastitis. Animals raised under semi-intensive systems stay most of the time in pickets, and soils are frequently contaminated with microorganisms, supporting a cycle of animal-environment transmission of these agents (Klaas; Zadoks, 2018).

Mastitis is determined by factors such as: hygiene practices of the pens, animal management and the potential of the bacteria to cause disease. Pathogenicity will depend on the intrinsic virulence characteristics of these bacteria and resistance to antimicrobial therapies to which the animals are subjected (Moraes et al., 2023).

The analysis of microbial richness and diversity in soils allows the establishment of the correlation between the variety of microorganisms in this environment with the incidence of mastitis in the herd, in order to identify the challenges that will be aligned and specific and strategic points of control of the disease (Liu et al., 2022).

The detection of resistance genes, in turn, complements the study of the diversity of bacteria in the soil, pointing out the impact that these bacteria have when in contact with animals and humans. This impact is noticeable in the difficulty of antimicrobial treatments established on the farm, and in the risks to public health that the persistence of this type of bacteria can promote. Antimicrobial resistance is currently considered a threat to public health worldwide, and the indiscriminate use of antimicrobials in animal production contributes to the selection of resistant bacteria (Marques et al., 2017). The excrements from these animals may contain residues of antimicrobials, resistant bacteria and resistance genes, and once in the soil, they can modify the resistome of this environment and favor the dissemination of resistance genes for commensal bacteria and human pathogens. The occurrence of these bacteria in the soil can influence the persistence of mastitis on the farm, which is practically impossible to eradicate, in addition to the occurrence of other diseases in the herd and family workers (Delgado-Baquerizo et al., 2022).

The implementation of techniques independent of bacterial cultivation for the analysis of bacterial diversity and resistance genes, such as Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE), offers an effective alternative for examining the microbial composition of soils on dairy farms, which is not possible using conventional cultivation methods, offering the possibility to examine a large number of samples at once and in a relatively short time. In addition, they allow analyses of resistance of these bacteria to broad-spectrum antimicrobials used in routine production (Marques et al., 2013; Dibbern et al., 2015).

Thus, the objective of this work was to detect Gram negative bacteria present in the pasture soil of dairy family farms that may be involved in the etiology of bovine mastitis and to detect genes that encode broad spectrum betalactamases in these soils, which could be associated with the maintenance of the disease on the farm.

MATERIAL AND METHODS

A dairy farm located in the city of Barra do Piraí-RJ, with a herd of about 40 cows, was selected. Soil samples were collected from the rotational pasture areas where the animals roam. The samples were collected in four pickets and in different seasons of the year (spring and fall of 2014, summer and winter of 2015), taking into account the last picket used by the animals before collection. Each picket sampled was represented by three sub-samples, referring to randomly selected areas within the picket, totaling 12 sub-samples, taken at a depth of 0-10 cm and then kept under refrigeration to perform the following steps (Table 1). After the collection period, they were stored at -20°C for further analysis in the Veterinary Bacteriology Laboratory of the Veterinary Institute of Federal Rural University of Rio de Janeiro.

Total DNA extraction from soil was performed using the PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc), according to the protocol provided by the manufacturer. The extracted DNA was used as a template for amplification of the *rrs* gene that encodes the 16S rRNA of bacteria using the Nested PCR (Polymerase Chain Reaction) technique. In the first reaction, primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1512r (5'-ACGGCTACCTTGTTACGACT-3') were used, which generate fragments of approximately 1500 bp (Suzuki: Giovannoni, 1996). The products generated in the 1st reaction were used as template for the 2nd PCR reaction using the primer pair: GC-338f (5'-ACTCCTACGGGAGG CAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3'), which generate final product around 198 bp (Kane; Poulsen; Stahl, 1993). The products of the PCR reactions were visualized by 1.5% agarose gel electrophoresis under UV light in the L-PIX EX photodocumentation system (Loccus Biotechnology).

Nested-PCR products were separated by DGGE (Denaturing Gradient Gel Electrophoresis) in 8% polyacrylamide gel and concentration gradient between 45% and 65%, defined from the mixture of urea and deionized formamide solutions. Electrophoresis was performed at 70 V and 60°C for 16 hours in a vertical DcodeTM "Universal Mutation Detection System" (BIO-Rad, Richmond, USA) previously added and heated with 0.5% TAE buffer at 60°C. The electrophoretic run was conducted at 70 V for 16 hours. After the gel run, it was stained with silver nitrate (Duarte; Krautter; Soares, et al., 2001). The gels were photographed and the images were analyzed with Bionumerics software

Table 1. Representation of the samples collected from each area of the dairy farm

Pickets	Area 1	Area 2	Area 3	Season
Picket 9	1P9	2P9	3P9	spring
Picket 10	1P10	2P10	3P10	autumn
Picket 4	1P4	2P4	3P4	summer
Picket 6	1P6	2P6	3P6	winter

(AppliedMaths, Saint-Martens-Latem), for band counting to analyze the richness of bacterial populations, and to determine the differences using the Dice coefficient and for cluster analysis with the UPGMA algorithm (Unweighted Pair-Group Method with Arithmetic Mean).

In each DGGE gel a marker composed of PCR products of the bacteria Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, and Enterobacter cloacae was included. With this, the absolute and relative frequency of the bacterial groups observed was established. In addition, it was possible to establish a semi-quantitative analysis of each species detected in the gels, according to the greater or lesser degree of intensity of the bands formed, from a numerical pattern, where 1: formation of weak bands representing a small quantity, 2: slightly more evident bands, reasonable quantity, 3: bands of medium intensity, medium quantity, and 4: intense bands, large quantity.

The presence of genes encoding broad-spectrum betalactamases (ESBL), related to betalactam resistance, was also evaluated. To amplify the genes that encode TEM-type betalactamases (blaTEM gene), we used the primers blaTEM F (5' - ATGAGTATTCAACATTTCCGTG - 3') and blaTEM R (5' -TTACCAATGCTTAATCAGTGAG - 3'), which generate fragments of approximately 861 bpb (Minarini et al., 2007). For amplification of genes encoding SHV-type betalactamases (blaSHV gene), the primers blaSHV F (5' - TTTATCGGCCCTCACTCAAGG - 3') and blaSHV R (5' -GCTGCGGGCCGGATAACG - 3') were used, which generate fragments of approximately 931 bpb (Shahid, 2010). For amplification of genes encoding CTX-M-type betalactamases (blaCTX-M gene), the primers blaCTX-M F (5' - AAAAATCACTGCGCCAGTTC - 3') and blaCTX-M R (5' -CCGTCGGTGACGATTTTAGCC - 3') were used, which generate fragments of approximately 862 bpb (Geser; Stephan; Hächler, 2012). The products of the PCR reactions were visualized by 1.5% agarose gel electrophoresis under UV light in the L-PIX EX photodocumentation system (Loccus Biotechnology), and the presence and absence of bands were analyzed. The presence of the band of the expected size indicates the presence of the gene, and absence of the band indicates absence of the gene.

RESULTS

The PCR generated a product of approximately 1500 bp in 100% (16/16) of the samples. The second reaction (Nested-PCR) generated a product of approximately 198 bp in the same samples. In the DGGE gel, different taxonomic operational units (UTOs) of bacteria were observed, expressed by the bands in the gel. The higher number of UTOs is an indication of higher species richness of bacteria in the soil, and the different positioning of the bands along the gel indicate different gene sequences belonging to different bacterial groups (Table 2).

The dendograms were assembled from the gels, and the bacterial diversity of the sampled areas could be verified. In Figure 1, it is possible to observe that the profile of bacteria present in certain areas differ from the others, to a greater or lesser degree. Areas with greater similarity of bands generated are grouped in brackets, indicating similarity in the bacterial profile.

The marker containing amplification products of the bacteria of interest was added to all gels, allowing comparison of the height of the bands generated in the samples with the marker bands, as shown in Figure 2.

With the DGGE maker, it was possible to verify in the samples the presence of Gram negative bacteria involved in the etiology of environmental mastitis, with emphasis on *E. coli* and *Pseudomonas* spp., both detected in 100%. *Proteus* spp. and *Enterobacter* spp. species were detected in 88.9% and 72.2%, respectively.

In Table 3, the result of the band intensity analysis was expressed, based on a numerical standard, which shows the highest and lowest prevalence for the bacteria present in the production environment. We have in descending order, from highest to lowest occurrence, *E. coli*, *Pseudomonas* spp, *Proteus* spp. and *Enterobacter* spp.

Eight of the 12 soil samples were tested for genes encoding betalactamases. In three of the eight soil samples the *blaSHV* gene was found to be present 37.5% (3/8) with a product size of approximately 931 bp. The *blaTEM* and *blaCTX-M* genes were not detected in any of the samples. The results are expressed in Figure 3 and Table 4.

DISCUSSIONS

The presence of these microorganisms in the soil, justify the relation of environmental mastitis with the cases of disease in the property and the drop in milk production. The DGGE molecular technique allowed the analysis of a large number of samples at the same time and a more reliable and detailed analysis of the bacteria present, informing us of the intensity with which these bacteria are present in the soils, the bacterial richness present in them and the bacterial diversity in general.

Regarding the season of the year, there may be some correlation with the results shown, due to the higher humidity and lower light incidence during rainy periods. There was a greater amount of UTOs in the samples of pickets 9 and 10,

Table 2. Species richness. Average number of bands (UTOs) from the samples of the three gel repetitions and the total number of bands per picket/season of the year

Pickets	Area 1	Area 2	Area 3	TOTAL
Picket 9 (spring)	19	21	34	74
Picket 10 (autumn)	14	16	33	63
Picket 4 (summer)	17	6	17	40
Picket 6 (winter)	8	11	9	28

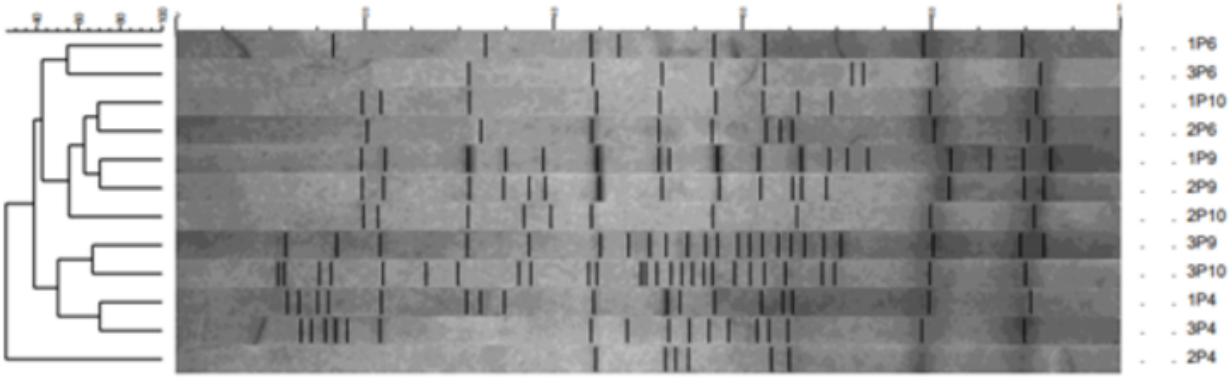

Figure 1. Dendogram. Analysis of bacterial diversity. The dashes represent the operational taxonomic units and the keys beside represent the similarity in the bacterial profile of the sampled areas.

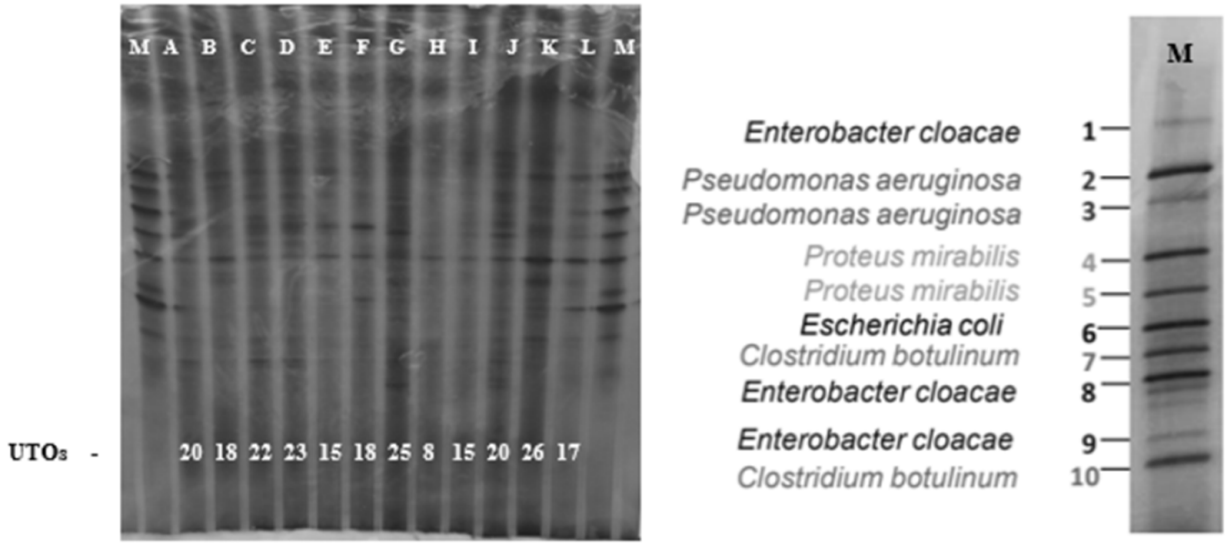

Figure 2. Denaturing gradient polyacrylamide gel electrophoresis of the amplification products of the 16S rDNA region. A. 1P9. B. 2P9. C. 3P9. D. 1P10. E. 2P10. F. 3P10. G. 1P4. H. 2P4. I. 3P4. J. 1P6. K. 2P5. L. 3P6. M. DGGE maker.

Table 3. Analysis of band intensity and occurrence of bacterial species. The following arabic numerals were considered: 1 = bands with low intensity, 2 = bands with reasonable intensity, 3 = bands with medium intensity, 4 = bands with high intensity. T = total intensity value

Bacteria	1P9	2P9	3P9	1P10	2P10	3P10	1P4	2P4	3P4	1P6	2P6	3P6	T
Enterobacter	2	2	1	2	2	1	2	2	1	3	3	3	24
E. coli	4	4	4	4	4	4	4	4	4	4	4	4	48
Pseudomonas	3	3	2	3	3	2	2	2	1	2	4	4	31
Proteus	3	2	1	3	2	1	3	2	1	4	3	3	28

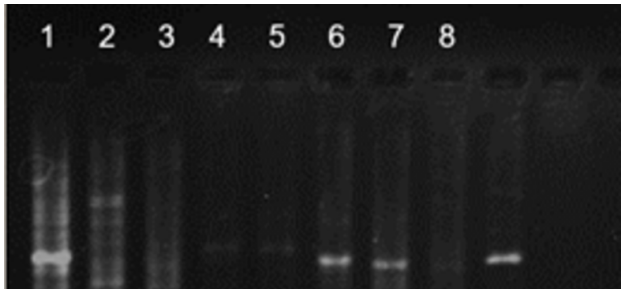

Figure 3. Agarose gel electrophoresis of blaSHV gene amplification products. 1. 1P9. 2. 2P9. 3. 1P10. 4. 2P10. 5. 1P4. 6. 2P4. 7. 1P6. 8. 2P6. CP. Positive control. CN. Negative control.

and this may have occurred due to the better adaptation of these bacteria in soils under conditions of high humidity, low light incidence and mild temperatures (Silva et al., 2022).

The analysis of the gels by means of dendograms allowed the identification of areas of the property with higher bacterial diversity, as well as tracing a bacterial profile of the local soil. Areas that were more distant, in separate keys, as is the case of area 2 of picket 4 (2P4), can be understood as areas with higher bacterial diversity due to the greater dissimilarity with the others. Diversity can be associated with variable soil characteristics, such as plant diversity, chemical and physical

Table 4. Presence of beta-lactamase coding genes in soil samples

Resistance Genes	1P9	2P9	1P10	2P10	1P4	2P4	1P6	2P6
blaTEM	-	-	-	-	-	-	-	-
blaSHV	+	-	-	-	-	+	+	-
blaCTX-M	-	-	-	-	-	-	-	-

Gene presence in the sample (+). Gene absence in the sample (-).

characteristics. In general, as the areas studied belong to the same property, the profiles did not differ much among the samples. Areas with a higher diversity of microorganisms require a more directed control, so that these bacteria do not contaminate other areas of the pasture (Yang et al., 2021).

Broad-spectrum betalactamases of the SHV type are among the most widespread in the environment, and they can hydrolyze several betalactams, generating resistance to these antimicrobials. Bacteria producing broad-spectrum betalactamases can also be resistant to other non-beta-lactam drugs such as quinolones and aminoglycosides, making treatment difficult in case of infections. In addition, plasmids carrying the genes encoding the production of broad-spectrum betalactamases usually contain genes for resistance to other antimicrobials such as aminoglycosides, sulfonamides, tetracyclines, and chloramphenicol. Due to their low toxicity, the betalactams are among the most prescribed worldwide for the treatment of infections such as bovine mastitis, a frequent infection in production and commonly responsible for drug therapies in animals. The detection of genes encoding broad-spectrum betalactamases in dairy cattle pasture soils is of concern, because once in this environment, they can be transferred to pathogenic bacteria of different species and genera through the transfer of mobile genetic material (Zheng et al., 2022).

Because it is difficult to disinfect the environment, the eradication of the disease is practically impossible. What is recommended in the case of this type of mastitis is the control and hygiene of the environment to avoid a too high concentration of microorganisms in the enclosure. Farms that use specific prophylactic techniques in management, such as post-dipping, have reduced the risk of environmental mastitis in the herd (Soutelino et al., 2022). The problem of soil resistome is an important issue to be discussed, because it is necessary to consider other factors besides the management and disinfection of milking facilities in the control of mastitis and antimicrobial resistance. The pickets and other rearing environments may be important amplifiers and responsible for maintaining these genes on farms (Delgado-Baquerizo et al., 2022).

The Normative Instruction n°77 of 2018 from the Ministry of Agriculture, Livestock and Supply (MAPA), establishes rules and techniques for the production of raw milk (Brasil, 2018). The reality in Brazil shows that in many regions and municipalities products of animal origin are sold at a lower cost to the population, which in most cases is unaware of the origin (Vasconcelos et al. 2022). This fact alerts to a public health problem, where, regardless of the inspection system in force, milk in very poor sanitary conditions, as is the case of milk from mastitic cows, is commercialized freely and on a large scale in the country (Vasconcelos et al., 2021). Another problem is that of the small rural producer, who, in most cases, does not have access to laboratories to determine the occurrence of mastitis and lacks modern methodologies to control production (Delgado; Bergamasco, 2017).

Although comparisons between soil bacterial profiles can only be concerned with massive sequencing of all the microorganisms in question, the DGGE technique is a good alternative for making general analyses and comparisons of these soil communities and assessing their impacts.

CONCLUSIONS

The DGGE technique for soil bacterial diversity analysis detected the existence of enterobacteria involved in the etiology of mastitis, where E. coli and Pseudomonas were detected in all samples, while Proteus and Enterobacter were detected in most samples. The *blaSHV* gene, responsible for encoding betalactamases, was detected in soil samples from the property.

REFERENCES

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução normativa Nº 77 de 26 de novembro de 2018. Regulamento Técnico de produção, acondicionamento, conservação, transporte, seleção e recepção do leite cru. **Diário Oficial da União**, Brasília, DF. Edição 230, seção 1, p. 10.

DELGADO, G. C.; BERGAMASCO, S. M. P. P. Agricultura familiar brasileira: desafios e perspectivas de futuro. In **Ministério do Desenvolvimento Agrário**. 2017.

DELGADO-BAQUERIZO, M. et al. The global distribution and environmental drivers of the soil antibiotic resistome. **Microbiome**, v. 10, n. 219, 2022.

DIBBERN, A. G. et al. Evaluation of methods of DNA extraction from *Staphylococcus aureus* in milk for use in real-time PCR. **Genetics and Molecular Research**, v. 14, n. 1, p. 227-233, 2015.

DUARTE, L. V.; KRAUTTER, M.; SOARES, A. F. Bioconstructions à spongiaires siliceux dans le Lias terminal du Bassin lusitanien (Portugal): stratigraphie, sédimentologie et signification paléogéographique. **Bulletin de la Société Géologique de France**. 172, p. 637-646, 2001.

GESER, N.; STEPHAN, R.; HÄCHLER, H. Occurrence and characteristics of ESBL producing Enterobacteriaceae in food producing animals, minced meat and raw milk. **BMC Veterinary Research**, v. 8, n. 21. 2012.

KANE, M.D.; POULSEN, L.K.; STAHL, D.A. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16s ribosomal-rna sequences. **Applied Environmental Microbiology**. v. 59, p. 682-686, 1993.

KLAAS, I. C.; ZADOKS, R. N. An update on environmental mastitis: Challenging perceptions. **Transboundary and Emerging Diseases**. v. 1, p. 166-185, 2018.

LIU, S. et al. Composition and Diversity of Soil Microbial Community Associated With Land Use Types in the Agro–Pastoral Area in the Upper Yellow River Basin. **Frontiers in Plant Science**. v. 13, 2022.

MARQUES, V. F. et al. Análise fenotípica e genotípica da virulência de Staphylococcus spp. e de sua dispersão clonal como contribuição ao estudo da mastite bovina1. **Pesquisa Veterinária Brasileira**, v. 33, n. 2, p. 161-170, 2013.

MARQUES, V. F. et al. Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. **Brazilian Journal of Microbiology**, v. 48, n. 1, p. 118-124, 2017.

MORAES, G. N. et al. Antimicrobial susceptibility profile of *Enterococcus* species isolated from cows with clinical mastitis and from bulk milk tanks in Brazil. **African Journal of Microbiology Research**, v. 17, n. 2, p. 29-34, 2023.

MINARINI, L. A. R. et al. Multilocus sequence typing of uropathogenic ESBL-producing *E. coli* isolated in a Brazilian community. **Current Microbiology**, v. 55, p. 524-9, 2007.

SHAHID, M. *Citrobacter* spp. simultaneously harboring bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{ampC}, and insertion sequences IS26 and orf513: an evolutionary phenomenon of recent concern for antibiotic resistance. **Journal of Clinical Microbiology**, v. 48, n. 5, p. 1833-1838, 2010.

SILVA, I. et al. Short-Term Responses of Soil Microbial Communities to Changes in Air Temperature, Soil Moisture and UV Radiation. **Genes**, v. 13, n. 850, 2022.

SOUTELINO, M. E. M. et al. Economic and production aspects of milking management in dairy farming. **Acta Veterinária Brasilica**. v. 16, n. 2, 161-165, 2022.

SUZUKI, M. T.; GIOVANNONI, S. J. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. **Applied Environmental Microbiology**. v. 62, p. 625-630, 1996.

VASCONCELOS, A. B. et al. Occurrence and antimicrobial resistance profile of Salmonella spp. in calves from the Mesoregion Sertão of Alagoas, Brazil. **Acta Veterinaria Brasilica**, v. 15, p. 36-40, 2021.

VASCONCELOS, A. B. et al. Detection of *Salmonella* spp. in eggs for human consumption from poultry farms in municipalities of Alagoas State, Brazil. **Acta Veterinaria Brasilica**. v. 16, p. 190-195, 2022.

YANG, Y. et al. Do Long-Term Conservation Pasture Management Practices Influence Microbial Diversity and Antimicrobial Resistant Genes in Runoff? **Frontiers in Microbiology**. v. 12, 2021.

ZHENG. D. et al. Global biogeography and projection of soil antibiotic resistance genes. **Science Advances**. v. 8, n. 46, 2022.