MARCILENE DANIEL DAMASCENO

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Prof. Dr. Alessandro de Sá Guimarães
Orientador
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APROVADA em 19 de fevereiro de 2025.

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LAVRAS-MG

2025

Dedico este trabalho a minha mãe, Cláudia, que mesmo diante de todas as dificuldades sempre me incentivou a voar!

“Pais que tiveram as oportunidades interrompidas criam filhos com propósito de vencer.”

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RESUMO

Mastite e brucelose bovina são duas importantes doenças que oneram a pecuária leiteira no Brasil e no mundo. A mastite é causada principalmente por patógenos infecciosos como *Escherichia coli* e *Staphylococcus aureus*, estando associada ao frequente e extensivo uso de antimicrobianos no gado leiteiro, o que contribui para o aumento da resistência bacteriana. A brucelose é uma doença zoonótica que afeta o trato reprodutivo dos animais e é transmitida principalmente por meio do contato com membranas fetais e fômites contaminados. Devido à importância dessas duas enfermidades, um dos trabalhos realizados nesse estudo avaliou a eficácia *in vitro* de agentes antimicrobianos frequentemente utilizados como *dipping* para prevenção da mastite bovina a cepas sensíveis e multidrogas resistentes (MDR) de *S. aureus* e *E. coli*. O segundo experimento avaliou a susceptibilidade dessas cepas MDR de *S. aureus* e *E. coli* a quatro extratos alcoólicos de própolis, como composto antimicrobiano alternativo para a prevenção da mastite. O terceiro estudo investigou a presença da brucelose bovina em vacas leiteiras alojadas em sistema compost barn, um tipo de confinamento intensivo para a criação de rebanho leiteiro, que é constituído por uma cama com matéria orgânica revolvida diariamente. Os resultados encontrados no primeiro trabalho demonstram que em geral, todas as cepas de *E. coli* e *S. aureus* foram susceptíveis em concentrações abaixo da recomendada para uso para todos os antissépticos utilizados como *dipping*, exceto para o hipoclorito de sódio. Também exibiram um aumento da concentração inibitória mínima de acordo com o ano de isolamento dos patógenos, sugerindo a emergência de cepas resistentes em resposta a pressão de seleção exercida pela exposição aos desinfetantes. Os resultados contidos no segundo trabalho revelam ação antimicrobiana do extrato de própolis a 5,0 mg/mL para inibição da maior parte dos isolados MDR de *E. coli* e *S. aureus*. O terceiro estudo demonstrou amplificação de gene específico para *Brucella* spp. em amostras de [17/20 (85 %)] propriedades que utilizam sistema compost barn para criação. Como conclusão, os achados desses experimentos sugerem fortemente que a exposição contínua aos antissépticos pode levar à seleção de cepas menos suscetíveis na pecuária leiteira, além de indicar o potencial do extrato de própolis para uso como agente antimicrobiano para controle e prevenção da mastite bovina. Por fim, também demonstrou a presença de *Brucella* spp. nos animais alojados em sistema compost barn.

Palavras-chave: *Staphylococcus aureus*; *Escherichia coli*; brucelose; resistência bacteriana.

ABSTRACT

Mastitis and bovine brucellosis are two important diseases that burden dairy farming in Brazil and around the world. Mastitis is mainly caused by infectious pathogens such as *Escherichia coli* and *Staphylococcus aureus*, and is associated with the frequent and extensive use of antimicrobials in dairy cattle, which contributes to increased bacterial resistance. *Brucellosis* is a zoonotic disease that affects the reproductive tract of animals and is transmitted mainly through contact with contaminated fetal membranes and fomites. Due to the importance of these two diseases, one of the works carried out in this study evaluated the *in vitro* efficacy of antimicrobial agents frequently used as dipping to prevent bovine mastitis to sensitive and multidrug resistant (MDR) strains of *S. aureus* and *E. coli*. The second experiment evaluated the susceptibility of these MDR strains of S. aureus and *E. coli* to four alcoholic extracts of propolis, as an alternative antimicrobial compound for the prevention of mastitis. The third study investigated the presence of bovine brucellosis in dairy cows housed in a compost barn system, a type of intensive confinement for raising dairy herds, which consists of bedding with organic matter turned over daily. The results found in the first work demonstrate that in general, all strains of *E. coli* and *S. aureus* were susceptible at concentrations below those recommended for use for all antiseptics used as dipping, except for sodium hypochlorite. The increase in the minimum inhibitory concentration according to the year of isolation of the pathogens, suggesting the emergence of resistant strains in response to the selection pressure exerted by exposure to disinfectants. Results contained in the second work reveal the antimicrobial action of propolis extract at 5.0 mg/mL to inhibit most MDR isolates of *E. coli* and *S. aureus*. The third study demonstrated gene amplification specific for *Brucella* spp. in samples from [17/20 (85%)] properties that use the compost barn system for breeding. In conclusion, the findings of these experiments strongly suggest that continuous exposure to antiseptics can lead to the selection of less susceptible strains in dairy farming, in addition to indicating the potential of propolis extract for use as an antimicrobial agent for the control and prevention of bovine mastitis. Finally, it also demonstrated the presence of *Brucella* spp. in animals housed in a compost barn system.

Keywords: *Staphylococcus aureus; Escherichia coli;* brucellosis; bacterial resistance.

IMPACT INDICATORS

Mastitis and bovine brucellosis are two diseases of public health relevance. Mastitis is directly related to the extensive use of antimicrobials in dairy farming. Brucellosis is a zoonotic disease that has reproductive symptoms. Due to the importance of these two diseases, the work carried out in this study evaluated the susceptibility of antimicrobial agents frequently used as dipping to sensitive and multidrug resistant strains isolated from bovine mastitis. The potential of alcoholic propolis extracts as an alternative microbial compound for the prevention of this same disease. This work also investigated the presence of bovine brucellosis in dairy cows housed in a compost barn system, a type of intensive confinement for raising dairy cows that consists of bedding with organic matter turned over daily. The results obtained in this work present sanitary and social impacts on public health by demonstrating the bacterial resistance found in bovine mastitis isolates, which makes the treatment of infected animals difficult and contributes to the contamination of the environment with antimicrobial residues. The gene amplification specific for *Brucella* spp. in samples from dairy cows housed in a compost barn is also an impact factor, which becomes a potential risk of transmission to the population from the consumption of unpasteurized milk from these animals or from the contact of professionals and rural workers with contaminated postpartum secretions. This research also has technological appeal as it emphasizes the importance of developing alternative disinfectant formulas that are effective and do not contribute to bacterial resistance, as well as demonstrating the effectiveness of alternative techniques for diagnosing bovine brucellosis. Furthermore, based on the findings of this work, we can infer that there are economic losses in production caused by the presence and/or resistance of the aforementioned pathogens. Finally, cultural impacts are seen through discussions on the rational use of antimicrobials and sustainable production in dairy farming.

SUMMARY

FIRST SECTION

INTRODUCTION

Bovine mastitis is one of the most prevalent infectious diseases that places the greatest burden on dairy farming in Brazil and around the world, due to a decrease in milk production and quality, disposal of milk with residues and expenses with veterinary treatments (Blosser 1979). Mastitis is characterized as inflammation of the mammary gland, with multifactorial etiology and most frequently caused by agents of bacterial origin, and can be classified as environmental or contagious mastitis (Gonçalves et al. 2018).

Antimicrobials are used in large scale for mastitis prevention and treatment, through the used to disinfect equipment, antiseptics are applied to the teats during pre- and post-dipping or through the administration of systemic or intramammary antibiotics (Yanuartono et al. 2020). The high and often indiscriminate use of antimicrobials contributes to increased tolerance and resistance of pathogens that cause bovine mastitis to antibiotics and disinfectants (Naranjo-Lucena and Slowey 2023; Kampf 2018). In view of this, antimicrobial alternatives, as well as the rational use of drugs and monitoring and investigating the effectiveness of techniques and doses already used, become fundamental (NMC 2005; Tomanić, Samardžija, and Kovačević 2023).

Another prevalent infectious disease associated with economic losses in dairy farming is bovine brucellosis (Pal et al. 2017). Bovine brucellosis is a zoonotic disease caused by bacteria of the genus *Brucella* spp., especially *Brucella abortus* (Alton et al. 1988). It is transmitted to animals mainly through contact with contaminated fetal membranes and fomites; infected animals, in turn, develop reproductive problems such as abortion, retained placenta and infertility (Megid, Mathias, and Robles 2010; Kiros, Asgedom, and Duguma 2016). Due to the way brucellosis is transmitted, the compost barn (or compost-bedded pack barn), an intensive and new production system in Brazil, which consists of a bed with organic matter turned over daily, raises questions about the spread of diseases among the animals housed, once the animals give birth on the bed (Leso et al. 2020; Emanuelson et al. 2022).

Due to the problems presented, this dissertation studies the susceptibility of strains of *Staphylococcus aureus* and *Escherichia coli*, which represent, respectively, the main bacteria of contagious and Gram-positive and environmental and Gram-negative origin, related to bovine mastitis, against antiseptics commonly used as dipping (Cheng and Han 2020). It also investigates the antimicrobial activity of the alcoholic extract of propolis on multidrug-resistant strains of *S. aureus* and *E. coli*. Furthermore, it also analyzes the presence of *Brucella* spp. in dairy cows housed in a compost barn system.

Article 1 of this dissertation is titled “Susceptibility of mastitis-causing pathogens (*Escherichia coli* and *Staphylococcus aureus*) to disinfectants used as teat dipping” and aims to determine *in vitro* the susceptibility of *S. aureus* and *E. coli* isolated from bovine mastitis in Brazil, between 1994 and 2016, to disinfectants commonly used as dipping for the control and prevention of bovine mastitis. Article 2 is titled “Antimicrobial activity of propolis against multidrug-resistant bovine mastitis pathogens” and aims to investigate the antimicrobial activity of alcoholic extract of Brazilian propolis produced by bees of the species *Appis melifera* and *Melipona quadrifasciata* against multidrug-resistant *E. coli* and *S. aureus* isolated from bovine mastitis in Brazil between the years 1998 and 2016. The Article 3 is named “Detection of *Brucella* spp. dairy cows in the transition period housed in a compost-bedded pack barn system” and aims to investigate the presence of brucellosis in cows dairy farms housed in compost -bedded pack barn.

The studies were written in article format, with the intention of being submitted to the indicated journals after corrections suggested by the panel.

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CONCLUSION

Bovine mastitis and brucellosis are infectious diseases of great importance for public health and dairy farming. The results obtained in these studies indicate that continuous exposure of bovine mastitis isolates to antiseptics can lead to the selection of less susceptible bacterial strains. Demonstrate the antimicrobial capacity of the alcoholic extract of propolis against isolated multi-resistant strains of bovine mastitis, indicating the potential of this compound as an alternative antimicrobial agent for the control and prevention of the disease. Furthermore, it also demonstrates the presence of *Brucella* spp. in samples from dairy cows housed in compost-bedded pack barn system barn.

REFERENCES

ALTON, Godfrey Greenset e colab. **Techniques for the brucellosis laboratory.** [S.l: s.n.], 1988.

BLOSSER, T. H. **Economic Losses from and the National Research Program on Mastitis in the United States**. Journal of Dairy Science, v. 62, n. 1, p. 119–127, 1979. Disponível em: <http://dx.doi.org/10.3168/jds.S0022-0302(79)83213-0>.

CHENG, Wei Nee e HAN, Sung Gu. **Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — A review**. Asian-Australasian Journal of Animal Sciences, v. 33, n. 11, p. 1699–1713, 2020.

EMANUELSON, Ulf e colab. **Animal Health in Compost-Bedded Pack and Cubicle Dairy Barns in Six European Countries**. Animals, v. 12, n. 3, p. 1–9, 2022.

GONÇALVES, J. L. e colab. **Bovine subclinical mastitis reduces milk yield and economic return**. Livestock Science, v. 210, n. January, p. 25–32, 2018.

KAMPF, Günter. **Biocidal agents used for disinfection can enhance antibiotic resistance in gram-negative species**. Antibiotics, v. 7, n. 4, 2018.

KIROS, Ashenafi e ASGEDOM, Hagos e DUGUMA, Reta. **A Review on Bovine Brucellosis: Epidemiology, Diagnosis and Control Options**. ARC Journal of Animal and Veterinary Sciences, v. 2, n. 3, 2016.

LESO, L. e colab. **Invited review: Compost-bedded pack barns for dairy cows**. Journal of Dairy Science, v. 103, n. 2, p. 1072–1099, 2020. Disponível em: <http://dx.doi.org/10.3168/jds.2019-16864>.

MEGID, Jane e MATHIAS, LA e ROBLES, CA. **Clinical manifestations of brucellosis in domestic animals and humans**. Open Veterinary Science, v. 4, p. 119–126, 2010. Disponível em: <http://benthamscience.com/open/tovsj/articles/V004/SI0045TOVSJ/119TOVSJ.pdf>.

NARANJO-LUCENA, Amalia e SLOWEY, Rosemarie. **Invited review: Antimicrobial resistance in bovine mastitis pathogens: A review of genetic determinants and prevalence of resistance in European countries**. Journal of Dairy Science, v. 106, n. 1, p. 1–23, 2023. Disponível em: <http://dx.doi.org/10.3168/jds.2022-22267>.

NMC. **Summary of Peer-Reviewed Publications on Egficacy of Premilking and Postmilking Teat Disinfectants Published Since 1980**. v. 1980, n. 2011, p. 26–39, 2005.

PAL, Mahendra e colab. **Public Health and Economic Importance of Bovine Brucellosis: An Overview**. American Journal of Epidemiology and Infectious Disease, v. 5, n. 2, p. 27–34, 2017.

TOMANIĆ, Dragana e SAMARDŽIJA, Marko e KOVAČEVIĆ, Zorana. **Alternatives to Antimicrobial Treatment in Bovine Mastitis Therapy: A Review**. Antibiotics, v. 12, n. 4, 2023.

YANUARTONO e colab. **The Benefits of Teat Dipping as Prevention of Mastitis**. Journal of Livestock Science and Production, v. 4, n. 1, p. 231–249, 2020.

SECOND SECTION - ARTICLES

ARTICLE 1

Target journal: Veterinary Microbiology

SUSCEPTIBILITY OF MASTITIS-CAUSING PATHOGENS (*Escherichia coli* AND *Staphylococcus aureus*) TO DISINFECTANTS USED AS TEAT DIPPING

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ABSTRACT

Mastitis is one of the most important diseases for dairy farming and *Escherichia coli* and *Staphylococcus aureus* are among the mainly mastitis-causing pathogens. Aiming to prevent mastitis, pre- and post-milking disinfectant dips are used, but this constant exposure can contribute to the emergence of antimicrobial resistant strains. Thus, this study aimed to determine the *in vitro* susceptibility of mastitis-causing *S. aureus* and *E. coli* to disinfectants used as teat dipping. The disinfectants tested were hydrogen peroxide, sodium hypochlorite, chlorhexidine digluconate, lactic acid, quaternary ammonium, and iodine. Susceptibility was assessed through the technique of microdilution in broth to obtain minimal inhibitory concentration (MIC). In general, all *E. coli* and *S. aureus* strains were susceptible to chlorhexidine digluconate, hydrogen peroxide, iodine, lactic acid, and quaternary ammonium with concentrations lower than those used in field. Regarding sodium hypochlorite, 80.77% (42/52) of the *E. coli* isolates were not susceptible to the concentration recommended for use as teat dipping, while 34.5% (138/400) of the *S. aureus* had the MIC equal or higher than this same concentration. In addition, an increase in the MIC according to the year of isolation of the pathogens was observed, with the latest isolates being more tolerant to all disinfectants, with exception of chlorhexidine digluconate. These results strongly suggest that continuous exposure to disinfectants can lead to the selection of less susceptible strains in dairy farming, which is a major issue for animal and public health.

Keywords: *E. coli*, *S. aureus*, resistance, pre-milking disinfectant, post-milking disinfectant, minimum inhibitory concentration, MIC.

1. INTRODUCTION

Bovine mastitis is defined as the inflammation of the mammary gland and it is mainly caused due to the presence of an infectious agent (Zigo et al. 2021). This disease has a complex and multifactorial etiology, being highly prevalent worldwide and considered one of the most expensive illness for dairy industry (Ruegg 2017). The economic losses are principally due to the decrease in milk yield and quality, milk disposal and expenses with treatments, such as medicines and veterinary service (Guimarães et al. 2017).

Among the main mastitis-causing pathogens, *Staphylococcus aureus* and *Escherichia coli* are the most prevalent Gram-positive and Gram-negative bacteria causing intramammary infections in cattle, respectively (Armstrong 2019; Cheng and Han 2020). *S. aureus* is considered a contagious pathogen that can cause subclinical or clinical mastitis, being capable of forming abscesses and biofilms in the mammary gland, which difficult the treatment with antimicrobial agents (Pérez et al. 2020). *E. coli*, on the other hand, is a coliform and belongs to the natural intestinal microbiota of cattle, being known as an environmental and opportunistic mastitis pathogen associated with clinical and acute infections (Bradley 2002).

Due to the high prevalence, mastitis leads to a high and, frequently, indiscriminate use of antimicrobials drugs in dairy farms (Nobrega et al. 2017), contributing to the emergence of *S. aureus* and *E. coli* resistant strains. This represents an important and emerging issue for human and animal health, since both agents are zoonotic pathogens (Maity and Ambatipudi 2021). In fact, mastitis is considered the main responsible for the use of antimicrobial therapies in dairy farming, representing 42% of all antimicrobial treatments in the farms (Nobrega et al. 2017). Due this frequent exposure, several studies have been conducted to determine the susceptibility of *S. aureus* and *E. coli*, as well as other mastitis-causing pathogens, to antimicrobials used for mastitis treatments (Molineri et al. 2021; Goulart and Mellata 2022; Naranjo-Lucena and Slowey 2023).

Additionally to antimicrobials, in order to prevent intramammary infections, mastitis pathogens are also constantly exposed to other antimicrobial substances, such as antiseptics and disinfectants used for disinfection of environment and animals (Nacional Mastitis Council (NMC) 2016). In this context, the “dipping” technique, which consists in to dip cows teats in an disinfectant solution before (pre dipping) and after (post dipping) each milking process, is one of the major procedures recommended by the National Mastitis Council (NMC) for mastitis control (Nacional Mastitis Council (NMC) 2016). Among the main disinfectants used for this purpose, are those based on iodine, chlorhexidine, lactic acid, sodium chlorine, quaternary ammonium and hydrogen peroxide (Nacional Mastitis Council (NMC) 2014).

Since cows are milked at least once a day, there is a very frequent exposure to these antimicrobials principles in the farms that adopt dipping, which can contribute to the emergence of resistant strains, prejudicing the effectiveness of the used disinfectants (Maillard and Pascoe 2024). Indeed, resistance to antiseptics and disinfectants have been studied in microorganisms isolated from other environments, as food industry and human hospitals (Langsrud et al. 2003; Köhler et al. 2018; Caro-Hernández et al. 2022; Rodr et al. 2022). However, in mastitis-causing pathogens, it is still poorly investigated, with only a few studies reporting reduced susceptibility to different disinfectants, including in *S. aureus* and *E. coli* (Fitzpatrick et al. 2019; Behiry et al. 2012; R. P. Santos et al. 2016).

In addition, studies have shown that the bacterial resistance to some disinfectants also contributes to increased resistance to antibiotics by cross-resistance, as mechanisms of resistance to disinfectants tends to be less specific, as efflux-pumps (Maillard and Pascoe 2024; Tong et al. 2021; Rodr et al. 2022). In fact, frequent exposure to disinfectants can impose selective pressure and contribute to the emergence of multidrug-resistant pathogens (Azizoglu, Lyman, and Anderson 2013; Maillard and Pascoe 2024). Therefore, the understanding of the levels of susceptibility among mastitis-causing pathogens to the disinfectants commonly used for teat dipping contributes to the surveillance in antimicrobial resistance, as well as to establish adequate concentration for its use in the farms. In this sense, the aim of the present study was to determine the *in vitro* susceptibility of *S. aureus* and *E. coli* strains isolated from bovine mastitis in Brazil, between 1994 and 2016, to disinfectants commonly used as teat dipping for the control and prevention of bovine mastitis.

2. MATERIALS AND METHODS

2.1. Strains

Four hundred (400) *S. aureus* strains were used. These strains belong to the Collection of Microorganisms of Interest to Agroindustry and Livestock from Brazilian Agricultural Research Corporation (Embrapa) and were isolated from milk of cows with mastitis in four Brazilian states between 1994 and 2016 [74.25 % (297/400) from Minas Gerais, 16.25 % (65/400) from Rio de Janeiro, 8 % (32/400) from São Paulo and 1.5 % (6/400) from Goiás state].

Fifth-two (52) *E. coli* strains isolated between 2004 and 2016 from cows with mastitis in Minas Gerais, Brazil, were also used. These strains belong to the Collection of Microorganism of the

Laboratórios Integrados de Sanidade Animal e Saúde Coletiva (LISASC), from Universidade Federal de Lavras (UFLA).

2.2. Antimicrobial susceptibility test

Six disinfectants were evaluated: hydrogen peroxide (Synth, Brazil), sodium hypochlorite (Orion, Brazil), chlorhexidine digluconate (Merck, Germany), lactic acid (Merck, Germany), quaternary ammonium (Chemitec, Brazil), and iodine (Alphatec, Brazil). Most of the disinfectant solutions were obtained commercially, with the exception of iodide. Iodine solution was produced in an initial concentration of 5% using 5 g of iodine, 10 g of potassium iodide and deionized water, for a final volume of 100 mL. The ranges of concentration tested for each disinfectant (Table 1) were chosen based on the commonly and effective concentrations used as dipping in dairy farms, according to a survey of the effectiveness of disinfectants used as dipping conducted by the NMC (Nacional Mastitis Council (NMC) 2014) (Table 1).

The minimal inhibitory concentration (MIC) for each disinfectant was tested by the technique of broth microdilution determined by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute (CLSI) 2018; CLSI 2013), adapting the protocols recommended for evaluate antibiotic susceptibility in bacteria that grow aerobically. Briefly, fresh culture of each strain grown on Brain Heart Infusion (BHI) (Merck, Germany) agar and incubated at 37 ºC for 24 h were used for inoculum preparation. Colonies were suspended in 0.85% saline solution and adjusted to turbidity equivalent of 0.5 McFarland standard. Suspensions were diluted in order to obtain a final inoculum of 10^5 colony forming units (CFU)/well. Each disinfectant was diluted in sterile cation-adjusted Mueller Hinton (Becton Dickson, France) broth to obtain the maximum concentration tested (Table 1). Plates were incubated at 37 ºC for 16-20 h, until results interpretation by growth visual observation. All analyses were performed in duplicate.

As there are no quality controls established for interpretation of susceptibility tests carried out with disinfectants, five consecutive assays (repeatability) were conducted with the reference strains *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, in order to obtain MIC ranges for each of them.

All reference strains were evaluated for all disinfectants and the one with the MIC range more appropriate to the tested concentration range for each disinfectant was chosen for quality control and adopted in all assays.

2.3 Statistical analysis

All analyses were carried out using software R (v. 4.2.2) and graphs were built using the basic package and ggplot2 (Wickham 2016).

Descriptive analyses were conducted to obtain the percentage of isolates inhibited in each concentration according to species and year of isolation. MIC average, standard deviation, median, interquartile range (IQR) for each disinfectant according to the species was also obtained. The MIC_{50} and MIC_{90} values were defined as the lowest concentration of the antimicrobial in which 50% and 90% of the strains were inhibited, respectively. The Mann Whitney U test was performed to compare the distribution of the MIC of each disinfectant between the two bacterial species (*E. coli* and *S. aureus*), considering a level of significance of 95% ($\alpha = 0.05$).

Mixed linear models were built using MIC results of *S. aureus* for each disinfectant (lactic acid, hydrogen peroxide, quaternary ammonium and sodium hypochlorite) as dependent variable and year of isolation (fixed effect) and farm (random effect) as independent variables, to assess factors possibly associated with greater tolerance to disinfectants. For *E. coli*, linear models were built using MIC results for each disinfectant (lactic acid, hydrogen peroxide, quaternary ammonium and sodium hypochlorite) as dependent variable and year of isolation as independent variable, since strains did not have information about farm where the strain was isolated. Chlorhexidine results were not evaluated due to the absence of variability in the values. Box-cox transformation was applied to transform values of all dependent variables using the MASS package on R (Ripley et al. 2025).

3. RESULTS

Reference microorganisms for each disinfectant and their MIC ranges used for quality control are shown in Table 1.

Table 1 - Recommended concentration for use and tested concentrations of disinfectants and Minimum Inhibitory Concentration for the reference microorganisms used as quality control strains for the tests.

Disinfectant	Recommended concentration of use (%)	Tested concentration range (%)	Microorganism	MIC^a for reference microorganisms (mean ± standard deviation)*
Chlorhexidine	0.35	0.002 - 1.4	*P. aeruginosa* ATCC 27853	≤ 0.003 (0.003)
Hydrogen peroxide	0.5	0.002 – 1.0	*E. faecalis* ATCC 29212	0.008 - 0.016 (0.011 ± 0.005)

Iodine	1.0	0.002 – 1.0	*P. aeruginosa* ATCC 27853	0.007 - 0.015 (0.010 ± 0.004)
Lactic acid	2.64	0.021 - 10.56	*P. aeruginosa* ATCC 27853	0.082 - 0.165 (0.144 ± 0.042)
Quaternary Ammonia	0.5	0.004 – 2.0	*P. aeruginosa* ATCC 27853	0.031 - 0.062 (0.037 ± 0.014)
Sodium hypochlorite	0.6	0.004-2.5	*P. aeruginosa* ATCC 27853	0.62-1.25 (0.872 ± 0.345)

[a] MIC: minimum inhibitory concentration.

*Values from five tests.

Table 2 shows the MIC required to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the microorganisms tested for each of the disinfectants evaluated, as well as the average, standard deviation, and median values according to each species. In general, both *E. coli* and *S. aureus* were inhibited *in vitro* by lower concentrations of chlorhexidine, hydrogen peroxide, iodine, lactic acid and quaternary ammonium than those recommended by the NMC for teat dipping. On the other hand, the recommended concentration for sodium hypochlorite was not sufficient to inhibit 80.77% of the *E. coli* isolates (42/52) and only 32.75 % (131/400) of the *S. aureus* isolates were inhibited using the recommended concentration of sodium hypochlorite (Table 2 and Figure 1). The concentration required to inhibit *S. aureus* and *E. coli* isolates in the present study against the six tested disinfectants is shown in Figure 1.

Comparing the two species studied, it is possible to observe that the MIC mean needed to inhibit E. *coli* strains was greater than the needed for *S. aureus* for tested disinfectants ($p < 0.05$), with exception of chlorhexidine ($p > 0.05$) (Table 2). The susceptibility of the *S. aureus* and *E. coli* strains according to the year of isolation was also evaluated, showing lower susceptibility to the disinfectants according to the years, especially for lactic acid and sodium hypochlorite (Figure 2 and 3). The linear models showed the positive linear effect of time (year of isolation) in the concentrations of lactic acid and sodium hypochlorite needed to inhibit *S. aureus* ($p > 0.05$) (Tables 3 and 4), corrected for the effect of farm.

Table 2 – Minimum inhibitory concentration (MIC) values of disinfectant, used as dipping, tested against *Escherichia coli* and *Staphylococcus aureus* strains isolated from bovine mastitis in Brazil, 1994 – 2016.

Disinfectants	**Recommended concentration (%)**	**MIC[a]$_{50}$**		**MIC$_{90}$**		**MIC mean ([d]SD)**		**MIC median ([e]IQR)**		**p-value***
		EC[b]	**SA[c]**	**EC**	**SA**	**EC**	**SA**	**EC**	**SA**	
Chlorhexidine	0.350	≤ 0.002	≤ 0.002	≤ 0.002	≤ 0.002	0.002	0.002	≤ 0.002	≤ 0.002	0.230
Hydrogen peroxide	0.640	≤ 0.002	≤ 0.002	0.004	≤ 0.002	0.003 (± 0.002)	0.002 (± 0.001)	0.003 (0.002)	≤ 0.002	0.000
Iodine	1.000	0.016	0.008	0.016	0.016	0.014 (± 0.004)	0.008 (± 0.004)	0.016 (0.008)	0.008 (0.004)	0.000
Latic acid	2.640	0.165	0.082	0.330	0.165	0.190 (± 0.082)	0.133 (± 0.071)	0.165	0.082 (0.082)	0.000
Quaternary ammonium	0.640	0.016	≤ 0.004	0.031	≤ 0.004	0.019 (± 0.017)	0.004 (± 0.002)	0.016	≤ 0.004	0.000
Sodium hypochlorite	0.500	1.250	0.310	1.250	0.620	1.171 (± 0.455)	0.392 (± 0.216)	1.250	0.310 (0.310)	0.000

[a]MIC: minimum inhibitory concentration; [b]EC: *Escherichia coli*; [c]SA: *Staphylococcus aureus*, [d]SD: standard deviation, [e]IQR: interquartile range.

*Mann Whitney U test performed to compare the distribution of the MIC of each disinfectant between the two bacterial species.

Table 3. Analysis of variance for assessment of effect of year of isolation on the minimal inhibitory concentrations (MIC) of disinfectants used as teat dip disinfectants, tested against *Escherichia coli* and *Staphylococcus aureus* strains isolated from bovine mastitis in Brazil, 1994 – 2016.

Dependent Variable	**[a]Sum Sq**	**[b]Mean Sq**	**[c]F value**	**[d]Pr (>F)**
MIC *Escherichia coli*				
Hydrogen peroxide	0.9090	0.2273	1.4120	0.2497
Iodine	0.0000	0.0000	0.6111	0.6573
Latic acid	71.7900	17.9480	0.7548	0.5615
Quaternary ammonium	0.0348	0.0087	0.0240	0.9988
Sodium hypochlorite	2.4298	0.6074	2.3091	0.0766
MIC *Staphylococcus aureus*				
Hydrogen peroxide	2567.9000	112.2800	0.4710	0.9760
Iodine	72.2420	3.4401	2.9453	0.0000
Latic acid	1287.5000	61.3100	4.4240	0.0000
Quaternary ammonium	52.5790	2.5038	0.5382	0.9499
Sodium hypochlorite	1453.0000	69.1890	2.8244	0.0001

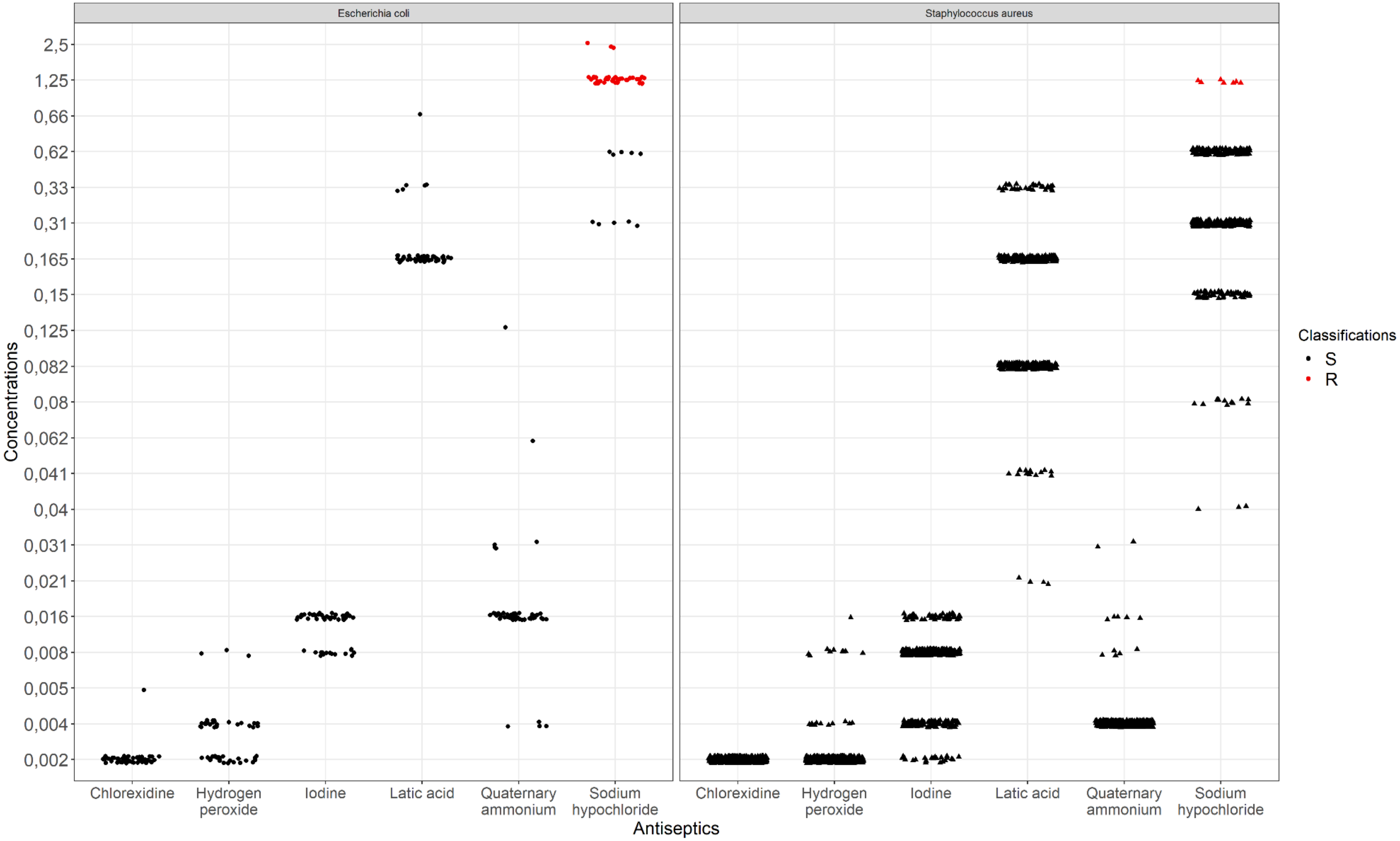
[a]Sum Sq: sum of squares; [b]Mean Sq: mean of squares; [c]F value: results of variance comparisons; [d]Pr (>F): p value.

Table 4. Results of linear mixed models for assessment of linear effect of year of isolation on the minimal inhibitory concentrations (MIC) of disinfectants used as teat dip disinfectants, tested against *Staphylococcus aureus* strains isolated from bovine mastitis in Brazil, 1994 – 2016.

Parameter	**Estimate Latic Acid**	**[a]Std. Error**	**[b]df**	**t value**	**Pr(>|t|)**
Intercept	-19,8987	0,5119	113,6391	-38,8710	0,0000
Year Linear	12,6115	2,4610	201,2348	5,1250	0,0000
	Estimate Sodium Hypochlorite	**Std. Error**	**df**	**t value**	**Pr(>|t|)**
Intercept	-10,3552	0,6023	111,6232	-14,1440	0,0000
Year Linear	8,0037	3,0209	207,8045	2,6490	0,0087

[a]Std. Error: standard error; [b]df: degrees of freedom.

Figure 1 - Minimum inhibitory concentration (MIC) values of disinfectant, used as teat dipping, tested against *Escherichia coli* and *Staphylococcus* aureus strains isolated from bovine mastitis in Brazil, 1994 and 2016.



203 **Figure 2** – Percentage of *Escherichia coli* strains isolated from bovine mastitis inhibited by different concentrations of disinfectants used as teat
204 dipping in dairy farms, according to the year of isolation.

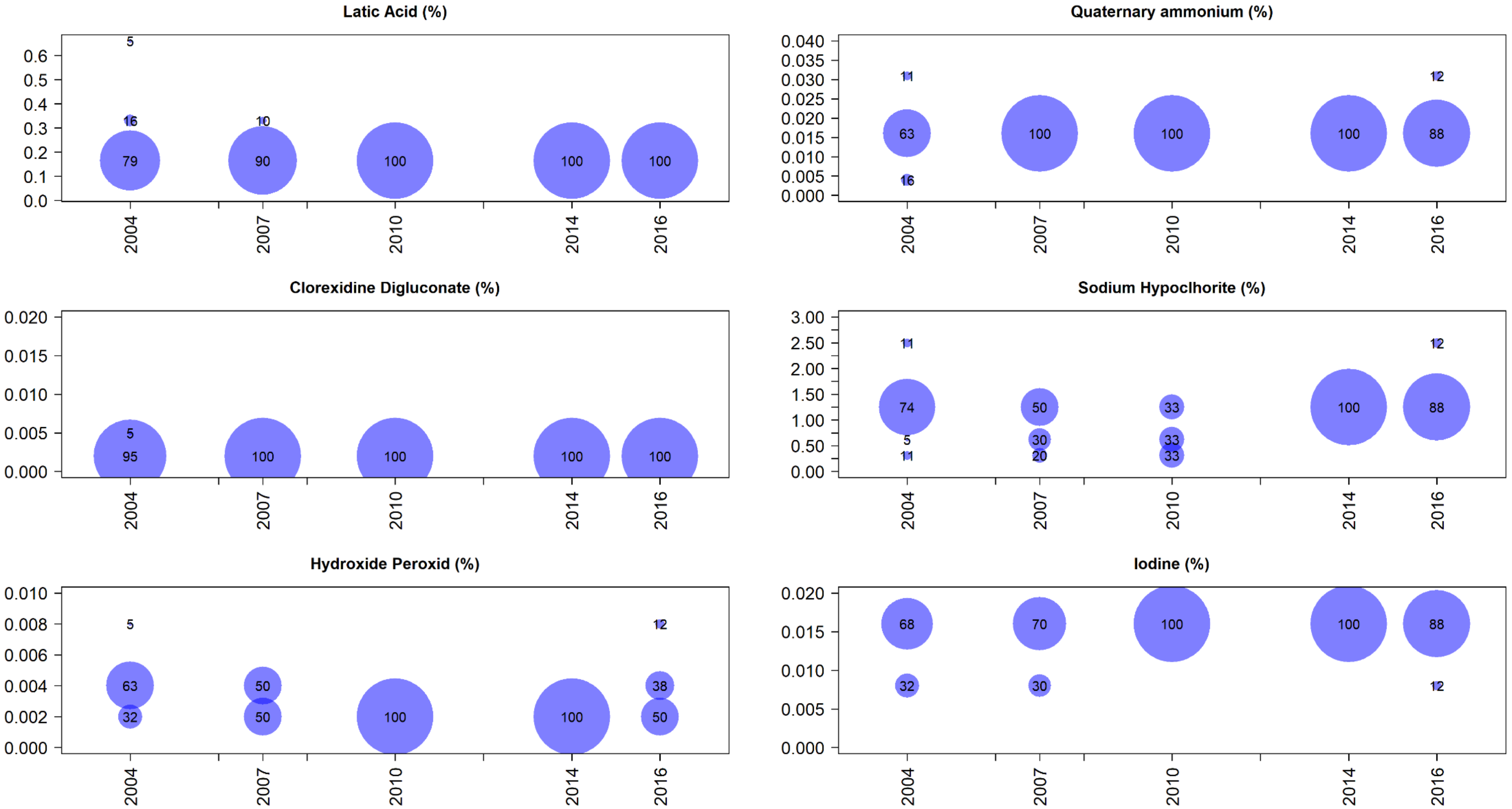


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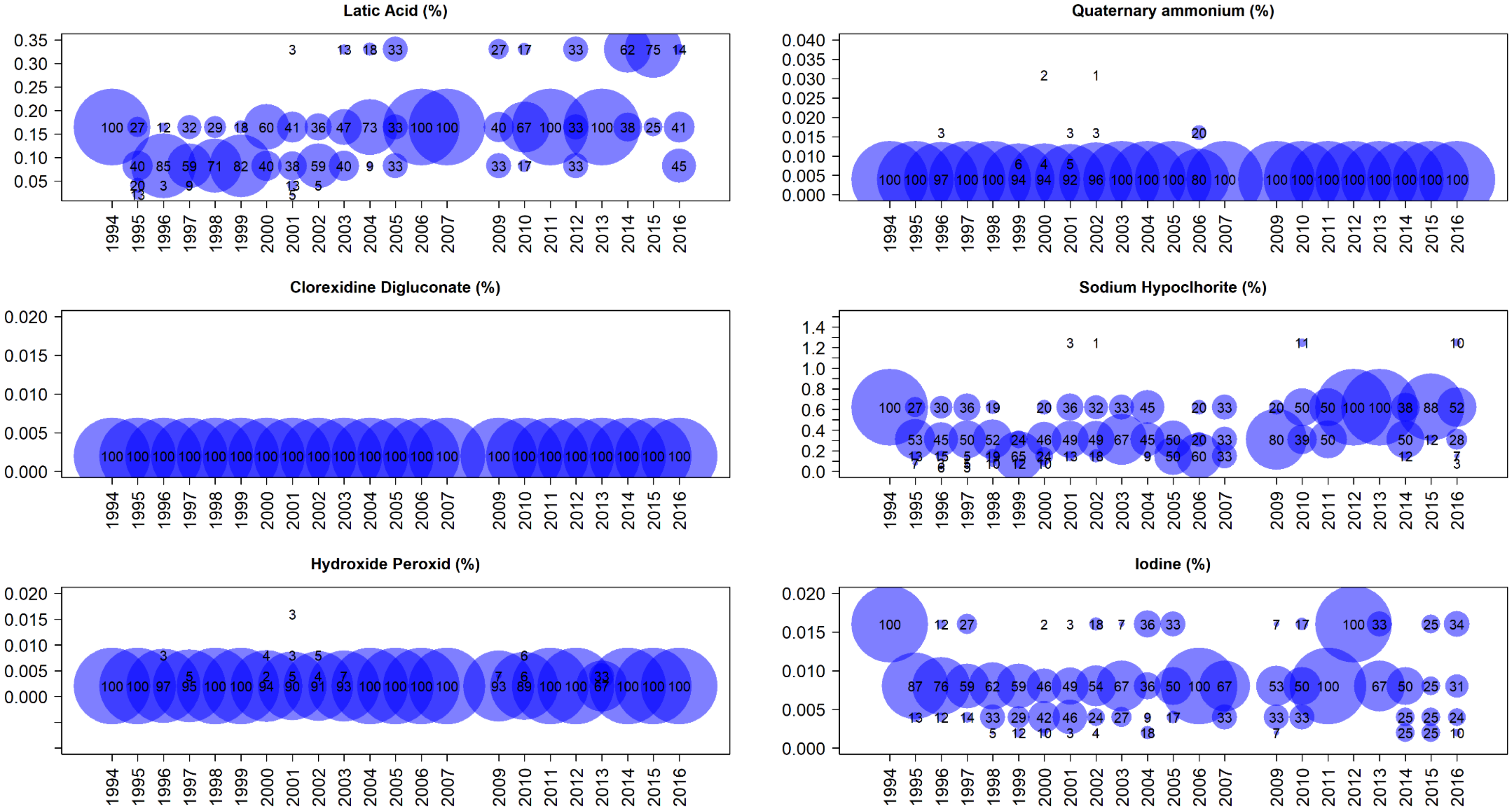
Figure 3 - Percentage of *S. aureus* strains isolated from bovine mastitis inhibited in different concentrations of disinfectants used as teat dipping in dairy farms, according to the year of isolation.



4. DISCUSSION

Since antimicrobial resistance is a major issue for public and animal health (Prestinaci, Pezzotti, and Pantosti 2015) and mastitis is the main disease associated with the use of antimicrobial drugs in dairy farms, several studies have been conducted to determine the antimicrobial susceptibility of mastitis-causing pathogens in the last decades (Naranjo-Lucena and Slowey 2023; Goulart and Mellata 2022; Molineri et al. 2021). However, the efforts have been mainly concentrated on the drugs used for mastitis treatment and knowledge about susceptibility of the pathogens to other antimicrobials used for mastitis prevention, as disinfectants, is still scarce. In this scenario, our results demonstrated that 80.77% (42/52) of the *E. coli* isolates were not susceptible to the concentration of sodium hypochlorite (0.6 %) recommended by NMC for use as teat dipping and 34.5% (138/400) of the *S. aureus* had a MIC equal or higher than this same concentration. In addition, although the isolates have been susceptible to the other tested disinfectants (chlorhexidine, hydrogen peroxide, iodine, lactic acid, and quaternary ammonium), there appears to be a tendency towards lower susceptibility in isolates from more recent years, especially to lactic acid and sodium hypochlorite. These results suggest that the intense and continuous use of disinfectants is accelerating to the selection of less susceptible strains.

Although sodium hypochlorite is one of the most used disinfectants worldwide (Maillard and Pascoe 2024) and tolerance to chlorine disinfectants has already been reported (Xiao et al. 2022; Caro-Hernández et al. 2022), the mechanisms responsible for this phenotype are still not completely elucidated (Tong et al. 2021). Indeed, in dairy farming, sodium hypochlorite is daily used as a teat disinfectant, especially pre-milking, and to disinfect equipment and environment (Ózsvári and Ivanyos 2022; Nacional Mastitis Council (NMC) 2014). This constant exposure can accelerate the selection or trigger the development of more tolerant strains over the years, as demonstrated by our findings, with alarming levels of tolerance to sodium hypochlorite in both, *E. coli* and *S. aureus* strains, especially in the recent isolates. Likewise, studies conducted with other pathogens demonstrated that the exposure to sodium hypochlorite triggers a SOS response by inducing oxidative stress in the bacteria (Nam and Yoo 2024; da Cruz Nizer et al. 2023; Tong et al. 2021). In *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* this oxidative stress alter the transcriptional response, leading to an up-regulation of genes associated with efflux-pumps, antioxidant enzymes, and beta-lactamases, while genes related to membrane permeability are down-regulated (Nam and Yoo 2024; da Cruz Nizer et al. 2023; Tong et al. 2021). These alterations are possibly responsible for the phenotype of resistance to several disinfectants and antibiotics, as already reported for *Salmonella* spp. (Xiao et al. 2022), *P. aeruginosa* and *K. pneumoniae* (Nam and Yoo 2024; Tong et al. 2021). In this sense, emergence of chlorite tolerant strains (especially *E. coli*, as observed in our study) in dairy farms can jeopardize not only the efficacy of sodium hypochlorite as disinfectant but also contribute to the resistance to other antimicrobial drugs, used both for treatment and prevention of mastitis. In order to minimize this issue, an alternative to reduce the use of this drug in dairy farms would be the alternate the disinfectants bases used, reducing the selective pressure in the strains.

In general, *S. aureus* and *E. coli* strains exhibited susceptibility to the other tested disinfectants in concentrations much lower than those usually used as teat dipping in the milking routine (Nacional Mastitis Council (NMC) 2014), especially to chlorhexidine digluconate, which was the disinfectant with best performance to inhibit both species. However, it is worth noting that *in vitro* assays were conducted in controlled conditions, with established concentrations (CFU) of the inoculum, in addition to an exposure of at least 18 h to the active principle (Clinical and Laboratory Standards Institute (CLSI) 2018). On the other hand, during the teat dipping, cow udder can have organic matter (feces, mud, milk, etc), the challenge (CFU) is variable, and the teats are usually exposed to disinfectants for a short period (30 seconds to 1 min) (Nacional Mastitis Council (NMC) 2016), which perhaps makes necessary a greater dose than that observed *in vitro* to guarantee the disinfection or completely prevent the action of the drug. Given that, it is possible to speculate if the *S. aureus* strains inhibited *in vitro* with the recommended concentration of sodium hypochlorite [32.75 % (131/400)] would be inhibited in field conditions. Additionally, even though *E. coli* and *S. aureus* strains were susceptible to the other disinfectants in the concentration of use, it was observed that the more recent isolates required greater MIC of iodine, hydrogen peroxide, lactic acid, and quaternary ammonium to be inhibited, suggesting an acquisition of partial resistance mechanisms to the tested disinfectants, especially lactic acid, to which was observed a significative effect of year of isolation in the need concentrations to inhibit *S. aureus*. Furthermore, it is worth to mention that the disinfectants tested in the present study are also used for human asepsis, disinfection of surfaces in the food industry and in hospitals, and many other applications, which increase concern about resistance (Maillard and Pascoe 2024). These findings highlight the need for surveillance not only for sodium hypochlorite, but also for other tested disinfectants, aiming both animal and human health.

It was also observed that there was a difference in susceptibility according to the bacterial species, *S. aureus* strains showed greater susceptibility compared to *E. coli* isolates, for all tested disinfectants. This difference was expected and can be explained by the presence of the lipid bilayer in Gram-negative bacteria (Zeinab, Buthaina, and Rafik 2023). As mentioned before, changes in the outer membrane of the Gram-negative bacteria, as decreasing in the permeability (Tong et al. 2021), can prevent the entry and action of antimicrobials, consequently turning the isolates more resistant to the action of disinfectants and antibiotics (Miller 2016). Considering that *E. coli* is the major Gram-negative pathogen causing bovine mastitis and the need of greater doses to be inhibited compared to *S. aureus*, it is possible to consider that the disinfectant concentrations defined for this pathogen will be sufficient to inhibit other mastitis-causing pathogens.

Among the limitations of this study is the evaluation of the inhibition of bacterial strains to disinfectants only after 16-20 h of exposure. The evaluation at more time frames would allow the creation of growth curves to determine the action of disinfectants, especially with short durations (30 seconds to 1 minute), in order to mimetize the time and duration of teat dipping. However, as discussed, if the pathogens show tolerance even after long periods of exposition, they probably would not be sensitive if the exposition period was shorter. Moreover, this methodology was chosen as can be compared to the international standards for other antimicrobial drugs. Another possible limitation of the present study is that we evaluated only two bacterial species, and, although they were the most important species among Gram-negative and Gram-positive mastitis-causing pathogens, further studies with other pathogens, such as *S. agalactiae, S. uberis*, *Staphylococcus* non-*aureus* etc., would be helpful to understand the magnitude of disinfectant tolerance issue in bovine mastitis context. In addition, another limitation of this study is the influence of organic matter in the action of sodium hypochlorite, since it was diluted in organic cultivation medium, possibly leading to the need of higher concentrations of the disinfectant to inhibit the strains (Köhler et al. 2018). Nonetheless, although it may influence the results obtained, there is no alternative method to evaluate *in vitro* susceptibility of bacteria to antimicrobials, since it is necessary to provide bacteria with nutrients to evaluate its growth. In this sense, sodium hypochlorite efficacy could be reduced and the MIC observed for the strains overestimated, this effect would be the same for all strains evaluated and results about the effect of year of isolation in the tolerance remain relevant.

5. CONCLUSION

This study demonstrated a high tolerance of mastitis-causing *E. coli* and *S. aureus* to sodium hypochlorite. Additionally, it was observed that the strains that were isolated more recently were more tolerant to the disinfectants compared to oldest strains, especially lactic acid and sodium hypochlorite considering *S. aureus* strains. These findings highlight the urgence of surveillance on disinfectant resistance on livestock pathogens, especially in dairy farming.

6. REFERENCES

Armstrong, D., 2019. Control of contagious mastitis Contents. Agric. Hortic. Dev. Board 28.

Azizoglu, R.O., Lyman, R., Anderson, K.L., 2013. Bovine Staphylococcus aureus: Dose response to iodine and chlorhexidine and effect of iodine challenge on antibiotic susceptibility. J. Dairy Sci. 96, 993–999. doi:10.3168/jds.2012-5857

Behiry, A. El, Schlenker, G., Szabo, I., Roesler, U., 2012. In vitro susceptibility of Staphylococcus aureus strains isolated from cows with subclinical mastitis to different antimicrobial agents. J. Vet. Sci. 13, 153–161. doi:10.4142/jvs.2012.13.2.153

Bradley, A.J., 2002. Bovine mastitis: An evolving disease. Vet. J. 164, 116–128. doi:10.1053/tvjl.2002.0724

Caro-Hernández, P.A., Orozco-Mera, J.C., Castaño-Henao, O.L., Quimbaya-Gómez, M.A., 2022. Evaluating bacterial resistance to antimicrobials in isolated bacteria from food contact surfaces. Entramado 18, 1–14. doi:10.18041/1900-3803/entramado.1.7331

Cheng, W.N., Han, S.G., 2020. Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — A review. Asian-Australasian J. Anim. Sci. 33, 1699–1713. doi:10.5713/ajas.20.0156

Clinical and Laboratory Standards Institute (CLSI), 2018. Performance Standards for Antimicrobial Susceptibility Testing, 28th ed, Journal of Services Marketing. Clinical and Laboratory Standards Institute, Wayne.

CLSI, 2013. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for. Clinical and Laboratory Standards Institute, Wayne.

da Cruz Nizer, W.S., Adams, M.E., Inkovskiy, V., Beaulieu, C., Overhage, J., 2023. The secondary metabolite hydrogen cyanide protects Pseudomonas aeruginosa against sodium hypochlorite-induced oxidative stress. Front. Microbiol. 14, 1–16. doi:10.3389/fmicb.2023.1294518

Fitzpatrick, S.R., Garvey, M., Jordan, K., Flynn, J., O'Brien, B., Gleeson, D., 2019. Screening commercial teat disinfectants against bacteria isolated from bovine milk using disk diffusion. Vet. World 12, 629–637. doi:10.14202/vetworld.2019.629-637

Goulart, D.B., Mellata, M., 2022. Escherichia coli Mastitis in Dairy Cattle: Etiology, Diagnosis, and Treatment Challenges. Front. Microbiol. 13, 1–15. doi:10.3389/fmicb.2022.928346

Guimarães, J.L.B., Brito, M.A.V.P., Lange, C.C., Silva, M.R., Ribeiro, J.B., Mendonça, L.C., Mendonça, J.F.M., Souza, G.N., 2017. Estimate of the economic impact of mastitis: A case study in a Holstein dairy herd under tropical conditions. Prev. Vet. Med. 142, 46–50. doi:10.1016/j.prevetmed.2017.04.011

Köhler, A.T., Rodloff, A.C., Labahn, M., Reinhardt, M., Truyen, U., Speck, S., 2018. Efficacy of sodium hypochlorite against multidrug-resistant Gram-negative bacteria. J. Hosp. Infect. 100, 40–46. doi:10.1016/j.jhin.2018.07.017

Langsrud, S., Sidhu, M.S., Heir, E., Holck, A.L., 2003. Bacterial disinfectant resistance - a challenge for the food industry. Int. Biodeterior. Biodegrad. 51, 283–290. doi:10.1016/S0964-8305(03)00039-8

Maillard, J.Y., Pascoe, M., 2024. Disinfectants and antiseptics: mechanisms of action and resistance. Nat. Rev. Microbiol. 22, 4–17. doi:10.1038/s41579-023-00958-3

Maity, S., Ambatipudi, K., 2021. Mammary microbial dysbiosis leads to the zoonosis of bovine mastitis: A One-Health perspective. FEMS Microbiol. Ecol. 97, 1–17.

doi:10.1093/femsec/fiaa241

Miller, S.I., 2016. Antibiotic resistance and regulation of the Gram-negative bacterial outer membrane barrier by host innate immune molecules. MBio 7, 1–3. doi:10.1128/mBio.01541-16

Molineri, A.I., Camussone, C., Zbrun, M.V., Suárez Archilla, G., Cristiani, M., Neder, V., Calvinho, L., Signorini, M., 2021. Antimicrobial resistance of Staphylococcus aureus isolated from bovine mastitis: Systematic review and meta-analysis. Prev. Vet. Med. 188. doi:10.1016/j.prevetmed.2021.105261

Nacional Mastitis Council (NMC), 2016. Recommended Mastitis Control Program.

Nacional Mastitis Council (NMC), 2014. Summary Of Peer-Reviewed Publications On Efficacy Of Premilking And Postmilking Teat Disinfectants Published Since 1980.

Nam, J.-H., Yoo, J.S., 2024. Sublethal Sodium Hypochlorite Exposure: Impact on Resistance-Nodulation-Cell Division Efflux Pump Overexpression and Cross-Resistance to Imipenem. Antibiotics 13, 828. doi:10.3390/antibiotics13090828

Naranjo-Lucena, A., Slowey, R., 2023. Invited review: Antimicrobial resistance in bovine mastitis pathogens: A review of genetic determinants and prevalence of resistance in European countries. J. Dairy Sci. 106, 1–23. doi:10.3168/jds.2022-22267

Nobrega, D.B., De Buck, J., Naqvi, S.A., Liu, G., Naushad, S., Saini, V., Barkema, H.W., 2017. Comparison of treatment records and inventory of empty drug containers to quantify antimicrobial usage in dairy herds. J. Dairy Sci. 100, 9736–9745. doi:10.3168/jds.2017-13116

Ózsvári, L., Ivanyos, D., 2022. The use of teat disinfectants and milking machine cleaning products in commercial Holstein-Friesian farms. Front. Vet. Sci. 9, 1–15.

doi:10.3389/fvets.2022.956843

Pérez, V.K.C., Costa, G.M. da, Guimarães, A.S., Heinemann, M.B., Lage, A.P., Dorneles, E.M.S., 2020. Relationship between virulence factors and antimicrobial resistance in Staphylococcus aureus from bovine mastitis. J. Glob. Antimicrob. Resist. 22, 792–802. doi:10.1016/j.jgar.2020.06.010

Prestinaci, F., Pezzotti, P., Pantosti, A., 2015. Antimicrobial resistance: A global multifaceted phenomenon. Pathog. Glob. Health 109, 309–318. doi:10.1179/2047773215Y.0000000030

Ripley, B., Venables, B., Bates, D.M., Hornik, K., Gebhardt, A., Firth, D., 2025. MASS: Support Functions and Datasets for Venables and Ripley’s MASS. doi:10.32614/CRAN.package.MASS

Rodr, C., Alonso-calleja, C., Garc, C., Carballo, J., Capita, R., 2022. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for Twelve Antimicrobials (Biocides and Antibiotics) in Eight Strains of Listeria monocytogenes. Biology (Basel). 11, 46.

Ruegg, P.L., 2017. A 100-Year Review: Mastitis detection, management, and prevention. J. Dairy Sci. 100, 10381–10397. doi:10.3168/jds.2017-13023

Santos, R.P., Souza, F.N., Vasconcelos, C.G.C., Cortez, A., Rosa, D.L.S.O., Jardim, A.B., Cunha, A.F., Lana, Â.M.Q., Heinemann, M.B., Cerqueira, M.M.O.P., 2016. In vitro efficacy of teat antiseptics against Staphylococcus aureus strains isolated from bovine mastitis. Ciencias Agrar. 37, 1997–2002. doi:10.5433/1679-0359.2016v37n4p1997

Tong, C., Hu, H., Chen, G., Li, Z., Li, A., Zhang, J., 2021. Chlorine disinfectants promote microbial resistance in Pseudomonas sp. Environ. Res. 199, 111296. doi:10.1016/j.envres.2021.111296

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Xiao, X., Bai, L., Wang, S., Liu, L., Qu, X., Zhang, J., Xiao, Y., Tang, B., Li, Y., Yang, H., Wang, W., 2022. Chlorine Tolerance and Cross-Resistance to Antibiotics in Poultry-Associated Salmonella Isolates in China. Front. Microbiol. 12, 1–11. doi:10.3389/fmicb.2021.833743

Zeinab, B., Buthaina, J., Rafik, K., 2023. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. Molecules 25, 1–23.

Zigo, F., Vasil', M., Ondrašovičová, S., Výrostková, J., Bujok, J., Pecka-Kielb, E., 2021. Maintaining Optimal Mammary Gland Health and Prevention of Mastitis. Front. Vet. Sci. 8, 1–17. doi:10.3389/fvets.2021.607311

ARTICLE 2

Target journal: Brazilian Journal of Microbiology

ANTIMICROBIAL ACTIVITY OF PROPOLIS AGAINST MULTIDRUG-RESISTANT BOVINE MASTITIS PATHOGENS

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ABSTRACT

Bovine mastitis is characterized as inflammation of the mammary gland and is one of the main causes of economic losses in dairy farming. It is mainly caused by infectious pathogens such as *Escherichia coli* and *Staphylococcus. aureus*. For the prevention and treatment of this infection, a frequent and extensive use of antimicrobials is reported on dairy farms, which contributes to the decrease in tolerance and increase in bacterial resistance to antibiotics and disinfectants, becoming a serious problem for human and animal health. Thus, this study aimed to investigate *in vitro* the antimicrobial activity of alcoholic extracts of Brazilian propolis produced by *Appis melifera* and *Melipona quadrifasciata* bees against multi-drug resistant (MDR) *E. coli* and *S. aureus* strains isolated from bovine mastitis in Brazil, between 1998 and 2016. Susceptibility was assessed through the technique of both microdilution to obtain minimal inhibitory concentration (MIC). The results demonstrate that 5.0 mg/mL concentration of propolis extract was effective in inhibiting all MDR isolates, except for one MDR *E. coli*, which was not inhibited after exposure to a propolis extract. This study demonstrated antimicrobial activity of propolis extract against mastitis-causing MDR *E.*

coli and *S. aureus*. These results demonstrate the potential of propolis extract for use as an antimicrobial agent for the prevention and treatment of bovine mastitis.

Keywords: *Escherichia coli*, *Staphylococcus aureus*, alcoholic extracts, minimum inhibitory concentration, antibiotic, multi-drug resistant.

1. INTRODUCTION

Bovine mastitis is the main infectious disease affecting dairy herds in Brazil and worldwide (Guimarães et al. 2017; Gonçalves et al. 2018; Ruegg 2017). The disease is responsible for great economic losses in dairy farming, related to treatment of animals and milk disposal costs, in addition it reduces the yield and quality of produced milk, and compromises the health of the mammary gland for future lactations, leading to increased animal culling rates (Guimarães et al. 2017; Blosser 1979; Halasa et al. 2007; Janzen 1970).

The disease is mainly caused by bacterial pathogens that can have environmental or contagious origin (Kibebew 2017). Among the main mastitis-causing bacteria are *Staphylococcus aureus*, Coagulase Negative *Staphylococcus* (CoNS), *Streptococcus agalactiae*, *Streptococcus uberis*, and *Escherichia coli* (Bogni et al. 2011; Heikkilä et al. 2018). Due to the bacterial etiology, mastitis demands high levels of antimicrobial use in the farms, both to prevent and to treat intramammary infections (Bogni et al. 2011; Barlow 2011). Intramammary antimicrobials are used either to treat infections developed during lactation, as well as dry cow therapy (to treat older and prevent new infections) (Bogni et al. 2011; Barlow 2011). In addition, disinfectants are routinely used for pre and post-milking teat disinfections to prevent, respectively, environmental and contagious mastitis (Nacional Mastitis Council (NMC) 2016; Oliver et al. 1993).

Despite antimicrobials are fundamental to control bovine mastitis, frequent and not judicious use contributes to the emergence of resistant strains (Catry 2017; White and McDermott 2001). In fact, tolerance and resistance to antibiotics and disinfectants in mastitis-causing pathogens have been reported by many studies, with cases of multi-drug resistant (MDR) (resistant to three or more antimicrobial classes) (Pérez et al. 2020; Silva et al. 2017; Idriss et al. 2014; Rato et al. 2013; Dorneles et al. 2019; Fitzpatrick et al. 2019; Behiry et al.

2012; R. P. Santos et al. 2016; Enger et al. 2015), which is a major issue for both public and animal health (Catry 2017). In this sense, proposing alternative antimicrobials together with the rational use of the existing drugs is crucial to guarantee the effective treatment and prevention of infectious diseases (Tomanić, Samardžija, and Kovačević 2023; El-Sayed and Kamel 2021).

In this context, propolis, a resinous product produced by bees from different parts of plants, stands out as a very promising alternative (L. M. Santos et al. 2020; Manav et al. 2020). It is a blend of several chemical substances and has several biological properties, including antimicrobial activity (L. M. Santos et al. 2020; Mašek et al. 2018; De Groot 2013). This property is a result of the synergic action of several compounds, mainly phenols and flavonoids, which hamper the development of bacterial resistance to propolis (El-Guendouz et al. 2018; Nandre et al. 2021; Wang et al. 2021; Bouchelaghem et al. 2022). In view of this, propolis has been proposed as antimicrobial to prevent and treat several human and animal illnesses (Zullkiflee, Taha, and Usman 2022; L. M. Santos et al. 2020), including bovine mastitis (Pasca et al. 2020; Klhar et al. 2019; PETER et al. 2021; Fiordalisi et al. 2016; Šuran et al. 2020; Amarante et al. 2019; Niculae et al. 2015; Bacic 2016; Hegazi, Abdou, and Allah 2014; Machado et al. 2019), as well as an alternative to MDR strains (Nandre et al. 2021; Wang et al. 2021; Amarante et al. 2019).

Therefore, the aim of the present the study was to investigate *in vitro* the antimicrobial activity of alcoholic extracts of Brazilian propolis produced by *Appis melifera* and *Melipona quadrifasciata* bees against MDR *E. coli* and *S. aureus* strains isolated from bovine mastitis in Brazil, between 1998 and 2016.

2. MATERIAL AND METHODS

2.1. Strains

MDR strains of *E. coli* (n = 5) and *S. aureus* (n = 4) isolated from mastitis cases, in Brazil, between 1998 and 2016, were used. These samples were selected for being resistant to four or more antimicrobial classes (Pérez et al. 2020) and belong to the Collection of Microorganisms of the Laboratórios Integrados de Sanidade Animal e Saúde Coletiva (LISASC) from Universidade Federal de Lavras (UFLA) and to the Collection of Microorganisms of Interest to Agroindustry and Livestock from Brazilian Agricultural Research-Corporation (Embrapa). In addition, six American Type Culture Collection (ATCC) strains were evaluated: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus agalactiae* ATCC 13813, and *Streptococcus uberis* ATCC 700407.

2.2. Propolis

Three propolis samples were obtained *in natura* during summer (December 2021 to January 2022) from commercial apiaries in the state of Minas Gerais, Brazil. The samples were produced by bees of the species *Apis mellifera* in the municipalites of Barbacena (Propolis B), Lavras (Propolis L) and São Vicente de Minas (Propolis S), using alecrim do campo (*Baccharis dracunculifolia*), and plus copaiba (*Copaifera langsdorffii*), respectively, as source of nutrition.

Propolis M was produced by *Melipona quadrifasciata* bees in Lavras region, Minas Gerais, Brazil, and collected in March 2023. Bee's nutrition was based on basil (*Ocimum basilicum*), orange tree (*Citrus sinensis L*), boldo (*Peumus boldus*) and lavender (*Lavandula* spp).

2.3. *Ethanolic Extraction of Propolis (EEP)*

Propolis samples *in natura* were ground in 70% ethanol at a concentration of 10% (10 g for 100 mL of 70% ethanol). Solutions were shaken for 24 h at 250 rpm and at 28º C, subsequently, submitted to ultrasonic bath treatment at 40Hz for 20 min, as proposed by Pobiega et al. (Pobiega et al. 2019). Samples were then centrifuged at 3900 x g for 10 min at room temperature and the supernatant was removed and filtered by gravity using nº 4 filters. Rotary evaporation was carried out to remove ethanol, followed by a drying step at room temperature to evaporate residual water. The dry extract was lyophilized and resuspended in 70% ethanol (v/v) at a concentration of 10 mg/mL.

2.4. Biochemical tests

2.4.1. Total Phenolic Compounds

The total phenolic content of the ethanolic extracts and fractions was determined using Folin-Ciocalteau reagent (Sigma-Aldrich, USA) according to Kim et al. (Kim, Jeong, and Lee 2003) with modifications. Briefly, 50 μL of extract and fractions or gallic acid standard solution (0.5% in PA ethanol) was mixed in 500 μL of Folin-Ciocalteau reagent followed by 7% sodium bicarbonate (Na_2CO_3). Then, the mixture was incubated for 120 min at room temperature in the dark and centrifuged at 5.000 rpm for 5 min at 25° C. An aliquot of 275 μL of each sample in triplicate was added to 96 polystyrene microplates. A curve ranging from 0.062 to 0.004

mg/mL in a gallic acid ethanol solution (Sigma-Aldrich, Brazil) was obtained. The total phenolic content was expressed in mg gallic acid equivalent (GAE) per mg dry weight extract, calculated using the formula $y = 6.32x + 0.1635$ ($R^2 = 0.9985$), and the result was expressed in mg gallic acid equivalent/g propolis (mg EqAG/g).

2.4.2. *Total Flavonoid Content*

The evaluation of the total flavonoid content of the crude extract and fractions was carried out according to the method determined by Ahn et al. (Ahn et al. 2007). An aliquot of 100 μL of crude extracts and fractions was mixed with 100 μL of $AlCl_3$ (10% w/v). After 40 min, the absorbance was taken at 420 nm. The total flavonoid content was determined by means of a quercetin standard curve ($y = 20.053x + 01095$, $R^2 = 0.9964$) and was expressed in in milligrams of quercetin equivalents per g of dry leaf (mg EqQ/g).

2.4.3. Total Antioxidant Capacity

The total antioxidant capacity test was determined by evaluating the molybdenum complexation, measured according to Prieto et al. (Prieto, Pineda, and Aguilar 1999) through the reduction of ammonium molybdate. The extracts (200 μL of 1:2 dilutions) were mixed with 1500 μL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium monobasic phosphate, 4 mM ammonium molybdate). After 90 min of incubation at 95 °C, samples were cooled to room temperature and their absorbances were measured at 695 nm. The total antioxidant capacity tests were performed in triplicate and determined using an ascorbic acid standard curve ($y = 2.4077x - 0.0405$ and $R^2 = 0.9961$) and the results expressed in mg ascorbic acid equivalents/g dry leaf (mg EqAA/g).

2.5. Antimicrobial susceptibility test

The Minimum Inhibitory Concentration (MIC) for each alcoholic extract was performed by the technique microdilution in broth according to the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute (CLSI) 2018), adapting the protocols recommended for evaluate antibiotic susceptibility in bacteria that grow aerobically. Briefly, fresh cultures of each strain were grown on Mueller Hinton agar (MH) (Kasvi, Brazil), with the addition of 5% of sheep blood for *Streptococcus* spp., by incubation at 37 ºC for 24 h. Inoculum was prepared using saline solution (0.85% NaCl, pH 7.0) and adjusted to turbidity equivalent of 0.5 McFarland standard. Suspensions were diluted in order to obtain a final inoculum of 10^5 colony forming units (CFU)/well. The assays were carried out in duplicates using 96-well microplates containing Mueller Hinton broth (Himedia, United States). Ten two-fold dilutions were tested, ranging from 0.01 to 5 mg/mL. Plates were incubated at 37 ºC for 16-20 h, until results were interpreted visually. Contents of the wells in which no bacterial growth was observed were inoculated on Mueller Hinton agar (Kasvi, Brazil) (MIC), in order to determine bactericidal or bacteriostatic action. Tests using only 70% alcohol were also performed to evaluate the antimicrobial effect of alcohol in the concentrations contained in the extract. Gentamicin was used for quality control of the bacterial inoculum.

3. RESULTS

3.1. Biochemical tests

Table 1 shows the concentration of phenolics, flavonoids and the total antioxidant capacity of all tested propolis extracts. The results demonstrate that the L and S propolis extract exhibited the higher concentration of phenolic compounds (4.32± 0.23 and 3.75± 0.11 EqQ/g, respectively). The M and L propolis extract exhibited the higher concentration of flavonoid compounds (3.48± 0.03 and 2.53± 0.00 mg EqAG/g, respectively), whereas the B propolis extract exhibited the highest value of the total antioxidant capacity (105.04 ± 2.30mg EqAA/g).

3.2. Antimicrobial susceptibility test

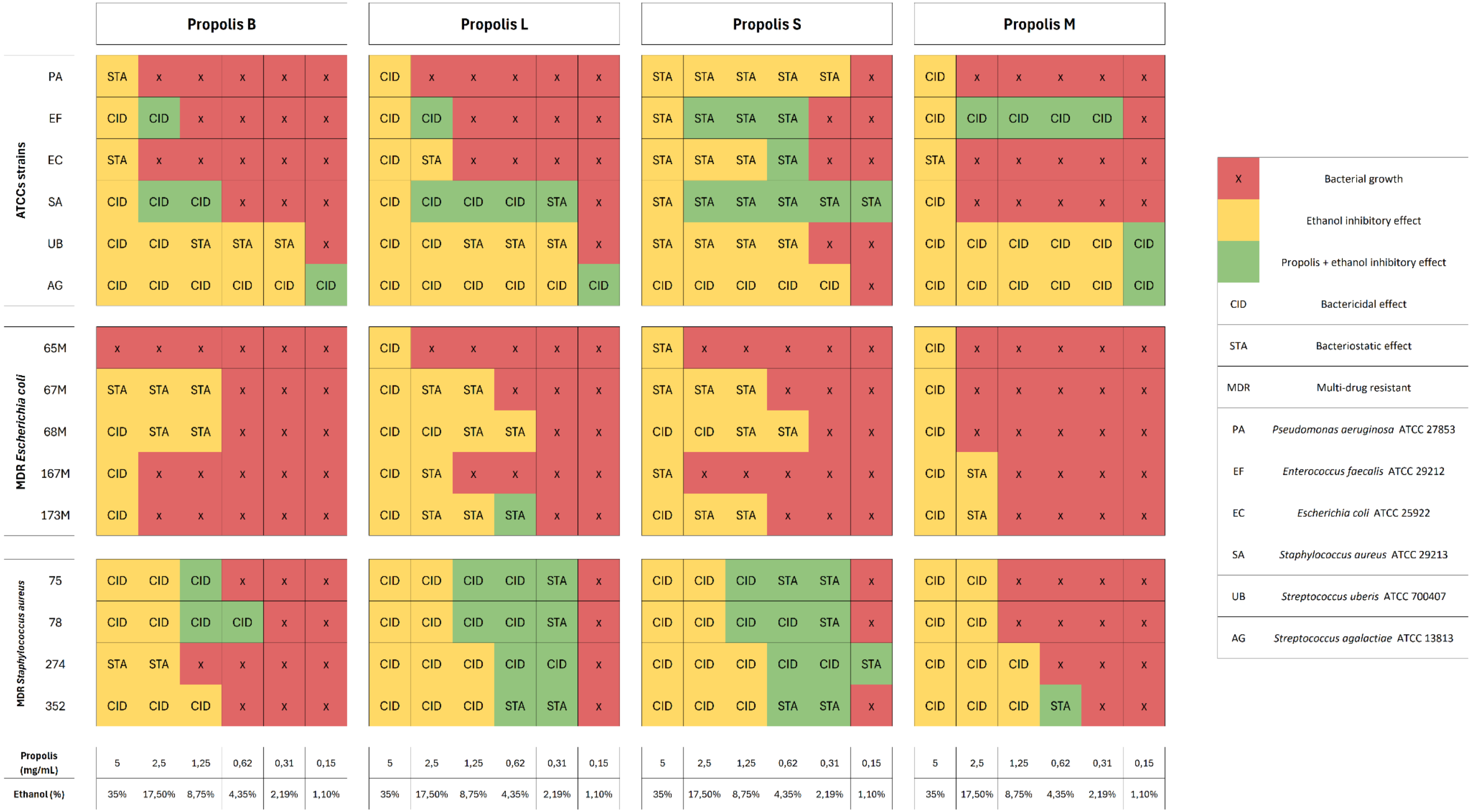
The concentration of 5.0 mg/mL of all tested propolis extract were sufficient to inhibited most of the strains *in vitro*: 100% (6/6) of the ATCC strains, 100% (4/4) of MDR *S. aureus* and 80% (4/5) of the MDR *E. coli*. Only one MDR *E. coli* isolate was not susceptible to all concentration of Propolis B extract (Table and Figure 1). In general, it is also possible to observe that MDR *S. aureus* strains were inhibited at lower concentrations that *E. coli* strains, for all tested propolis extracts, and that reference strains and MDR strains were inhibited at similar concentrations.

Table 1 – Biochemical tests and Minimum Inhibitory Concentration (MIC) results of Brazilian propolis extracts tested against reference strains and multidrug resistant strains of *E. coli* and *S. aureus* isolated from bovine mastitis in Brazil, 1998 – 2016.

	Biochemical tests			**MIC ATCC strains (mg/mL)**						**MIC multidrug-resistant strains (mg/mL)**								
										Escherichia coli					***Staphylococcus aureus***			
Propolis extracts	**Total phenolic compounds (mg EqQ/g)**	**Total flavonoid content (mg EqAG/g)**	**Total antioxidant capacity (mg EqAA/g)**	**EC**	**SA**	**EF**	**PA**	**AG**	**UB**	**65M**	**67M**	**68M**	**167M**	**173M**	**75**	**78**	**274**	**352**
Propolis B	3.26 (± 0.15)	0.23 (± 0.01)	105.04 (± 2.30)	5.0	1.25	2.5	5.0	0.62	0.62	> 5.0	1.25	1.25	5.0	5.0	1.25	0.62	2.5	1.25
Propolis L	4.32 (± 0.23)	2.53 (± 0.00)	25.07 (± 0.64)	2.5	0.31	2.5	5.0	0.15	0.31	5.0	1.25	0.62	2.5	0.62	0.31	0.31	0.31	0.31
Propolis S	3.75 (± 0.11)	2.09 (± 0.06)	4.93 (± 0.94)	0.62	0.15	0.62	0.31	0.31	0.62	5.0	1.25	0.62	5.0	1.25	0.31	0.31	0.15	0.31
Propolis M	3.35 (± 0.18)	3.48 (± 0.03)	56.90 (± 3.91)	5.0	5.0	0.31	5.0	0.15	0.15	5.0	5.0	5.0	2.5	2.5	2.5	2.5	1.25	0.62

ATCC: American Type Culture Collection
Propolis B: Propolis produced by bees of the species *Apis mellifera* in the Barbacena, Minas Gerais, Brazil
Propolis L: Propolis produced by bees of the species *Apis mellifera* in the Lavras, Minas Gerais, Brazil
Propolis S: Propolis produced by bees of the species *Apis mellifera* in the São Vicente de Minas, Minas Gerais, Brazil
Propolis M: Propolis produced by bees of the species *Melipona quadrifasciata* in the Lavras, Minas Gerais, Brazil
EC: *Escherichia coli* ATCC 25922
SA: *Staphylococcus aureus* ATCC 29213
EF: *Enterococcus faecalis* ATCC 29212
PA*: Pseudomonas aeruginosa* ATCC 2921
AG: *Streptococcus agalactiae* ATCC 13813
UB: *Streptococcus uberis* ATCC 700407
65M: Multidrug-resistant *Escherichia coli* isolated from subclinical bovine mastitis in Minas Gerais, Brazil, 2004
67M: Multidrug-resistant *Escherichia coli* isolated from subclinical bovine mastitis in Minas Gerais, Brazil, 2004
68M: Multidrug-resistant *Escherichia coli* isolated from subclinical bovine mastitis in Minas Gerais, Brazil, 2004
167M: Multidrug-resistant *Escherichia coli* isolated from clinical bovine mastitis, region and year unknow
173M: Multidrug-resistant *Escherichia coli* isolated from clinical bovine mastitis in Minas Gerais, Brazil, 2016
75: Multidrug-resistant *Staphylococcus aureus* isolated from bovine mastitis in Minas Gerais, Brazil, 1998
78: Multidrug-resistant *Staphylococcus aureus* isolated from bovine mastitis in Minas Gerais, Brazil, 1998
274: Multidrug-resistant *Staphylococcus aureus* isolated from bovine mastitis in Minas Gerais, Brazil, 2004
352: Multidrug-resistant *Staphylococcus aureus* isolated from bovine mastitis in Minas Gerais, Brazil, 2015

Figure 1 – Minimum Inhibitory Concentration (MIC) results of Brazilian propolis extract tested against reference strains and multidrug-resistant strains of *E. coli* and *S. aureus* isolated from bovine mastitis in Brazil, 1998 – 2016.



Propolis B

	Propolis (mg/mL)	5	2,5	1,25	0,62	0,31	0,15
	Ethanol (%)	35%	17,50%	8,75%	4,35%	2,19%	1,10%
ATCCs strains	PA	STA	x	x	x	x	x
	EF	CID	CID	x	x	x	x
	EC	STA	x	x	x	x	x
	SA	CID	CID	CID	x	x	x
	UB	CID	CID	STA	STA	STA	x
	AG	CID	CID	CID	CID	CID	CID
MDR *Escherichia coli*	65M	x	x	x	x	x	x
	67M	STA	STA	STA	x	x	x
	68M	CID	STA	STA	x	x	x
	167M	CID	x	x	x	x	x
	173M	CID	x	x	x	x	x
MDR *Staphylococcus aureus*	75	CID	CID	CID	x	x	x
	78	CID	CID	CID	CID	x	x
	274	STA	STA	x	x	x	x
	352	CID	CID	CID	x	x	x

Propolis L

	Propolis (mg/mL)	5	2,5	1,25	0,62	0,31	0,15
	Ethanol (%)	35%	17,50%	8,75%	4,35%	2,19%	1,10%
ATCCs strains	PA	CID	x	x	x	x	x
	EF	CID	CID	x	x	x	x
	EC	CID	STA	x	x	x	x
	SA	CID	CID	CID	CID	STA	x
	UB	CID	CID	STA	STA	STA	x
	AG	CID	CID	CID	CID	CID	CID
MDR *Escherichia coli*	65M	CID	x	x	x	x	x
	67M	CID	STA	STA	x	x	x
	68M	CID	CID	STA	STA	x	x
	167M	CID	STA	x	x	x	x
	173M	CID	STA	STA	STA	x	x
MDR *Staphylococcus aureus*	75	CID	CID	CID	CID	STA	x
	78	CID	CID	CID	CID	STA	x
	274	CID	CID	CID	CID	CID	x
	352	CID	CID	CID	STA	STA	x

Propolis S

	Propolis (mg/mL)	5	2,5	1,25	0,62	0,31	0,15
	Ethanol (%)	35%	17,50%	8,75%	4,35%	2,19%	1,10%
ATCCs strains	PA	STA	STA	STA	STA	STA	x
	EF	STA	STA	STA	STA	x	x
	EC	STA	STA	STA	STA	x	x
	SA	STA	STA	STA	STA	STA	STA
	UB	STA	STA	STA	STA	x	x
	AG	CID	CID	CID	CID	CID	x
MDR *Escherichia coli*	65M	STA	x	x	x	x	x
	67M	STA	STA	STA	x	x	x
	68M	CID	CID	STA	STA	x	x
	167M	STA	x	x	x	x	x
	173M	CID	STA	STA	x	x	x
MDR *Staphylococcus aureus*	75	CID	CID	CID	STA	STA	x
	78	CID	CID	CID	CID	STA	x
	274	CID	CID	CID	CID	CID	STA
	352	CID	CID	CID	STA	STA	x

Propolis M

	Propolis (mg/mL)	5	2,5	1,25	0,62	0,31	0,15
	Ethanol (%)	35%	17,50%	8,75%	4,35%	2,19%	1,10%
ATCCs strains	PA	CID	x	x	x	x	x
	EF	CID	CID	CID	CID	CID	x
	EC	STA	x	x	x	x	x
	SA	CID	x	x	x	x	x
	UB	CID	CID	CID	CID	CID	CID
	AG	CID	CID	CID	CID	CID	CID
MDR *Escherichia coli*	65M	CID	x	x	x	x	x
	67M	CID	x	x	x	x	x
	68M	CID	x	x	x	x	x
	167M	CID	STA	x	x	x	x
	173M	CID	STA	x	x	x	x
MDR *Staphylococcus aureus*	75	CID	CID	x	x	x	x
	78	CID	CID	x	x	x	x
	274	CID	CID	CID	x	x	x
	352	CID	CID	CID	STA	x	x

🟥 x	Bacterial growth
🟨	Ethanol inhibitory effect
🟩	Propolis + ethanol inhibitory effect
CID	Bactericidal effect
STA	Bacteriostatic effect
MDR	Multi-drug resistant
PA	*Pseudomonas aeruginosa* ATCC 27853
EF	*Enterococcus faecalis* ATCC 29212
EC	*Escherichia coli* ATCC 25922
SA	*Staphylococcus aureus* ATCC 29213
UB	*Streptococcus uberis* ATCC 700407
AG	*Streptococcus agalactiae* ATCC 13813

4. DISCUSSION

Mastitis is an infectious disease that involves extensive use of antimicrobial drugs, including both antibiotics and disinfectants, in dairy farms, which contributes to the emergence of the multidrug-resistant strains (Pérez et al. 2020; Silva et al. 2017; Idriss et al. 2014; Rato et al. 2013; Dorneles et al. 2019; Fitzpatrick et al. 2019; Behiry et al. 2012; R. P. Santos et al. 2016; Enger et al. 2015; Damasceno et al., n.d.), one of the main contemporary threats for the public and animal health (Catry 2017). Hence, research into antimicrobial alternatives is hugely necessary (Simões, Bennett, and Rosa 2009; El-Sayed and Kamel 2021). In this sense, studies using natural compounds, such as propolis, were developed to investigate their antimicrobial capacity, and demonstrated the efficacy of these substances against bacterial pathogens of importance to human and animal health, including pathogens that cause mastitis. Indeed, our results demonstrated that S, L, M and B propolis extracts inhibited all MDR strains, but one MDR *E. coli* (1/5) that was not inhibited by B propolis extract. These results suggest that propolis extracts could be used as an antimicrobial alternative for prevent and may be also to treat bovine mastitis, effective against susceptible and MDR pathogens. Corroborating these findings previous works have also observed antimicrobial activity of propolis compounds against *S. aureus*, *E. coli*, Coagulase-negative *staphylococci*, *S. agalactiae*, *S. dysgalactiae*, *P. aeruginosa*, *E. faecalis*, *Klebsiella* spp. and *Proteus* spp. among other pathogens of importance for bovine mastitis (Mašek et al. 2018; El-Guendouz et al. 2018; Klhar et al. 2019; Fiordalisi et al. 2016; Amarante et al. 2019; Niculae et al. 2015; Bacic 2016; Hegazi, Abdou, and Allah 2014). Moreover, these studies also revealed the ability of propolis extracts to inhibit MDR pathogens and not induce bacterial resistance (Nandre et al. 2021; El-Guendouz et al. 2018), as well as to be safe and efficient, which can be contribute to the reduction in economic losses in the sector (Pasca et al. 2020). Among the potential uses of propolis against mastitis-causing pathogens, stands out intramammary infusion and teat dipping that has already been tested elsewhere with promising results (Machado et al. 2019; Manav et al. 2020; Klhar et al. 2019; Bacic et al. 2016; Niculae et al. 2015; Šuran et al. 2020; Pasca et al. 2020). However, more tests are necessary to determine their efficacy and safe concentration *in vivo*, since toxicity can occur by contact with the animal's skin (Pasca et al. 2020; Machado et al. 2019).

The use of propolis extract becomes even more relevant due to its action against MDR strains (Amarante et al. 2019; Nandre et al. 2021), which represent a severe problem for the dairy industry and public health (Awandkar, Kulkarni, and Khode 2022). Furthermore, the frequent exposure to disinfectants and antibiotics further contributes to the worsening of this problem, due to the induction of increased tolerance and resistance of microorganisms (Maillard and Pascoe 2024; Azizoglu, Lyman, and Anderson 2013). Because of this, antimicrobial alternatives that are capable of acting on MDR pathogens are important and increasingly investigated (Tomanić, Samardžija, and Kovačević 2023). In this sense, the efficacy of propolis extract against MDR strains of *E. coli* and *S. aureus* demonstrated in our study may contribute to the reduction of the use of conventional antimicrobials through the use of this alternative compound for the prevention and treatment of mastitis, gaining even greater importance for organic milk production, since this restricts the use of antibiotics and the search for antimicrobial alternatives such as the demonstrated in this study (Do Nascimento et al. 2022).

It is worth to mention that propolis composition varies according to local flora, region, collection period, genetics of bees (L. M. Santos et al. 2020; Fiordalisi et al. 2016; Mašek et al. 2018; Ahn et al. 2007), as well as propolis extraction method (Deolindo et al. 2021; H. C. Dos Santos et al. 2019; Mašek et al. 2018). Nevertheless, the components such as phenols and flavonoids that are supposed to be related to the antimicrobial property are found in all types of propolis (Amarante et al. 2019; H. C. Dos Santos et al. 2019; Fiordalisi et al. 2016; Ahn et al. 2007; Mašek et al. 2018; Pobiega et al. 2019; Kim, Jeong, and Lee 2003; L. M. Santos et al. 2020; Bacic et al. 2016; Niculae et al. 2015). In fact, our results support this concept, demonstrating that propolis extracts with higher concentration of these compounds (Propolis S and L), especially phenols, were the extracts that best inhibited reference and MDR strains.

It was also observed that there was a difference in the susceptibility to propolis according to the bacterial species, since *S. aureus* strains were inhibited at lower concentrations than *E. coli* isolates, which was also demonstrated by other similar studies (Manav et al. 2020; Klhar et al. 2019; Deolindo et al. 2021; Hegazi, Abdou, and Allah 2014). This difference in susceptibility is probably due to the presence of the lipid bilayer in Gram-negative bacteria, which makes them naturally more resistant to antimicrobial agents than Gram-positive bacteria (Zeinab, Buthaina, and Rafik 2023). Additionally, Gram-negative bacteria are more likely to have mechanisms that can increase their tolerance to antimicrobials, such as performing alterations of the membrane, formation of vesicles with toxic compounds and efflux pumps mechanisms (Ramos et al. 2002).

The presence of alcohol in the extract composition, as well as the sample size and the use of only two bacterial genera are limitations of this work. However, the efficacy of propolis extract against MDR strains may indicate antimicrobial capacity of this compound against other pathogens that cause bovine mastitis.

5. CONCLUSION

This study demonstrated antimicrobial activity of propolis extract against mastitis-causing MDR *E. coli* and *S. aureus*. Our results demonstrate the potential of propolis extract for use as an antimicrobial agent for the prevention and treatment of bovine mastitis.

6. REFERENCES

Ahn, Mok Ryeon, Shigenori Kumazawa, Yumiko Usui, Jun Nakamura, Mitsuo Matsuka, Fang Zhu, and Tsutomu Nakayama. 2007. “Antioxidant Activity and Constituents of Propolis Collected in Various Areas of China.” *Food Chemistry* 101 (4): 1383–92. https://doi.org/10.1016/j.foodchem.2006.03.045.

Alton, Godfrey Greenset, Lois M Jones, R D Angus, and J M Verger. 1988. *Techniques for the Brucellosis Laboratory.*

Amarante, Jarbas F., Márcia F. Ribeiro, Mateus M. Costa, Fredson G. Menezes, Tania M.S. Silva, Talita A.B. Amarante, Adriana Gradela, and Liliane M.D. Moura. 2019. “Chemical Composition and Antimicrobial Activity of Two Extract of Propolis against Isolates of Staphylococcus Spp. and Multiresistant Bacterials.” *Brazilian Journal of Veterinary Research* 39 (9): 734–43. https://doi.org/10.1590/1678-5150-PVB-6128.

Andrade, Rafaella Resende, Ilda de Fátima Ferreira Tinôco, Flávio Alves Damasceno, Carlos Eduardo Alves Oliveira, Mariana Silva Concha, Ozana de Fátima Zacaroni, Gianluca Bambi, and Matteo Barbari. 2024. “Understanding Compost-Bedded Pack Barn Systems in Regions with a Tropical Climate: A Review of the Current State of the Art.” *Animals* 14 (12). https://doi.org/10.3390/ani14121755.

Andrade, Rafaella Silva, Marina Martins de Oliveira, Júlio Sílvio de Sousa Bueno Filho, Fernando Ferreira, Jacques Godfroid, Andrey Pereira Lage, and Elaine Maria Seles Dorneles. 2024. “Accuracy of Serological Tests for Bovine Brucellosis: A Systematic Review and Meta-Analysis.” *Preventive Veterinary Medicine* 222 (May 2023): 106079. https://doi.org/10.1016/j.prevetmed.2023.106079.

Armstrong, Derek. 2019. “Control of Contagious Mastitis Contents.” *Agriculture and Horticulture Development Board (AHDB)*, 28.

Awandkar, Sudhakar P., Mahesh B. Kulkarni, and Narendra V. Khode. 2022. “Bacteria from Bovine Clinical Mastitis Showed Multiple Drug Resistance.” *Veterinary Research Communications* 46 (1): 147–58. https://doi.org/10.1007/s11259-021-09838-8.

Azizoglu, Reha Onur, Roberta Lyman, and Kevin L. Anderson. 2013. “Bovine Staphylococcus Aureus: Dose Response to Iodine and Chlorhexidine and Effect of Iodine Challenge on Antibiotic Susceptibility.” *Journal of Dairy Science* 96 (2): 993–99. https://doi.org/10.3168/jds.2012-5857.

Bacic, Goran. 2016. “Intramammary Propolis Formulation for Subclinical Mastitis Prevention and Treatment in Dairy Cows.” *Journal of Dairy, Veterinary & Animal Research* 3 (5): 15406. https://doi.org/10.15406/jdvar.2016.03.00091.

Bacic, Goran, Nino Macesic, Lada Radin, Jasna Aladrovic, Kresimir Matanovic, Tomislav Masek, Diana Brozic, et al. 2016. “Intramammary Propolis Formulation for Subclinical Mastitis Prevention and Treatment in Dairy Cows.” *Journal of Dairy, Veterinary & Animal Research* 3 (5): 15406. https://doi.org/10.15406/jdvar.2016.03.00091.

Baily, G. G., J. B. Krahn, B. S. Drasar, and N. G. Stoker. 1992. “Detection of Brucella Melitensis and Brucella Abortus by DNA Amplification.” *Journal of Tropical Medicine and Hygiene* 95:271–75.

Barlow, John. 2011. “Mastitis Therapy and Antimicrobial Susceptibility: A Multispecies Review with a Focus on Antibiotic Treatment of Mastitis in Dairy Cattle.” *Journal of Mammary Gland Biology and Neoplasia* 16 (4): 383–407. https://doi.org/10.1007/s10911-011-9235-z.

Behiry, Ayman El, Gerd Schlenker, Istvan Szabo, and Uwe Roesler. 2012. “In Vitro Susceptibility of Staphylococcus Aureus Strains Isolated from Cows with Subclinical Mastitis to Different Antimicrobial Agents.” *Journal of Veterinary Science* 13 (2): 153–61. https://doi.org/10.4142/jvs.2012.13.2.153.

Bewley, J. M., L. M. Robertson, and E. A. Eckelkamp. 2017. “A 100-Year Review: Lactating Dairy Cattle Housing Management.” *Journal of Dairy Science* 100 (12): 10418–31. https://doi.org/10.3168/jds.2017-13251.

Biancifiori, F, D. Nannini, A. Di Matteo, and P. Belfiore. 1996. “Assessment of an Indirect

ELISA in Milk for the Diagnosis of Ovine Brucellosis." *Comparative Immunology, Microbiology and Infectious Diseases* 19 (1): 17–24. https://doi.org/10.1016/0147-9571(95)00024-0.

Blosser, T. H. 1979. "Economic Losses from and the National Research Program on Mastitis in the United States." *Journal of Dairy Science* 62 (1): 119–27. https://doi.org/10.3168/jds.S0022-0302(79)83213-0.

Bogni, Cristina, Liliana Odierno, Claudia Raspanti, José Giraudo, Alejandro Larriestra, Elina Reinoso, Mirta Lasagno, et al. 2011. "War against Mastitis: Current Concepts on Controlling Bovine Mastitis Pathogens." *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances.*, 483–494. http://www.formatex.info/microbiology3/book/483-494.pdf.

Bouchelaghem, Sarra, Sourav Das, Romen Singh Naorem, Lilla Czuni, Gábor Papp, and Marianna Kocsis. 2022. "Evaluation of Total Phenolic and Flavonoid Contents, Antibacterial and Antibiofilm Activities of Hungarian Propolis Ethanolic Extract against Staphylococcus Aureus." *Molecules* 27 (2). https://doi.org/10.3390/molecules27020574.

Bradley, A. J. 2002. "Bovine Mastitis: An Evolving Disease." *Veterinary Journal* 164 (2): 116–28. https://doi.org/10.1053/tvjl.2002.0724.

Caro-Hernández, Paola Andrea, Juan Camilo Orozco-Mera, Olga Lucia Castaño-Henao, and Mauricio Alberto Quimbaya-Gómez. 2022. "Evaluating Bacterial Resistance to Antimicrobials in Isolated Bacteria from Food Contact Surfaces." *Entramado* 18 (1): 1–14. https://doi.org/10.18041/1900-3803/entramado.1.7331.

Catry, Boudewijn. 2017. "Antimicrobial Policies in Animals and Human Health." *Archives of Public Health* 75 (1): 1–5. https://doi.org/10.1186/s13690-017-0231-7.

Cheng, Wei Nee, and Sung Gu Han. 2020. "Bovine Mastitis: Risk Factors, Therapeutic Strategies, and Alternative Treatments — A Review." *Asian-Australasian Journal of Animal Sciences* 33 (11): 1699–1713. https://doi.org/10.5713/ajas.20.0156.

Çiftci, Alper, Tuba İça, Serap Savaşan, Barış Sareyyüpoğlu, Mehmet Akan, and Kadir Serdar Diker. 2017. "Evaluation of PCR Methods for Detection of Brucella Strains from Culture and Tissues." *Tropical Animal Health and Production* 49 (4): 755–63. https://doi.org/10.1007/s11250-017-1256-1.

Clinical and Laboratory Standards Institute (CLSI). 2018. *Performance Standards for*

Antimicrobial Susceptibility Testing. *Journal of Services Marketing*. 28th ed. Wayne: Clinical and Laboratory Standards Institute.

CLSI. 2013. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests For*. Wayne: Clinical and Laboratory Standards Institute.

Corbel, M.J., S.S. Elberg, and O. Cosivi. 2006. "Brucellosis in Humans and Animals." *Geneva: World Health Organization Press*, 89 p.

Cordes, D. O., and Margery E. Carter. 1979. "Persistence of Brucella Abortus Infection in Six Herds of Cattle under Brucellosis Eradication." *New Zealand Veterinary Journal* 27 (12): 255–59. https://doi.org/10.1080/00480169.1979.34666.

Corner, L A. 1983. "Three Aspects of Bovine Brucellosis: Epidemiology the Role of Bulls and Vaccines." *New South Wales Veterinary Proceedings,* 19.

Cruz Nizer, Waleska Stephanie da, Madison Elisabeth Adams, Vasily Inkovskiy, Carole Beaulieu, and Joerg Overhage. 2023. "The Secondary Metabolite Hydrogen Cyanide Protects Pseudomonas Aeruginosa against Sodium Hypochlorite-Induced Oxidative Stress." *Frontiers in Microbiology* 14:1–16. https://doi.org/10.3389/fmicb.2023.1294518.

Dadar, Maryam, Ruchi Tiwari, Khan Sharun, and Kuldeep Dhama. 2021. "Importance of Brucellosis Control Programs of Livestock on the Improvement of One Health." *Veterinary Quarterly* 41 (1): 137–51. https://doi.org/10.1080/01652176.2021.1894501.

Dağ, Serpil, Fatih Büyük, Hasan Özen, Özgür Çelebi, Musa Karaman, Doğan Akça, and Mitat Şahin. 2012. "Detection of Brucella Spp. in Vaginal Swab Samples of Aborting Cattle: Comparison of Immunoperoxidase to Bacteriological Culture Technique." *Kafkas Universitesi Veteriner Fakultesi Dergisi* 18 (4): 617–22. https://doi.org/10.9775/kvfd.2012.6019.

Damasceno, Marcilene Daniel, Maysa Serpa Gonçalves, Bruna Henrique Pinto da Silva, Giovanna Botelho Carneiro, Alice Gonçalves Reis, Bruna Reis Pereira, Anna Cecília Trolesi Reis Borges Costa, Elaine Maria Seles Dorneles, and Alessandro de Sá Guimarães. n.d. "Susceptibility Od Mastitis-Causing Pathogens (Escherichia Coli and Staphylococcus Aureus) to Disinfectants Used as Teat Dipping."

Deolindo, Guilherme Luiz, Vitor Luiz Molosse, Amanda Dilda, Lilian Kolling Girardini, Marcelo Vedovatto, Aleksandro Schafer da Silva, and Denise Nunes Araujo. 2021.

"Lacaune Ewes with Subclinical Mastitis : Effects of Intramammary Application of Própolis." *Research, Society and Development* 10 (2): 1–12.

Dorneles, Elaine M.S., Mariana D.A.M. Fonseca, Juliana A.P. Abreu, Andrey P. Lage, Maria A.V.P. Brito, Carine R. Pereira, Humberto M. Brandão, Alessandro S. Guimarães, and Marcos B. Heinemann. 2019. "Genetic Diversity and Antimicrobial Resistance in Staphylococcus Aureus and Coagulase-Negative Staphylococcus Isolates from Bovine Mastitis in Minas Gerais, Brazil." *MicrobiologyOpen* 8 (5): 1–7. https://doi.org/10.1002/mbo3.736.

Eberl, Daniela T., Marshall J. Smith, Oliver J. Megram, Megan M. Mayhew, Debra Willoughby, Samuel J. White, and Philippe B. Wilson. 2024. "Innovative Bedding Materials for Compost Bedded Pack Barns: Enhancing Dairy Cow Welfare and Sustainable Dairy Farming." *Environment, Development and Sustainability*, no. 0123456789. https://doi.org/10.1007/s10668-024-05244-7.

El-Guendouz, Soukaïna, Smail Aazza, Badiaa Lyoussi, Vassya Bankova, Milena Popova, Luís Neto, Maria Leonor Faleiro, and Maria Da Graça Miguel. 2018. "Moroccan Propolis: A Natural Antioxidant, Antibacterial, and Antibiofilm against Staphylococcus Aureus with No Induction of Resistance after Continuous Exposure." *Evidence-Based Complementary and Alternative Medicine* 2018. https://doi.org/10.1155/2018/9759240.

El-Sayed, Amr, and Mohamed Kamel. 2021. "Bovine Mastitis Prevention and Control in the Post-Antibiotic Era." *Tropical Animal Health and Production* 53 (2). https://doi.org/10.1007/s11250-021-02680-9.

Emanuelson, Ulf, Kerstin Brügemann, Marija Klopčič, Lorenzo Leso, Wijbrand Ouweltjes, Andreas Zentner, and Isabel Blanco-Penedo. 2022. "Animal Health in Compost-Bedded Pack and Cubicle Dairy Barns in Six European Countries." *Animals* 12 (3): 1–9. https://doi.org/10.3390/ani12030396.

Enger, B. D., L. K. Fox, J. M. Gay, and K. A. Johnson. 2015. "Reduction of Teat Skin Mastitis Pathogen Loads: Differences between Strains, Dips, and Contact Times." *Journal of Dairy Science* 98 (2): 1354–61. https://doi.org/10.3168/jds.2014-8622.

Fiordalisi, Samira A.L., Luciana A. Honorato, Márcia R. Loiko, César A.M. Avancini, Maria B.R. Veleirinho, Luiz C.P.Machado Filho, and Shirley Kuhnen. 2016. "The Effects of Brazilian Propolis on Etiological Agents of Mastitis and the Viability of Bovine Mammary Gland Explants." *Journal of Dairy Science* 99 (3): 2308–18.

https://doi.org/10.3168/jds.2015-9777.

Fitzpatrick, Sarah Rose, Mary Garvey, Kieran Jordan, Jim Flynn, Bernadette O'Brien, and David Gleeson. 2019. "Screening Commercial Teat Disinfectants against Bacteria Isolated from Bovine Milk Using Disk Diffusion." *Veterinary World* 12 (5): 629–37. https://doi.org/10.14202/vetworld.2019.629-637.

Gonçalves, J. L., C. Kamphuis, C. M.M.R. Martins, J. R. Barreiro, T. Tomazi, A. H. Gameiro, H. Hogeveen, and M. V. dos Santos. 2018. "Bovine Subclinical Mastitis Reduces Milk Yield and Economic Return." *Livestock Science* 210 (January): 25–32. https://doi.org/10.1016/j.livsci.2018.01.016.

Goulart, Débora Brito, and Melha Mellata. 2022. "Escherichia Coli Mastitis in Dairy Cattle: Etiology, Diagnosis, and Treatment Challenges." *Frontiers in Microbiology* 13:1–15. https://doi.org/10.3389/fmicb.2022.928346.

Groot, Anton C. De. 2013. "Propolis: A Review of Properties, Applications, Chemical Composition, Contact Allergy, and Other Adverse Effects." *Dermatitis* 24 (6): 263–82. https://doi.org/10.1097/DER.0000000000000011.

Guimarães, Juliana L.B., Maria A.V.P. Brito, Carla C. Lange, Márcio R. Silva, João B. Ribeiro, Letícia C. Mendonça, Juliana F.M. Mendonça, and Guilherme N. Souza. 2017. "Estimate of the Economic Impact of Mastitis: A Case Study in a Holstein Dairy Herd under Tropical Conditions." *Preventive Veterinary Medicine* 142:46–50. https://doi.org/10.1016/j.prevetmed.2017.04.011.

Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. 2007. "Economic Effects of Bovine Mastitis and Mastitis Management: A Review." *Veterinary Quarterly* 29 (1): 18–31. https://doi.org/10.1080/01652176.2007.9695224.

Hegazi, Ahmed, Amr M Abdou, and Fyrouz Abd Allah. 2014. "Antimicrobial Activity of Propolis on the Bacterial Causes of Mastitis." *Life Science Journal* 11 (5): 572–76.

Heikkilä, A. M., E. Liski, S. Pyörälä, and S. Taponen. 2018. "Pathogen-Specific Production Losses in Bovine Mastitis." *Journal of Dairy Science* 101 (10): 9493–9504. https://doi.org/10.3168/jds.2018-14824.

Idriss, S.H E., V. Foltys, V. Tancin, K. Kirchnerova, D. Tancinova, and K. Zaujec. 2014. "Mastitis Pathogens and Their Resistance against Antimicrobial Agents in Dairy Cows in Nitra, Slovakia." *Slovak J. Anim. Sci.* 47 (1): 33–38.

Janzen, J. J. 1970. “Economic Losses Resulting from Mastitis. A Review.” *Journal of Dairy Science* 53 (9): 1151–60. https://doi.org/10.3168/jds.S0022-0302(70)86361-5.

Kampf, Günter. 2018. “Biocidal Agents Used for Disinfection Can Enhance Antibiotic Resistance in Gram-Negative Species.” *Antibiotics* 7 (4). https://doi.org/10.3390/antibiotics7040110.

Keid, L. B., R. M. Soares, S. A. Vasconcellos, D. P. Chiebao, V. R. Salgado, J. Megid, and L. J. Richtzenhain. 2007. “A Polymerase Chain Reaction for Detection of Brucella Canis in Vaginal Swabs of Naturally Infected Bitches.” *Theriogenology* 68 (9): 1260–70. https://doi.org/10.1016/j.theriogenology.2007.08.021.

Kibebew, Kinfe. 2017. “Bovine Mastitis: A Review of Causes and Epidemiological Point of View.” *Journal of Biology, Agriculture and Healthcare* 7 (2): 1–14.

Kim, Dae Ok, Seung Weon Jeong, and Chang Y. Lee. 2003. “Antioxidant Capacity of Phenolic Phytochemicals from Various Cultivars of Plums.” *Food Chemistry* 81 (3): 321–26. https://doi.org/10.1016/S0308-8146(02)00423-5.

Kiros, Ashenafi, Hagos Asgedom, and Reta Duguma. 2016. “A Review on Bovine Brucellosis: Epidemiology, Diagnosis and Control Options.” *ARC Journal of Animal and Veterinary Sciences* 2 (3). https://doi.org/10.20431/2455-2518.0203002.

Klhar, Gabriela Thais, José Victor Isola, Cintia Saydelles Da Rosa, Diciane Zeni Giehl, Anelise Afonso Martins, Monica Elisa Bartmer, and Luciane Rumpel Segabinazzi. 2019. “Antimicrobial Activity of the Ethanolic Extract of Propolis against Bacteria That Cause Mastitis in Cattle.” *Biotemas* 32 (1): 1–10. https://doi.org/10.5007/2175-7925.2019v32n1p1.

Köhler, A. T., A. C. Rodloff, M. Labahn, M. Reinhardt, U. Truyen, and S. Speck. 2018. “Efficacy of Sodium Hypochlorite against Multidrug-Resistant Gram-Negative Bacteria.” *Journal of Hospital Infection* 100 (3): 40–46. https://doi.org/10.1016/j.jhin.2018.07.017.

Langsrud, Solveig, Maan Singh Sidhu, Even Heir, and Askild L. Holck. 2003. “Bacterial Disinfectant Resistance - a Challenge for the Food Industry.” *International Biodeterioration and Biodegradation* 51 (4): 283–90. https://doi.org/10.1016/S0964-8305(03)00039-8.

Leso, L., M. Barbari, M. A. Lopes, F. A. Damasceno, P. Galama, J. L. Taraba, and A. Kuipers.

2020. “Invited Review: Compost-Bedded Pack Barns for Dairy Cows.” *Journal of Dairy Science* 103 (2): 1072–99. https://doi.org/10.3168/jds.2019-16864.

Machado, Gabriela Tasso Pinheiro, Maria Beatriz Veleirinho, Letícia Mazzarino, Luiz Carlos Pinheiro Machado Filho, Marcelo Maraschin, Ronaldo Luis Aoki Cerri, and Shirley Kuhnen. 2019. “Development of Propolis Nanoparticles for the Treatment of Bovine Mastitis: In Vitro Studies on Antimicrobial and Cytotoxic Activities.” *Canadian Journal of Animal Science* 99 (4): 713–23. https://doi.org/10.1139/cjas-2018-0173.

Maillard, Jean Yves, and Michael Pascoe. 2024. “Disinfectants and Antiseptics: Mechanisms of Action and Resistance.” *Nature Reviews Microbiology* 22 (1): 4–17. https://doi.org/10.1038/s41579-023-00958-3.

Maity, Sudipa, and Kiran Ambatipudi. 2021. “Mammary Microbial Dysbiosis Leads to the Zoonosis of Bovine Mastitis: A One-Health Perspective.” *FEMS Microbiology Ecology* 97 (1): 1–17. https://doi.org/10.1093/femsec/fiaa241.

Manav, S., M. Yilmaz, H. Baytekin, K. Çelik, and A. Çağli. 2020. “The Use of Propolis as an Antimicrobial in Livestock – an Overview.” *Agricultural Science and Technology* 12 (3): 205–9. https://doi.org/10.15547/ast.2020.03.032.

Mašek, Tomislav, Nataša Perin, Livio Racané, Maja Cindrić, Hana Čipčić Paljetak, Mihaela Perić, Mario Matijašić, et al. 2018. “Chemical Composition, Antioxidant and Antibacterial Activity of Different Extracts of Poplar Type Propolis.” *Croatica Chemica Acta* 91 (1): 81–88. https://doi.org/10.5562/cca3298.

Megid, Jane, LA Mathias, and CA Robles. 2010. “Clinical Manifestations of Brucellosis in Domestic Animals and Humans.” *Open Veterinary Science* 4:119–26. http://benthamscience.com/open/tovsj/articles/V004/SI0045TOVSJ/119TOVSJ.pdf.

Miller, Samuel I. 2016. “Antibiotic Resistance and Regulation of the Gram-Negative Bacterial Outer Membrane Barrier by Host Innate Immune Molecules.” *MBio* 7 (5): 1–3. https://doi.org/10.1128/mBio.01541-16.

Molineri, Ana Inés, Cecilia Camussone, M. Virginia Zbrun, Guillermo Suárez Archilla, Mariana Cristiani, Verónica Neder, Luis Calvinho, and Marcelo Signorini. 2021. “Antimicrobial Resistance of Staphylococcus Aureus Isolated from Bovine Mastitis: Systematic Review and Meta-Analysis.” *Preventive Veterinary Medicine* 188 (December 2020). https://doi.org/10.1016/j.prevetmed.2021.105261.

Mulligan, F. J., and M. L. Doherty. 2008. “Production Diseases of the Transition Cow.” *Veterinary Journal* 176 (1): 3–9. https://doi.org/10.1016/j.tvjl.2007.12.018.

Nacional Mastitis Council (NMC). 2014. “Summary Of Peer-Reviewed Publications On Efficacy Of Premilking And Postmilking Teat Disinfectants Published Since 1980.”

———. 2016. “Recommended Mastitis Control Program.”

Nam, Ji-Hyun, and Jung Sik Yoo. 2024. “Sublethal Sodium Hypochlorite Exposure: Impact on Resistance-Nodulation-Cell Division Efflux Pump Overexpression and Cross-Resistance to Imipenem.” *Antibiotics* 13 (9): 828. https://doi.org/10.3390/antibiotics13090828.

Nandre, Vinod S., Aditi V. Bagade, Deepak M. Kasote, Jisun H.J. Lee, Kisan M. Kodam, Mohan V. Kulkarni, and Aijaz Ahmad. 2021. “Antibacterial Activity of Indian Propolis and Its Lead Compounds against Multi-Drug Resistant Clinical Isolates.” *Journal of Herbal Medicine* 29 (May): 100479. https://doi.org/10.1016/j.hermed.2021.100479.

Naranjo-Lucena, Amalia, and Rosemarie Slowey. 2023. “Invited Review: Antimicrobial Resistance in Bovine Mastitis Pathogens: A Review of Genetic Determinants and Prevalence of Resistance in European Countries.” *Journal of Dairy Science* 106 (1): 1–23. https://doi.org/10.3168/jds.2022-22267.

Nascimento, Gabriel Michelutti Do, Marita Vedovelli Cardozo, Mylena Karoline Valmorbida, Natália Pereira, José Carlos Barbosa, Flávio Rubens Favaron, and Fernando Antônio De Ávila. 2022. “Propolis in the Control of Bacterial Bovine Mastitis: A Tool for the Production of Organic Milk.” *Semina:Ciencias Agrarias* 43 (2): 869–82. https://doi.org/10.5433/1679-0359.2022v43n2p869.

Niculae, Mihaela, Laura Stan, Emoke Pall, Anamaria Ioana Paştiu, Iulia Maria Balaci, Sevastiţa Muste, and Marina Spînu. 2015. “In Vitro Synergistic Antimicrobial Activity of Romanian Propolis and Antibiotics against Escherichia Coli Isolated from Bovine Mastitis.” *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 43 (2): 327–34. https://doi.org/10.15835/nbha43210074.

NMC. 2005. “Summary of Peer-Reviewed Publications on Egficacy of Premilking and Postmilking Teat Disinfectants Published Since 1980” 1980 (2011): 26–39.

Nobrega, Diego B., Jeroen De Buck, S. Ali Naqvi, Gang Liu, Sohail Naushad, Vineet Saini, and Herman W. Barkema. 2017. “Comparison of Treatment Records and Inventory of

Empty Drug Containers to Quantify Antimicrobial Usage in Dairy Herds." *Journal of Dairy Science* 100 (12): 9736–45. https://doi.org/10.3168/jds.2017-13116.

Oliveira, Luciana Faria De, Elaine Maria Seles Dorneles, Ana Lourdes Arrais De Alencar Mota, Vitor Salvador Picão Gonçalves, José Soares Ferreira Neto, Fernando Ferreira, Ricardo Augusto Dias, et al. 2016. "Seroprevalence and Risk Factors for Bovine Brucellosis in Minas Gerais State, Brazil." *Semina:Ciencias Agrarias* 37 (5): 3449–66. https://doi.org/10.5433/1679-0359.2016v37n5Supl2p3449.

Oliver, S. P., M. J. Lewis, T. L. Ingle, B. E. Gillespie, and K. R. Matthews. 1993. "Prevention of Bovine Mastitis by a Premilking Teat Disinfectant Containing Chlorous Acid and Chlorine Dioxide." *Journal of Dairy Science* 76 (1): 287–92. https://doi.org/10.3168/jds.S0022-0302(93)77348-8.

Ózsvári, László, and Dorottya Ivanyos. 2022. "The Use of Teat Disinfectants and Milking Machine Cleaning Products in Commercial Holstein-Friesian Farms." *Frontiers in Veterinary Science* 9 (1): 1–15. https://doi.org/10.3389/fvets.2022.956843.

Paiano, R. B., L. Z. Moreno, V. T.M. Gomes, B. M. Parra, M. R. Barbosa, M. I.Z. Sato, J. Bonilla, G. Pugliesi, P. S. Baruselli, and A. M. Moreno. 2022. "Assessment of the Main Pathogens Associated with Clinical and Subclinical Endometritis in Cows by Culture and MALDI-TOF Mass Spectrometry Identification." *Journal of Dairy Science* 105 (4): 3367–76. https://doi.org/10.3168/jds.2021-20642.

Pal, Mahendra, Fikru Gizaw, Gelane Fekadu, Gezahagn Alemayehu, and Venkataramana Kandi. 2017. "Public Health and Economic Importance of Bovine Brucellosis: An Overview." *American Journal of Epidemiology and Infectious Disease* 5 (2): 27–34. https://doi.org/10.12691/ajeid-5-2-2.

Pasca, Claudia, Liviu Alexandru Marghitas, Daniel Severus Dezmirean, Ioana Adriana Matei, Victorita Bonta, Ioan Pasca, Flore Chirila, Adrian Cîmpean, and Nicodim Iosif Fit. 2020. "Efficacy of Natural Formulations in Bovine Mastitis Pathology: Alternative Solution to Antibiotic Treatment." *Journal of Veterinary Research* 64 (4): 523–29. https://doi.org/10.2478/jvetres-2020-0067.

Pascottini, O. Bogado, S. J. Van Schyndel, J. F.W. Spricigo, J. Rousseau, J. S. Weese, and S. J. LeBlanc. 2020. "Dynamics of Uterine Microbiota in Postpartum Dairy Cows with Clinical or Subclinical Endometritis." *Scientific Reports* 10 (1): 1–11. https://doi.org/10.1038/s41598-020-69317-z.

Pérez, Verónica Karen Castro, Geraldo Márcio da Costa, Alessandro Sá Guimarães, Marcos Bryan Heinemann, Andrey Pereira Lage, and Elaine Maria Seles Dorneles. 2020. “Relationship between Virulence Factors and Antimicrobial Resistance in Staphylococcus Aureus from Bovine Mastitis.” *Journal of Global Antimicrobial Resistance* 22:792–802. https://doi.org/10.1016/j.jgar.2020.06.010.

PETER, Cristina Mendes, Lariane da Silva Barcelos, Matheus Iuri Frühauf, Yandra Nadalin Botton, João Luiz Zani, and Geferson Fischer. 2021. “Is Propolis a Promising Candidate on Bacterial Bovine Mastitis’ Treatment?” *Science and Animal Health* 9 (2): 89–103. https://doi.org/10.15210/sah.v9i2.21598.

Phillips, C J C, and S A Schofield. 1994. “The Effect of Cubicle and Straw Yard Housing on the Behaviour, Production and Hoof Health of Dairy Cows.” *Animal Welfare* 3 (1): 37–44. https://doi.org/10.1017/s0962728600016365.

Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. “Rapid Extraction of Bacterial Genomic DNA with Guanidium Thiocyanate.” *Letters in Applied Microbiology* 8 (4): 151–56. https://doi.org/10.1111/j.1472-765X.1989.tb00262.x.

Pobiega, Katarzyna, Karolina Kraśniewska, Dorota Derewiaka, and Małgorzata Gniewosz. 2019. “Comparison of the Antimicrobial Activity of Propolis Extracts Obtained by Means of Various Extraction Methods.” *Journal of Food Science and Technology* 56 (12): 5386–95. https://doi.org/10.1007/s13197-019-04009-9.

Prestinaci, Francesca, Patrizio Pezzotti, and Annalisa Pantosti. 2015. “Antimicrobial Resistance: A Global Multifaceted Phenomenon.” *Pathogens and Global Health* 109 (7): 309–18. https://doi.org/10.1179/2047773215Y.0000000030.

Prieto, Pilar, Manuel Pineda, and Miguel Aguilar. 1999. “Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E.” *Analytical Biochemistry* 269 (2): 337–41. https://doi.org/10.1006/abio.1999.4019.

Puppel, Kamila, Marcin Gołębiewski, Grzegorz Grodkowski, Jan Slósarz, Małgorzata Kunowska-Slósarz, Paweł Solarczyk, Monika Łukasiewicz, Marek Balcerak, and Tomasz Przysucha. 2019. “Composition and Factors Affecting Quality of Bovine Colostrum: A Review.” *Animals* 9 (12). https://doi.org/10.3390/ani9121070.

Ramos, Juan L., Estrella Duque, María Trinidad Gallegos, Patricia Godoy, María Isabel

Ramos-González, Antonia Rojas, Wilson Terán, and Ana Segura. 2002. “Mechanisms of Solvent Tolerance in Gram-Negative Bacteria.” *Annual Review of Microbiology* 56:743–68. https://doi.org/10.1146/annurev.micro.56.012302.161038.

Rato, Márcia G., Ricardo Bexiga, Carlos Florindo, Lina M. Cavaco, Cristina L. Vilela, and Ilda Santos-Sanches. 2013. “Antimicrobial Resistance and Molecular Epidemiology of Streptococci from Bovine Mastitis.” *Veterinary Microbiology* 161 (3–4): 286–94. https://doi.org/10.1016/j.vetmic.2012.07.043.

Redfern, Emma A., Liam A. Sinclair, and Philip A. Robinson. 2021. “Dairy Cow Health and Management in the Transition Period: The Need to Understand the Human Dimension.” *Research in Veterinary Science* 137 (April): 94–101. https://doi.org/10.1016/j.rvsc.2021.04.029.

Ripley, Brian, Bill Venables, Douglas M. Bates, Kurt Hornik, Albrecht Gebhardt, and David Firth. 2025. “MASS: Support Functions and Datasets for Venables and Ripley’s MASS.” https://doi.org/10.32614/CRAN.package.MASS.

Rodr, Cristina, Carlos Alonso-calleja, Camino Garc, Javier Carballo, and Rosa Capita. 2022. “Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for Twelve Antimicrobials (Biocides and Antibiotics) in Eight Strains of Listeria Monocytogenes.” *Biology* 11 (Mic): 46.

Ruegg, Pamela L. 2017. “A 100-Year Review: Mastitis Detection, Management, and Prevention.” *Journal of Dairy Science* 100 (12): 10381–97. https://doi.org/10.3168/jds.2017-13023.

Santos, Heidy C. Dos, Dielson S. Vieira, Sandra M. Yamamoto, Mateus M. Costa, Maria C.A. Sá, Eva M.S. Silva, and Tania M.S. Silva. 2019. “Antimicrobial Activity of Propolis Extract Fractions against Staphylococcus Spp. Isolated from Goat Mastitis.” *Brazilian Journal of Veterinary Research* 39 (12): 954–60. https://doi.org/10.1590/1678-5150-PVB-5940.

Santos, Laerte M., Maísa S. Fonseca, Ana R. Sokolonski, Kathleen R. Deegan, Roberto P.C. Araújo, Marcelo A. Umsza-Guez, Josiane D.V. Barbosa, Ricardo D. Portela, and Bruna A.S. Machado. 2020. “Propolis: Types, Composition, Biological Activities, and Veterinary Product Patent Prospecting.” *Journal of the Science of Food and Agriculture* 100 (4): 1369–82. https://doi.org/10.1002/jsfa.10024.

Santos, Renata Paoli, Fernando Nogueira Souza, Carla Gasparotto Chande Vasconcelos, Adriana Cortez, Dalila Lapinha Silva Oliveira Rosa, Annatachi Botelho Jardim, Adriano França Cunha, Ângela Maria Quintão Lana, Marcos Bryan Heinemann, and Mônica Maria Oliveira Pinho Cerqueira. 2016. "In Vitro Efficacy of Teat Antiseptics against Staphylococcus Aureus Strains Isolated from Bovine Mastitis." *Ciencias Agrarias* 37 (4): 1997–2002. https://doi.org/10.5433/1679-0359.2016v37n4p1997.

Silva, Juliana Rosa da, Glei Dos Anjos De Carvalho Castro, Maysa Serpa Gonçalves, Dircéia Aparecida Da Costa Custódio, Gláucia Frasnelli Mian, and Geraldo Márcio da Costa. 2017. "In Vitro Antimicrobial Susceptibility and Genetic Resistance Determinants of Streptococcus Agalactiae Isolated from Mastitic Cows in Brazilian Dairy Herds." *Semina:Ciencias Agrarias* 38 (4): 2581–94. https://doi.org/10.5433/1679-0359.2017v38n4Supl1p2581.

Simões, Manuel, Richard N. Bennett, and Eduardo A.S. Rosa. 2009. "Understanding Antimicrobial Activities of Phytochemicals against Multidrug Resistant Bacteria and Biofilms." *Natural Product Reports* 26 (6): 746–57. https://doi.org/10.1039/b821648g.

Šuran, Jelena, Jasna Aladrović, Blanka Beer Ljubić, Josipa Vlainić, Marija Mamić, Božo Radić, Goran Bačić, et al. 2020. "The Antioxidant Effect of the Novel Bee-Product Based Intramammary Formulation Apimast® in Dairy Cattle." *Veterinarski Arhiv* 90 (3): 225–33. https://doi.org/10.24099/VET.ARHIV.0865.

Sutherland, S. S. 1980. "Immunology of Bovine Brucellosis." *Veterinary Bulletin* 50 (5): 359–63. http://scioteca.caf.com/bitstream/handle/123456789/1091/RED2017-Eng-8ene.pdf?sequence=12&isAllowed=y%0Ahttp://dx.doi.org/10.1016/j.regsciurbeco.2008.06.005%0Ahttps://www.researchgate.net/publication/305320484_SISTEM_PEMBETUNGAN_TERPUSAT_STRATEGI_MELESTARI.

Tibbs-Cortes, Bienvenido W., Faith M. Rahic-Seggerman, Stephan Schmitz-Esser, Paola M. Boggiatto, Steven Olsen, and Ellie J. Putz. 2024. "Fecal and Vaginal Microbiota of Vaccinated and Non-Vaccinated Pregnant Elk Challenged with Brucella Abortus." *Frontiers in Veterinary Science* 11 (January): 1–16. https://doi.org/10.3389/fvets.2024.1334858.

Tomanić, Dragana, Marko Samardžija, and Zorana Kovačević. 2023. "Alternatives to Antimicrobial Treatment in Bovine Mastitis Therapy: A Review." *Antibiotics* 12 (4). https://doi.org/10.3390/antibiotics12040683.

Tong, Chaoyu, Hong Hu, Gang Chen, Zhengyan Li, Aifeng Li, and Jianye Zhang. 2021. "Chlorine Disinfectants Promote Microbial Resistance in Pseudomonas Sp." *Environmental Research* 199 (May): 111296. https://doi.org/10.1016/j.envres.2021.111296.

Wang, Fei, Hui Liu, Junya Li, Wenwen Zhang, Bin Jiang, and Hongzhuan Xuan. 2021. "Australian Propolis Ethanol Extract Exerts Antibacterial Activity against Methicillin-Resistant Staphylococcus Aureus by Mechanisms of Disrupting Cell Structure, Reversing Resistance, and Resisting Biofilm." *Brazilian Journal of Microbiology* 52 (4): 1651–64. https://doi.org/10.1007/s42770-021-00547-7.

White, D.G., and P.F. McDermott. 2001. "Emergence and Transfer of Antibacterial Resistance." *Journal of Dairy Science* 84:E151–55. https://doi.org/10.3168/jds.s0022-0302(01)70209-3.

Wickham, Hadley. 2016. *Ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Wray, C. 1975. "Survival and Spread of Pathogenic Bacteria of Veterinary Importance within the Environment" 45 (8): 6.

Xiao, Xingning, Li Bai, Sheng Wang, Lisha Liu, Xiaoyun Qu, Jianmin Zhang, Yingping Xiao, et al. 2022. "Chlorine Tolerance and Cross-Resistance to Antibiotics in Poultry-Associated Salmonella Isolates in China." *Frontiers in Microbiology* 12 (February): 1–11. https://doi.org/10.3389/fmicb.2021.833743.

Yan, Linlin. 2021. "Ggvenn: Draw Venn Diagram by 'Ggplot2.'" *R Package Version* 19.

Yanuartono, Alfarisa Nururrozi, Soedarmanto Indarjulianto, Hary Purnamaningsih, and Universitas Gadjah Mada. 2020. "The Benefits of Teat Dipping as Prevention of Mastitis." *Journal of Livestock Science and Production* 4 (1): 231–49. https://doi.org/10.31002/jalspro.v4i1.2796.

Zeinab, Breijyeh, Jubeh Buthaina, and Karaman Rafik. 2023. "Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It." *Molecules* 25 (6): 1–23.

Zhang, Ning, Desheng Huang, Wei Wu, Jing Liu, Feng Liang, Baosen Zhou, and Peng Guan. 2018. "Animal Brucellosis Control or Eradication Programs Worldwide: A Systematic Review of Experiences and Lessons Learned." *Preventive Veterinary Medicine* 160:105–

15. https://doi.org/10.1016/j.prevetmed.2018.10.002.

Zigo, František, Milan Vasil’, Silvia Ondrašovičová, Jana Výrostková, Jolanta Bujok, and Ewa Pecka-Kielb. 2021. “Maintaining Optimal Mammary Gland Health and Prevention of Mastitis.” *Frontiers in Veterinary Science* 8 (February): 1–17. https://doi.org/10.3389/fvets.2021.607311.

Zullkiflee, Nadzirah, Hussein Taha, and Anwar Usman. 2022. “Propolis: Its Role and Efficacy in Human Health and Diseases.” *Molecules* 27 (18). https://doi.org/10.3390/molecules27186120.

ARTICLE 3

Target journal: Comparative Immunology, Microbiology and Infectious Diseases

DETECTION OF *Brucella* spp. IN DAIRY COWS IN THE TRANSITION PERIOD HOUSED IN A COMPOST-BEDDED PACK BARN SYSTEM

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ABSTRACT

Compost-bedded pack barn (CBP) is an intensive system for dairy cow confinement, consisting of a shared bed with organic matter and cattle excrement that provides well-being to the animals housed. However, it is still considered recent, with scarce information about housed animal health. Bovine brucellosis is a reproductive zoonotic disease with prevalence and occurrence worldwide and the transmission between bovines occurs mainly through contact with fetal membranes and fomites contaminated. Due to the relevance and increase of the CBP production and the way brucellosis is transmitted, the objective of the present work was to investigate the presence of brucellosis in dairy cows in the transition period housed in CBP. The analyzes were conducted using samples of CBP bed, vaginal swab, uterine cytology and serum from animals from twenty (20) dairy farms using CBP, collected in the years 2023 and 2024, from the states of Goiás and Minas Gerais, Brazil. A total of 17 [17/20 (85 %)] proprieties exhibited at least one animal with positive result in at least one test, and seven [7/17 (41.20 %)] of these animals were positive in at least two tests. Additionality, eight [8/17 (47.06%)] of theses proprieties showed positive results in at least two tests analyzed. These results demonstrated the presence of *Brucella* spp. in sample of CBP bed and dairy cows in transition period housed in CBP, suggesting the CBP can aggravate problems with bacterial infections in the system, such as brucellosis, by favoring the spread of the agent and transmission to other housed cows.

Keywords: brucellosis, abortion, PCR, serological test, RBT, 2ME

1. INTRODUCTION

Compost-bedded pack barn (CBP) is an intensive system for dairy cow confinement, consisting of a shared bed with organic matter and cattle excrement (Leso et al. 2020). This system has the potential to improve animal welfare by providing comfort, foot and leg health and allowing more natural animal behavior (Eberl et al. 2024; Phillips and Schofield 1994). However, it is still considered a recent production system and information about its impact on animal health is scarce, including potential effects on reproductive and mammary gland health (Emanuelson et al. 2022). Furthermore, some characteristics of the bed may also contribute to disease transmission, since its litter is made up of organic matter, shared by all animals in the confinement and needs correct management and control of temperature and humidity to be functional (R. R. Andrade et al. 2024). Moreover, CBP bed must be turned 1 to 3 times a day to generate aerobic composting of the material and the incorrect management can increase the risk of spreading pathogens and thereby diseases within the system (Leso et al. 2020).

Due to the comfort and well-being provided by CBP, there is an increase number of facilities and animals housed in this system worldwide (Leso et al. 2020; R. R. Andrade et al. 2024; Bewley, Robertson, and Eckelkamp 2017). In addition to lactating cows, some farms have also housed pre-calving animals in these facilities, which increase the risk of diseases transmission among different animal categories in the system, particularly reproductive pathogens (Redfern, Sinclair, and Robinson 2021; Mulligan and Doherty 2008).

Among the most important reproductive disease of cattle is bovine brucellosis, a zoonotic disease endemic in several parts of the world and a major problem for the livestock sector due reproductive problems, such as abortion, retained placenta, infertility and nonspecific signs, such as fever (Megid, Mathias, and Robles 2010; Pal et al. 2017). Bovine brucellosis is caused by bacteria of the genus *Brucella* spp., especially *Brucella abortus* (Corbel, Elberg, and Cosivi 2006), and transmission between animals occurs mainly through contact with fetal membranes from infected animals or through ingestions of contaminated food or water (Kiros, Asgedom, and Duguma 2016). Indeed, pregnant cows are key in the transmission of the pathogen, as the fetus, genital membranes, placenta and postpartum vaginal discharge may contain up to 10^4 CFU of *Brucella* spp. per gram of material (Corner 1983). In this sense, due to the characteristic of CBP, the calving of *Brucella*-positive animals inside the system may enhance the disease transmission within this system.

Given the relevance and increase of the CBP production system and the scarcity of information about the health of animals housed in this system, combined with the importance and the contagious aspect of bovine brucellosis, the aim of the present study was to investigate the association between the presence of brucellosis in dairy cows housed in CBP and the detection of the pathogen in the bed.

2. MATERIAL AND METHODS

2.1 Sampling and collection of samples

Samples of CPB bed, vaginal swab, endometrial smear and serum from postpartum cows were collected between 2023 and 2024 from twenty (20) dairy farms with CBP system in two Brazilian states [95% (19/20) from Minas Gerais, and 5% (1/20) from Goiás state]. All CPB lots were collected and only cows housed in the CBP and up to twenty-one (21) days postpartum were sampled, with a limit of up to twenty (20) animals per property. When the property exceeded this number of cows, the selection of animals to be sampled occurred randomly.

CBP bed were collected in a representative way and sealed in sterile tubes of 50 mL capability and were frozen at -80 ºC until analysis.

Vaginal discharges were collected in swabs containing Stuart Transport Medium (FirstLab, Brazil). Approximately 10 cm of the swab were inserted in the vaginal canal by trained personnel taking care to avoid outer surface contamination as described (Tibbs-Cortes et al. 2024). Samples were frozen at -80º C until analysis.

Endometrial smear were collected by using a cytobrush (Kolplast, Brazil). Previously the perineal area was cleansed with 70% ethylic alcohol and dried using a paper towel as described by Paiano et al., (2022) (Paiano et al. 2022), then the cytobobrush was introduced into the vagina and guided through the cervix per rectum as described by Bogado Pascottini et al., (2020) (Pascottini et al. 2020). Likewise, all samples were frozen at -80 ºC until DNA extraction.

Blood samples were obtained by coccygian vein puncture, using one sterile disposable needle in a vacuum tube with clot activator and capacity of 9 mL. After clotting the blood samples, serum was harvested by centrifugation at 3500 rpm for 15 min and stored at -20 ºC until analysis.

2.2 *Isolation*

Swabs samples were processed in a Biosafety Level 3 (BSL-3) on Universidade Federal de Lavras (UFLA), Laboratory for *Brucella* spp. isolation as described by Alton et al 1988 (Alton et al. 1988). Briefly, the swab samples were transferred to a new microtube containing 1000 µL of the phosphate-buffered saline (PBS) (0.01 M, pH 7.4) for elution. Subsequently, 500 µL of the content was transferred to another tube containing 4.5 mL of trypticase soy broth (TSB) (HiMedia, India) with Farrell selective supplement (TM Media, India), which were incubated for 7 days at 5% CO_2 atmosphere at 37 ºC. Then, 100 µL of the content were inoculated in plates of trypticase soy agar (TSA) (HiMedia, India) and incubated at the same conditions. Bacterial growth was analyzed 48, 96, 144 and 168 h after incubation and up to five colonies per sample were selected for biochemical tests (Alton et al. 1988). *Brucella*-suggestive colonies were stored in 1 mL of PBS, inactivated at 80 ºC for 1 h, stored at -20 ºC and thereafter used for DNA extraction.

2.3 *DNA extraction*

CBP bed were submitted to genomic DNA extraction using QIAgen® Power Fecal Pro DNA following the manufacture's recommendations. Vaginal swab and isolated *Brucella*-suggestive colonies were suspended in 1000 µL of PBS (0.01 M, pH 7.4) and submitted to genomic DNA extraction using the guanidium thiocyanate method according to Pitcher et al. (1989) (Pitcher, Saunders, and Owen 1989). Endometrial samples were suspended in 200 µL of PBS and submitted to genomic DNA extraction using Wizard® Genomic DNA Purification Kit following the manufacture's recommendations.

The quantity and quality of all extracted DNA samples were assessed by spectrophotometry using NanoDrop Lite Plus spectrophotometer (Thermo Scientific, United States). DNA samples were kept at -20 ºC until the PCR analysis.

2.4 *Polymerase Chain Reaction (PCR)*

All DNA samples obtained from CBP bed, vaginal swabs, *Brucell*a-suggestive colonies and endometrial smear were tested for *Brucella* spp. by conventional PCR. The search was carried out by amplification of the gene *bscp31*, using the primers B4- 5'-TGG CTC GGT TGC CAA TAT CAA-3' and B5 5'-CGC GCT TGC CTT TCA GGT CTG-3' that amplify a product of 223 pb (Baily et al. 1992). Briefly, PCR reactions were performed in a final volume of 25 µL containing 1X I0 buffer (Phoneutria, Brazil), 200 µM of deoxyribonucleotide triphosphate (dNTP) (Ludwig Biotecnologia Ltda, Brazil), 1.0 µM for each primer (Merck, United States), 1.5 mM of $MgCl_2$ (Phoneutria, Brazil), 1.25 U of Taq polymerase (Phoneutria, Brazil) and 2.0 µL of DNA template. Amplification was done with initial denaturation at 94 ºC for 3 min, followed by 30 cycles for denaturation at 94 ºC for 30 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s, followed by a final extension at 72 ºC for 10 min. DNA extracted from *B. abortus* 2308 strain and PCR reagents without DNA were used as positive and negative controls in each PCR assay, respectively.

The amplicons were separated by electrophoresis 1.5% agarose gel (Ludwig Biotecnologia Ltda, Brazil) stained with 0.5 mg/mL ethidium bromide (Ludwig Biotecnologia Ltda., Brazil). The bands were visualized under UV light and photographed using the L-PIX software (Loccus, Brazil).

2.5 Serological tests

Serum samples were tested for anti-smooth *Brucella* antibodies using Rose Bengal Test (RBT) (Idexx, Brazil) as screening test and 2 mercaptoethanol test (2ME) as a confirmatory test. The test were carried out in accordance with the recommendations of Alton et al., 1988 (Alton et al. 1988).

2.6 Statistical analysis

The statistical analysis were obtained using the R software version in 4.4.2 with aid of the package “ggvenn” (Yan 2021).

3. RESULTS

A total of 44 CBP bed, 314 vaginal swabs, 307 endometrial smear and 308 serum samples from 20 different CBP-properties were analyzed. Endometrial samples from seven animals [7/314 (2.23%)] were not collected due to endometrial problems observed during sampling. Moreover, serum from six animals [6/314 (1.91%)] were lost during handling and transport. The origin of samples, tests employed and positive results for each test are summarized in Figure 1 and Table 1.

Figure 1 – Flow chart of sampling and results of the bovine brucellosis analysis carried out in vaginal swabs, endometrial smear and serum samples from dairy cows from properties that use the compost-bedded pack barn CBP.

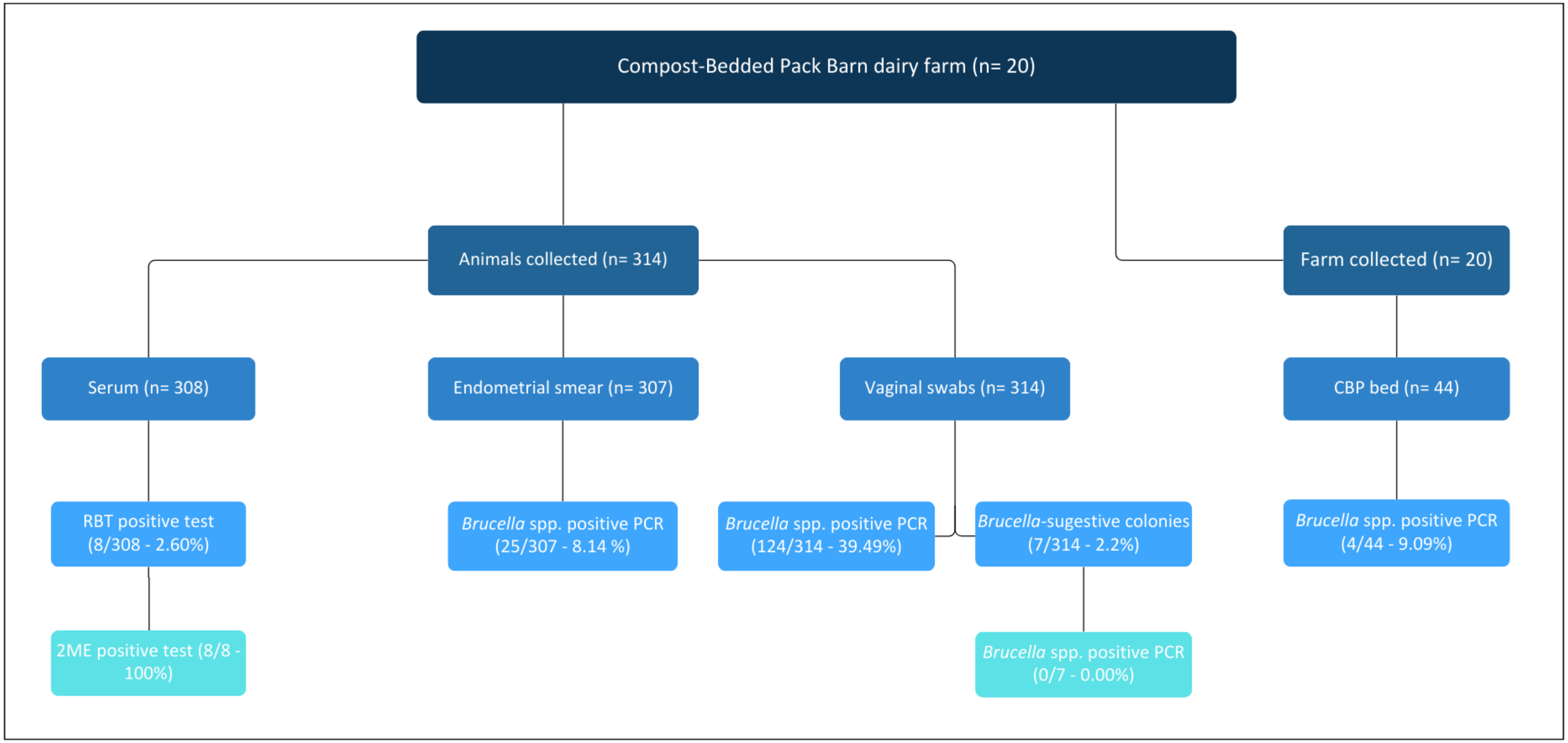


Table 1 – Frequency of anti-smooth *Brucella* antibodies (serum) and of *Brucella*-positive (bed, vaginal swabs and endometrial smear) in samples from dairy cows and from bed collected in properties with compost-bedded pack barn system.

Property (total n animals) *	CBP bed (n= 44)		Vaginal swabs (n = 314)				PCR endometrial smear (n = 307)		Serological tests (n = 308)			
	Brucella-PCR		Brucella-PCR		Brucella-isolation		Brucella-PCR		RBT		2ME	
	n	positive	n	Positive	n	positive	n	Positive	n	positive	n	positive
A (100)	2	0 (0.00%)	17	0 (0.00%)	17	0 (0.00%)	17	5 (29.41%)	16	0 (0.00%)	0	0 (0.00%)
B* (180)	2	0 (0.00%)	19	0 (0.00%)	19	0 (0.00%)	19	0 (0.00%)	19	0 (0.00%)	0	0 (0.00%)
C (500)	2	0 (0.00%)	20	13 (65.00%)	20	0 (0.00%)	20	4 (20.00%)	19	0 (0.00%)	0	0 (0.00%)
D (320)	3	0 (0.00%)	20	20 (100.00%)	20	0 (0.00%)	20	9 (45.00%)	20	0 (0.00%)	0	0 (0.00%)
E (110)	2	1 (50.00%)	15	11 (73.33%)	15	0 (0.00%)	15	4 (26.67%)	15	0 (0.00%)	0	0 (0.00%)
F (95)	2	0 (0.00%)	08	0 (0.00%)	8	0 (0.00%)	8	0 (0.0%)	07	1 (14.29%)	1	1 (100.00%)
G (330)	2	0 (0.00%)	18	2 (11.11%)	18	0 (0.00%)	17	0 (0.00%)	18	1 (5.55%)	1	1 (100.00%)
H (198)	2	1 (50.00%)	18	1 (5.56%)	18	0 (0.00%)	17	0 (0.00%)	18	0 (0.00%)	0	0 (0.00%)
I (220)	3	1 (33.33%)	20	0 (0.00%)	20	0 (0.00%)	20	1 (5.00%)	19	5 (26.32%)	5	5 (100.00%)
J (319)	2	0 (0.00%)	16	0 (0.00%)	16	0 (0.00%)	15	0 (0.00%)	16	0 (0.00%)	0	0 (0.00%)
K (350)	3	1 (50.00%)	8	3 (37.50%)	8	0 (0.00%)	8	0 (0.00%)	8	1 (12.50%)	1	1 (100.00%)
L (140)	2	0 (0.00%)	18	15 (83.33%)	18	0 (0.00%)	18	0 (0.00%)	18	0 (0.00%)	0	0 (0.00%)
M (170)	2	0 (0.00%)	16	11 (68.75%)	16	0 (0.00%)	16	0 (0.00%)	16	0 (0.00%)	0	0 (0.00%)
N (53)	2	0 (0.00%)	6	4 (66.67%)	6	0 (0.00%)	06	0 (0.00%)	6	0 (0.00%)	0	0 (0.00%)
O (295)	2	0 (0.00%)	17	0 (0.00%)	17	0 (0.00%)	14	0 (0.00%)	17	0 (0.00%)	0	0 (0.00%)
P (85)	2	0 (0.00%)	12	6 (50.00%)	12	0 (0.00%)	12	0 (0.00%)	12	0 (0.00%)	0	0 (0.00%)
Q (119)	2	0 (0.00%)	7	4 (57.14%)	7	0 (0.00%)	7	0 (0.00%)	6	0 (0.00%)	0	0 (0.00%)
R (273)	3	0 (0.00%)	19	14 (73.68%)	19	0 (0.00%)	19	0 (0.00%)	19	0 (0.00%)	0	0 (0.00%)
S (370)	2	0 (0.00%)	20	9 (45.00%)	20	0 (0.00%)	19	0 (0.00%)	19	0 (0.00%)	0	0 (0.00%)
T (215)	2	0 (0.00%)	20	11 (55.00%)	20	0 (0.00%)	20	2 (10.00%)	20	0 (0.00%)	0	0 (0.00%)
Total	**44**	**4 (9.09%)**	**314**	**124 (39.49%)**	**314**	**0 (0.00%)**	**307**	**25 (8.14%)**	**308**	**8 (2.60%)**	**8**	**8 (100%)**

*Only sample from the state of Goiás, Brazil. The remaining samples are from the state of Minas Gerais, Brazil.

In the present study, 22 *Brucella*-suggestive colonies were isolated from seven vaginal swabs samples [7/314 (2.3%)] (Alton et al. 1988); however, none of these colonies was positive in the genus-specific PCR for *Brucella* spp.

Using the same genus-specific PCR, a total of 4 CBP bed samples [4/44 (47.06%)]; from four different properties [4/20 (20%)] exhibited positive results for *Brucella* spp. Furthermore, 124 samples of vaginal swab [124/314 (39.49%)], from fourteen different properties [14/20 (70%)], and 25 samples of endometrial smear [25/307 (8.14%)], from five different properties [5/20 (25%)], were also positive for *Brucella* spp. (Table 1 and Figure 2). Anti-smooth *Brucella* antibodies were detected in eight serum samples [8/308 (2.6%)], from four properties [4/20 (20%)], considering the RBT. The reactive samples in the RBT were also tested in confirmatory test (2ME), being all positive [8/8 (100%)].

A total of 17 [17/20 (85%)] properties exhibited at least one animal with positive result in at least one test, and in seven [7/17 (41.2%)] of theses proprieties at least one animal was positive in at least two tests performed. The results demonstrated an average of 47.5% of cows in the herds with at least one positive result in the applied tests. Furthermore, eight [8/17 (47.06%)] of the properties showed positive results in at least two tests. Additionality, all the herds that showed positive results in the *Brucella*-PCR from the bed had also at least one cow positive in either serology or in the PCR from vaginal swabs / endometrial smears (Table 1 and Figure 3).

Figure 2 – Agarose 1% gel showing a representative PCR amplification for *bcsp31* gene (*Brucella* genus-specific) stained with ethidium bromide (0.5 mg / mL). Lanes L – 1Kb plus DNA Ladder molecular weight (Ludwig Biotecnologia Ltda, Brazil). Lanes 1, 2, 3, 4, 5 and 6 – tested samples; NC – negative control; PC – positive control *B. abortus* 2308 strain.

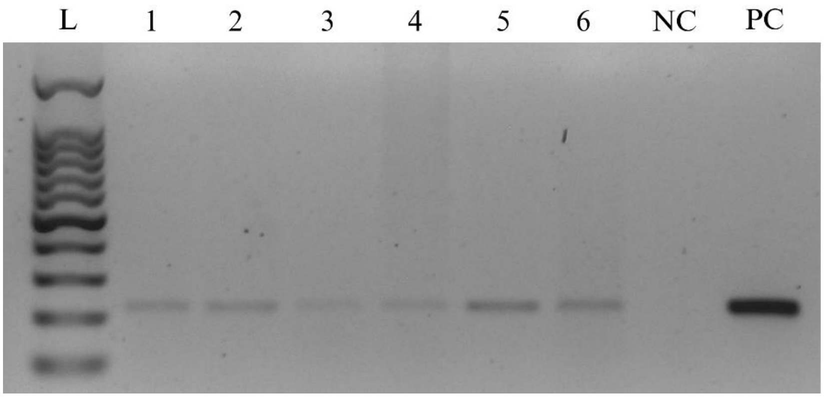
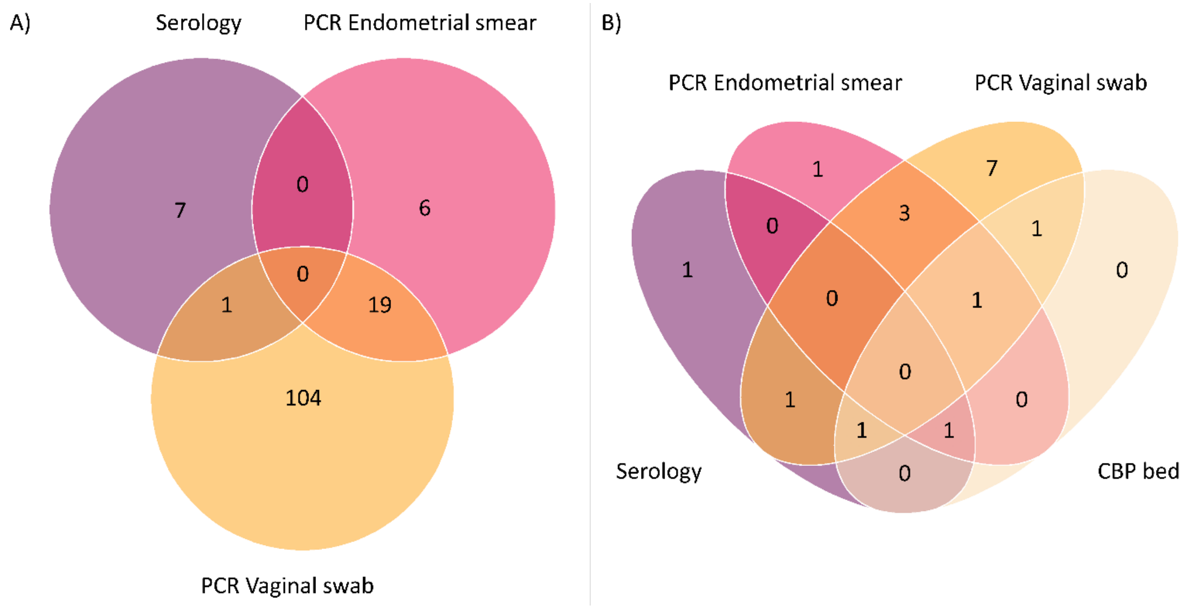


Figure 3 – Venn diagram describing positive results per animal (A) and per property (B). (A) There were ten positive animals in PCR of the endometrial smear and the vaginal swab and also one positive animal in the serology and vaginal swab. (B) Seven properties had positive animals in two test applied and eight properties had positive results in at least two tests.



4. DISCUSSION

The increased comfort and well-being provided by CBP have led to more facilities and animals housed in this system worldwide, including pre-calving animals, which raises the risk of disease transmission, particularly reproductive pathogens. One of the most significant reproductive diseases, bovine brucellosis, caused by *B. abortus*, spreads mainly through contact with infected fetal membranes or contaminated food and water, and the presence of *Brucella*-positive animals in CBP could enhance disease transmission. Therefore, this study is pioneering about the investigate of *Brucella* spp. from animal samples housed in CBP, revealing positive serological and molecular test results in both bed and cows.

Our finding of the presence of *Brucella* spp. in fluids of cows housed in CBP and in the litter of the system is epidemiologically important because demonstrated that the bed could be a potential source of infection to other animals. Indeed, all properties that exhibited *Brucella*-positive results in the PCR from bed (4/20) showed also positive results in other methods, this findings combined with the long viability of this pathogen in soils with shadow and humidity

(Wray 1975), may suggest that CBP can aggravate problems of bacterial infections, such as brucellosis within the system. This hypothesis becomes even more plausible considering that in all assessed properties the bed of the maternity is disturbed together with the other pens, which could further contribute to the dispersion of the agent throughout the system.

The high prevalence of brucellosis in cows housed in assessed CBP (17/20) revealed by our results (*Brucella* spp. DNA or reactive results in serological tests) reinforce the possible relevance of the CBP in the transmission of the disease. In fact, the last survey performed in Minas Gerais, where most of the assessed properties were localized, demonstrated a prevalence of positive herds of 3.59% (De Oliveira et al. 2016), which is much lower than that found in the present study. Therefore, the screening of new animals that will be introduced in the herd, combined with techniques such as vaccination, periodic monitoring and diagnosis of housed animals, is fundamental to prevent and control brucellosis in CBP as well as in other husbandry systems (Zhang et al. 2018; Dadar et al. 2021). Furthermore, it is important to mention that the strategy used, with the use of direct and indirect tests, also favors diagnostic accuracy (R. S. Andrade et al. 2024) and that 40% of the properties (8/20) displayed positive results in at least two different techniques, which all together corroborates the presence of brucellosis in these farms. Discrepancies between the results of the direct and indirect tests used may be due to the study design, as the reliability of serological tests in cows during the transition period may be negatively influenced by low levels of immunoglobulin in the blood, since there is recruitment and high excretion of these cells in the milk during the postpartum period (Biancifiori et al. 1996; Sutherland 1980; Puppel et al. 2019).

Likewise, the lower diagnostic sensitivity of culture of *Brucella* spp. compared to PCR could also explain the negative results in the isolation performed from vaginal swab samples. The number of *Brucella* viable cells in the vagina could also make bacterial cultivation difficult, although allow the detection of DNA by PCR (Keid et al. 2007). Indeed, infected cows can persistently eliminate great quantities of *Brucella* spp. in vaginal secretions for up to four weeks postpartum (Cordes and Carter 1979) and the PCR is characterized by speed and high sensibility and specificity method to detect the DNA pathogen (Dağ et al. 2012; Çiftci et al. 2017). These findings agree with the result of other study that also observed greater sensitivity of PCR for detecting *Brucella* spp. than bacterial isolation, in samples of aborted bovine fetus, milk and serum (Çiftci et al. 2017).

Additionally, our results also demonstrated the ability of PCR to detect the presence of *Brucella* DNA in vaginal swab and endometrial smear samples, indicating that these tests could be used to identify infected females in transition period. This work, however, has limitations such as the sample size (n = 20) and its representative (herds mainly from Minas Gerais), which hinders the generalization of the results found.

5. CONCLUSION

This study demonstrated the presence of *Brucella* spp. in samples of dairy cows and in transition period housed in CBP and in the bed of the system, suggesting the bed of CBP may act as source of the pathogen.

6. REFERENCES

Ahn, Mok Ryeon, Shigenori Kumazawa, Yumiko Usui, Jun Nakamura, Mitsuo Matsuka, Fang Zhu, and Tsutomu Nakayama. 2007. “Antioxidant Activity and Constituents of Propolis Collected in Various Areas of China.” *Food Chemistry* 101 (4): 1383–92. https://doi.org/10.1016/j.foodchem.2006.03.045.

Alton, Godfrey Greenset, Lois M Jones, R D Angus, and J M Verger. 1988. *Techniques for the Brucellosis Laboratory.*

Amarante, Jarbas F., Márcia F. Ribeiro, Mateus M. Costa, Fredson G. Menezes, Tania M.S. Silva, Talita A.B. Amarante, Adriana Gradela, and Liliane M.D. Moura. 2019. “Chemical Composition and Antimicrobial Activity of Two Extract of Propolis against Isolates of Staphylococcus Spp. and Multiresistant Bacterials.” *Brazilian Journal of Veterinary Research* 39 (9): 734–43. https://doi.org/10.1590/1678-5150-PVB-6128.

Andrade, Rafaella Resende, Ilda de Fátima Ferreira Tinôco, Flávio Alves Damasceno, Carlos Eduardo Alves Oliveira, Mariana Silva Concha, Ozana de Fátima Zacaroni, Gianluca Bambi, and Matteo Barbari. 2024. “Understanding Compost-Bedded Pack Barn Systems in Regions with a Tropical Climate: A Review of the Current State of the Art.” *Animals* 14 (12). https://doi.org/10.3390/ani14121755.

Andrade, Rafaella Silva, Marina Martins de Oliveira, Júlio Sílvio de Sousa Bueno Filho, Fernando Ferreira, Jacques Godfroid, Andrey Pereira Lage, and Elaine Maria Seles Dorneles. 2024. “Accuracy of Serological Tests for Bovine Brucellosis: A Systematic Review and Meta-Analysis.” *Preventive Veterinary Medicine* 222 (May 2023): 106079. https://doi.org/10.1016/j.prevetmed.2023.106079.

Armstrong, Derek. 2019. “Control of Contagious Mastitis Contents.” *Agriculture and Horticulture Development Board (AHDB)*, 28.

Awandkar, Sudhakar P., Mahesh B. Kulkarni, and Narendra V. Khode. 2022. “Bacteria from Bovine Clinical Mastitis Showed Multiple Drug Resistance.” *Veterinary Research Communications* 46 (1): 147–58. https://doi.org/10.1007/s11259-021-09838-8.

Azizoglu, Reha Onur, Roberta Lyman, and Kevin L. Anderson. 2013. “Bovine Staphylococcus Aureus: Dose Response to Iodine and Chlorhexidine and Effect of Iodine Challenge on Antibiotic Susceptibility.” *Journal of Dairy Science* 96 (2): 993–99. https://doi.org/10.3168/jds.2012-5857.

Bacic, Goran. 2016. “Intramammary Propolis Formulation for Subclinical Mastitis Prevention and Treatment in Dairy Cows.” *Journal of Dairy, Veterinary & Animal Research* 3 (5): 15406. https://doi.org/10.15406/jdvar.2016.03.00091.

Bacic, Goran, Nino Macesic, Lada Radin, Jasna Aladrovic, Kresimir Matanovic, Tomislav Masek, Diana Brozic, et al. 2016. “Intramammary Propolis Formulation for Subclinical Mastitis Prevention and Treatment in Dairy Cows.” *Journal of Dairy, Veterinary & Animal Research* 3 (5): 15406. https://doi.org/10.15406/jdvar.2016.03.00091.

Baily, G. G., J. B. Krahn, B. S. Drasar, and N. G. Stoker. 1992. “Detection of Brucella Melitensis and Brucella Abortus by DNA Amplification.” *Journal of Tropical Medicine and Hygiene* 95:271–75.

Barlow, John. 2011. “Mastitis Therapy and Antimicrobial Susceptibility: A Multispecies Review with a Focus on Antibiotic Treatment of Mastitis in Dairy Cattle.” *Journal of Mammary Gland Biology and Neoplasia* 16 (4): 383–407. https://doi.org/10.1007/s10911-011-9235-z.

Behiry, Ayman El, Gerd Schlenker, Istvan Szabo, and Uwe Roesler. 2012. “In Vitro Susceptibility of Staphylococcus Aureus Strains Isolated from Cows with Subclinical Mastitis to Different Antimicrobial Agents.” *Journal of Veterinary Science* 13 (2): 153–61. https://doi.org/10.4142/jvs.2012.13.2.153.

Bewley, J. M., L. M. Robertson, and E. A. Eckelkamp. 2017. “A 100-Year Review: Lactating Dairy Cattle Housing Management.” *Journal of Dairy Science* 100 (12): 10418–31. https://doi.org/10.3168/jds.2017-13251.

Biancifiori, F, D. Nannini, A. Di Matteo, and P. Belfiore. 1996. “Assessment of an Indirect ELISA in Milk for the Diagnosis of Ovine Brucellosis.” *Comparative Immunology, Microbiology and Infectious Diseases* 19 (1): 17–24. https://doi.org/10.1016/0147-9571(95)00024-0.

Blosser, T. H. 1979. “Economic Losses from and the National Research Program on Mastitis in the United States.” *Journal of Dairy Science* 62 (1): 119–27. https://doi.org/10.3168/jds.S0022-0302(79)83213-0.

Bogni, Cristina, Liliana Odierno, Claudia Raspanti, José Giraudo, Alejandro Larriestra, Elina Reinoso, Mirta Lasagno, et al. 2011. “War against Mastitis: Current Concepts on Controlling Bovine Mastitis Pathogens.” *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances.*, 483–494. http://www.formatex.info/microbiology3/book/483-494.pdf.

Bouchelaghem, Sarra, Sourav Das, Romen Singh Naorem, Lilla Czuni, Gábor Papp, and Marianna Kocsis. 2022. “Evaluation of Total Phenolic and Flavonoid Contents, Antibacterial and Antibiofilm Activities of Hungarian Propolis Ethanolic Extract against

Staphylococcus Aureus." *Molecules* 27 (2). https://doi.org/10.3390/molecules27020574.

Bradley, A. J. 2002. "Bovine Mastitis: An Evolving Disease." *Veterinary Journal* 164 (2): 116–28. https://doi.org/10.1053/tvjl.2002.0724.

Caro-Hernández, Paola Andrea, Juan Camilo Orozco-Mera, Olga Lucia Castaño-Henao, and Mauricio Alberto Quimbaya-Gómez. 2022. "Evaluating Bacterial Resistance to Antimicrobials in Isolated Bacteria from Food Contact Surfaces." *Entramado* 18 (1): 1–14. https://doi.org/10.18041/1900-3803/entramado.1.7331.

Catry, Boudewijn. 2017. "Antimicrobial Policies in Animals and Human Health." *Archives of Public Health* 75 (1): 1–5. https://doi.org/10.1186/s13690-017-0231-7.

Cheng, Wei Nee, and Sung Gu Han. 2020. "Bovine Mastitis: Risk Factors, Therapeutic Strategies, and Alternative Treatments — A Review." *Asian-Australasian Journal of Animal Sciences* 33 (11): 1699–1713. https://doi.org/10.5713/ajas.20.0156.

Çiftci, Alper, Tuba İça, Serap Savaşan, Barış Sareyyüpoğlu, Mehmet Akan, and Kadir Serdar Diker. 2017. "Evaluation of PCR Methods for Detection of Brucella Strains from Culture and Tissues." *Tropical Animal Health and Production* 49 (4): 755–63. https://doi.org/10.1007/s11250-017-1256-1.

Clinical and Laboratory Standards Institute (CLSI). 2018. *Performance Standards for Antimicrobial Susceptibility Testing*. *Journal of Services Marketing*. 28th ed. Wayne: Clinical and Laboratory Standards Institute.

CLSI. 2013. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests For*. Wayne: Clinical and Laboratory Standards Institute.

Corbel, M.J., S.S. Elberg, and O. Cosivi. 2006. "Brucellosis in Humans and Animals." *Geneva: World Health Organization Press*, 89 p.

Cordes, D. O., and Margery E. Carter. 1979. "Persistence of Brucella Abortus Infection in Six Herds of Cattle under Brucellosis Eradication." *New Zealand Veterinary Journal* 27 (12): 255–59. https://doi.org/10.1080/00480169.1979.34666.

Corner, L A. 1983. "Three Aspects of Bovine Brucellosis: Epidemiology the Role of Bulls and Vaccines." *New South Wales Veterinary Proceedings,* 19.

Cruz Nizer, Waleska Stephanie da, Madison Elisabeth Adams, Vasily Inkovskiy, Carole Beaulieu, and Joerg Overhage. 2023. "The Secondary Metabolite Hydrogen Cyanide Protects Pseudomonas Aeruginosa against Sodium Hypochlorite-Induced Oxidative Stress." *Frontiers in Microbiology* 14:1–16. https://doi.org/10.3389/fmicb.2023.1294518.

Dadar, Maryam, Ruchi Tiwari, Khan Sharun, and Kuldeep Dhama. 2021. "Importance of Brucellosis Control Programs of Livestock on the Improvement of One Health." *Veterinary Quarterly* 41 (1): 137–51. https://doi.org/10.1080/01652176.2021.1894501.

Dağ, Serpil, Fatih Büyük, Hasan Özen, Özgür Çelebi, Musa Karaman, Doğan Akça, and Mitat Şahin. 2012. "Detection of Brucella Spp. in Vaginal Swab Samples of Aborting Cattle: Comparison of Immunoperoxidase to Bacteriological Culture Technique." *Kafkas Universitesi Veteriner Fakultesi Dergisi* 18 (4): 617–22. https://doi.org/10.9775/kvfd.2012.6019.

Damasceno, Marcilene Daniel, Maysa Serpa Gonçalves, Bruna Henrique Pinto da Silva, Giovanna Botelho Carneiro, Alice Gonçalves Reis, Bruna Reis Pereira, Anna Cecília Trolesi Reis Borges Costa, Elaine Maria Seles Dorneles, and Alessandro de Sá Guimarães. n.d. "Susceptibility Od Mastitis-Causing Pathogens (Escherichia Coli and Staphylococcus Aureus) to Disinfectants Used as Teat Dipping."

Deolindo, Guilherme Luiz, Vitor Luiz Molosse, Amanda Dilda, Lilian Kolling Girardini, Marcelo Vedovatto, Aleksandro Schafer da Silva, and Denise Nunes Araujo. 2021. "Lacaune Ewes with Subclinical Mastitis : Effects of Intramammary Application of Própolis." *Research, Society and Development* 10 (2): 1–12.

Dorneles, Elaine M.S., Mariana D.A.M. Fonseca, Juliana A.P. Abreu, Andrey P. Lage, Maria A.V.P. Brito, Carine R. Pereira, Humberto M. Brandão, Alessandro S. Guimarães, and Marcos B. Heinemann. 2019. "Genetic Diversity and Antimicrobial Resistance in Staphylococcus Aureus and Coagulase-Negative Staphylococcus Isolates from Bovine Mastitis in Minas Gerais, Brazil." *MicrobiologyOpen* 8 (5): 1–7. https://doi.org/10.1002/mbo3.736.

Eberl, Daniela T., Marshall J. Smith, Oliver J. Megram, Megan M. Mayhew, Debra Willoughby, Samuel J. White, and Philippe B. Wilson. 2024. "Innovative Bedding Materials for Compost Bedded Pack Barns: Enhancing Dairy Cow Welfare and Sustainable Dairy Farming." *Environment, Development and Sustainability*, no. 0123456789. https://doi.org/10.1007/s10668-024-05244-7.

El-Guendouz, Soukaïna, Smail Aazza, Badiaa Lyoussi, Vassya Bankova, Milena Popova, Luís Neto, Maria Leonor Faleiro, and Maria Da Graça Miguel. 2018. "Moroccan Propolis: A Natural Antioxidant, Antibacterial, and Antibiofilm against Staphylococcus Aureus with No Induction of Resistance after Continuous Exposure." *Evidence-Based Complementary and Alternative Medicine* 2018. https://doi.org/10.1155/2018/9759240.

El-Sayed, Amr, and Mohamed Kamel. 2021. "Bovine Mastitis Prevention and Control in the Post-Antibiotic Era." *Tropical Animal Health and Production* 53 (2). https://doi.org/10.1007/s11250-021-02680-9.

Emanuelson, Ulf, Kerstin Brügemann, Marija Klopčič, Lorenzo Leso, Wijbrand Ouweltjes, Andreas Zentner, and Isabel Blanco-Penedo. 2022. "Animal Health in Compost-Bedded Pack and Cubicle Dairy Barns in Six European Countries." *Animals* 12 (3): 1–9. https://doi.org/10.3390/ani12030396.

Enger, B. D., L. K. Fox, J. M. Gay, and K. A. Johnson. 2015. "Reduction of Teat Skin Mastitis Pathogen Loads: Differences between Strains, Dips, and Contact Times." *Journal of Dairy Science* 98 (2): 1354–61. https://doi.org/10.3168/jds.2014-8622.

Fiordalisi, Samira A.L., Luciana A. Honorato, Márcia R. Loiko, César A.M. Avancini, Maria B.R. Veleirinho, Luiz C.P.Machado Filho, and Shirley Kuhnen. 2016. "The Effects of Brazilian Propolis on Etiological Agents of Mastitis and the Viability of Bovine Mammary Gland Explants." *Journal of Dairy Science* 99 (3): 2308–18. https://doi.org/10.3168/jds.2015-9777.

Fitzpatrick, Sarah Rose, Mary Garvey, Kieran Jordan, Jim Flynn, Bernadette O'Brien, and David Gleeson. 2019. "Screening Commercial Teat Disinfectants against Bacteria Isolated from Bovine Milk Using Disk Diffusion." *Veterinary World* 12 (5): 629–37. https://doi.org/10.14202/vetworld.2019.629-637.

Gonçalves, J. L., C. Kamphuis, C. M.M.R. Martins, J. R. Barreiro, T. Tomazi, A. H. Gameiro, H. Hogeveen, and M. V. dos Santos. 2018. "Bovine Subclinical Mastitis Reduces Milk Yield and Economic Return." *Livestock Science* 210 (January): 25–32. https://doi.org/10.1016/j.livsci.2018.01.016.

Goulart, Débora Brito, and Melha Mellata. 2022. "Escherichia Coli Mastitis in Dairy Cattle: Etiology, Diagnosis, and Treatment Challenges." *Frontiers in Microbiology* 13:1–15. https://doi.org/10.3389/fmicb.2022.928346.

Groot, Anton C. De. 2013. "Propolis: A Review of Properties, Applications, Chemical Composition, Contact Allergy, and Other Adverse Effects." *Dermatitis* 24 (6): 263–82. https://doi.org/10.1097/DER.0000000000000011.

Guimarães, Juliana L.B., Maria A.V.P. Brito, Carla C. Lange, Márcio R. Silva, João B. Ribeiro, Letícia C. Mendonça, Juliana F.M. Mendonça, and Guilherme N. Souza. 2017. "Estimate of the Economic Impact of Mastitis: A Case Study in a Holstein Dairy Herd under Tropical Conditions." *Preventive Veterinary Medicine* 142:46–50. https://doi.org/10.1016/j.prevetmed.2017.04.011.

Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. 2007. "Economic Effects of Bovine Mastitis and Mastitis Management: A Review." *Veterinary Quarterly* 29 (1): 18–31. https://doi.org/10.1080/01652176.2007.9695224.

Hegazi, Ahmed, Amr M Abdou, and Fyrouz Abd Allah. 2014. "Antimicrobial Activity of Propolis on the Bacterial Causes of Mastitis."

Life Science Journal 11 (5): 572–76.

Heikkilä, A. M., E. Liski, S. Pyörälä, and S. Taponen. 2018. “Pathogen-Specific Production Losses in Bovine Mastitis.” *Journal of Dairy Science* 101 (10): 9493–9504. https://doi.org/10.3168/jds.2018-14824.

Idriss, S.H E., V. Foltys, V. Tancin, K. Kirchnerova, D. Tancinova, and K. Zaujec. 2014. “Mastitis Pathogens and Their Resistance against Antimicrobial Agents in Dairy Cows in Nitra, Slovakia.” *Slovak J. Anim. Sci.* 47 (1): 33–38.

Janzen, J. J. 1970. “Economic Losses Resulting from Mastitis. A Review.” *Journal of Dairy Science* 53 (9): 1151–60. https://doi.org/10.3168/jds.S0022-0302(70)86361-5.

Kampf, Günter. 2018. “Biocidal Agents Used for Disinfection Can Enhance Antibiotic Resistance in Gram-Negative Species.” *Antibiotics* 7 (4). https://doi.org/10.3390/antibiotics7040110.

Keid, L. B., R. M. Soares, S. A. Vasconcellos, D. P. Chiebao, V. R. Salgado, J. Megid, and L. J. Richtzenhain. 2007. “A Polymerase Chain Reaction for Detection of Brucella Canis in Vaginal Swabs of Naturally Infected Bitches.” *Theriogenology* 68 (9): 1260–70. https://doi.org/10.1016/j.theriogenology.2007.08.021.

Kibebew, Kinfe. 2017. “Bovine Mastitis: A Review of Causes and Epidemiological Point of View.” *Journal of Biology, Agriculture and Healthcare* 7 (2): 1–14.

Kim, Dae Ok, Seung Weon Jeong, and Chang Y. Lee. 2003. “Antioxidant Capacity of Phenolic Phytochemicals from Various Cultivars of Plums.” *Food Chemistry* 81 (3): 321–26. https://doi.org/10.1016/S0308-8146(02)00423-5.

Kiros, Ashenafi, Hagos Asgedom, and Reta Duguma. 2016. “A Review on Bovine Brucellosis: Epidemiology, Diagnosis and Control Options.” *ARC Journal of Animal and Veterinary Sciences* 2 (3). https://doi.org/10.20431/2455-2518.0203002.

Klhar, Gabriela Thais, José Victor Isola, Cintia Saydelles Da Rosa, Diciane Zeni Giehl, Anelise Afonso Martins, Monica Elisa Bartmer, and Luciane Rumpel Segabinazzi. 2019. “Antimicrobial Activity of the Ethanolic Extract of Propolis against Bacteria That Cause Mastitis in Cattle.” *Biotemas* 32 (1): 1–10. https://doi.org/10.5007/2175-7925.2019v32n1p1.

Köhler, A. T., A. C. Rodloff, M. Labahn, M. Reinhardt, U. Truyen, and S. Speck. 2018. “Efficacy of Sodium Hypochlorite against Multidrug-Resistant Gram-Negative Bacteria.” *Journal of Hospital Infection* 100 (3): 40–46. https://doi.org/10.1016/j.jhin.2018.07.017.

Langsrud, Solveig, Maan Singh Sidhu, Even Heir, and Askild L. Holck. 2003. “Bacterial Disinfectant Resistance - a Challenge for the Food Industry.” *International Biodeterioration and Biodegradation* 51 (4): 283–90. https://doi.org/10.1016/S0964-8305(03)00039-8.

Leso, L., M. Barbari, M. A. Lopes, F. A. Damasceno, P. Galama, J. L. Taraba, and A. Kuipers. 2020. “Invited Review: Compost-Bedded Pack Barns for Dairy Cows.” *Journal of Dairy Science* 103 (2): 1072–99. https://doi.org/10.3168/jds.2019-16864.

Machado, Gabriela Tasso Pinheiro, Maria Beatriz Veleirinho, Letícia Mazzarino, Luiz Carlos Pinheiro Machado Filho, Marcelo Maraschin, Ronaldo Luis Aoki Cerri, and Shirley Kuhnen. 2019. “Development of Propolis Nanoparticles for the Treatment of Bovine Mastitis: In Vitro Studies on Antimicrobial and Cytotoxic Activities.” *Canadian Journal of Animal Science* 99 (4): 713–23. https://doi.org/10.1139/cjas-2018-0173.

Maillard, Jean Yves, and Michael Pascoe. 2024. “Disinfectants and Antiseptics: Mechanisms of Action and Resistance.” *Nature Reviews Microbiology* 22 (1): 4–17. https://doi.org/10.1038/s41579-023-00958-3.

Maity, Sudipa, and Kiran Ambatipudi. 2021. “Mammary Microbial Dysbiosis Leads to the Zoonosis of Bovine Mastitis: A One-Health Perspective.” *FEMS Microbiology Ecology* 97 (1): 1–17. https://doi.org/10.1093/femsec/fiaa241.

Manav, S., M. Yilmaz, H. Baytekin, K. Çelik, and A. Çağli. 2020. “The Use of Propolis as an Antimicrobial in Livestock – an Overview.” *Agricultural Science and Technology* 12 (3): 205–9. https://doi.org/10.15547/ast.2020.03.032.

Mašek, Tomislav, Nataša Perin, Livio Racané, Maja Cindrić, Hana Čipčić Paljetak, Mihaela Perić, Mario Matijašić, et al. 2018. “Chemical Composition, Antioxidant and Antibacterial Activity of Different Extracts of Poplar Type Propolis.” *Croatica Chemica Acta* 91 (1): 81–88. https://doi.org/10.5562/cca3298.

Megid, Jane, LA Mathias, and CA Robles. 2010. “Clinical Manifestations of Brucellosis in Domestic Animals and Humans.” *Open Veterinary Science* 4:119–26. http://benthamscience.com/open/tovsj/articles/V004/SI0045TOVSJ/119TOVSJ.pdf.

Miller, Samuel I. 2016. “Antibiotic Resistance and Regulation of the Gram-Negative Bacterial Outer Membrane Barrier by Host Innate Immune Molecules.” *MBio* 7 (5): 1–3. https://doi.org/10.1128/mBio.01541-16.

Molineri, Ana Inés, Cecilia Camussone, M. Virginia Zbrun, Guillermo Suárez Archilla, Mariana Cristiani, Verónica Neder, Luis Calvinho, and Marcelo Signorini. 2021. “Antimicrobial Resistance of Staphylococcus Aureus Isolated from Bovine Mastitis: Systematic Review and Meta-Analysis.” *Preventive Veterinary Medicine* 188 (December 2020). https://doi.org/10.1016/j.prevetmed.2021.105261.

Mulligan, F. J., and M. L. Doherty. 2008. “Production Diseases of the Transition Cow.” *Veterinary Journal* 176 (1): 3–9. https://doi.org/10.1016/j.tvjl.2007.12.018.

Nacional Mastitis Council (NMC). 2014. “Summary Of Peer-Reviewed Publications On Efficacy Of Premilking And Postmilking Teat Disinfectants Published Since 1980.”

———. 2016. “Recommended Mastitis Control Program.”

Nam, Ji-Hyun, and Jung Sik Yoo. 2024. “Sublethal Sodium Hypochlorite Exposure: Impact on Resistance-Nodulation-Cell Division Efflux Pump Overexpression and Cross-Resistance to Imipenem.” *Antibiotics* 13 (9): 828. https://doi.org/10.3390/antibiotics13090828.

Nandre, Vinod S., Aditi V. Bagade, Deepak M. Kasote, Jisun H.J. Lee, Kisan M. Kodam, Mohan V. Kulkarni, and Aijaz Ahmad. 2021. “Antibacterial Activity of Indian Propolis and Its Lead Compounds against Multi-Drug Resistant Clinical Isolates.” *Journal of Herbal Medicine* 29 (May): 100479. https://doi.org/10.1016/j.hermed.2021.100479.

Naranjo-Lucena, Amalia, and Rosemarie Slowey. 2023. “Invited Review: Antimicrobial Resistance in Bovine Mastitis Pathogens: A Review of Genetic Determinants and Prevalence of Resistance in European Countries.” *Journal of Dairy Science* 106 (1): 1–23. https://doi.org/10.3168/jds.2022-22267.

Nascimento, Gabriel Michelutti Do, Marita Vedovelli Cardozo, Mylena Karoline Valmorbida, Natália Pereira, José Carlos Barbosa, Flávio Rubens Favaron, and Fernando Antônio De Ávila. 2022. “Propolis in the Control of Bacterial Bovine Mastitis: A Tool for the Production of Organic Milk.” *Semina:Ciencias Agrarias* 43 (2): 869–82. https://doi.org/10.5433/1679-0359.2022v43n2p869.

Niculae, Mihaela, Laura Stan, Emoke Pall, Anamaria Ioana Paştiu, Iulia Maria Balaci, Sevastiţa Muste, and Marina Spînu. 2015. “In Vitro Synergistic Antimicrobial Activity of Romanian Propolis and Antibiotics against Escherichia Coli Isolated from Bovine Mastitis.” *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 43 (2): 327–34. https://doi.org/10.15835/nbha43210074.

NMC. 2005. “Summary of Peer-Reviewed Publications on Egficacy of Premilking and Postmilking Teat Disinfectants Published Since 1980” 1980 (2011): 26–39.

Nobrega, Diego B., Jeroen De Buck, S. Ali Naqvi, Gang Liu, Sohail Naushad, Vineet Saini, and Herman W. Barkema. 2017. “Comparison of Treatment Records and Inventory of Empty Drug Containers to Quantify Antimicrobial Usage in Dairy Herds." *Journal of Dairy Science* 100 (12): 9736–45. https://doi.org/10.3168/jds.2017-13116.

Oliveira, Luciana Faria De, Elaine Maria Seles Dorneles, Ana Lourdes Arrais De Alencar Mota, Vitor Salvador Picão Gonçalves, José Soares Ferreira Neto, Fernando Ferreira, Ricardo Augusto Dias, et al. 2016. "Seroprevalence and Risk Factors for Bovine Brucellosis in Minas Gerais State, Brazil." *Semina:Ciencias Agrarias* 37 (5): 3449–66. https://doi.org/10.5433/1679-0359.2016v37n5Supl2p3449.

Oliver, S. P., M. J. Lewis, T. L. Ingle, B. E. Gillespie, and K. R. Matthews. 1993. "Prevention of Bovine Mastitis by a Premilking Teat Disinfectant Containing Chlorous Acid and Chlorine Dioxide." *Journal of Dairy Science* 76 (1): 287–92. https://doi.org/10.3168/jds.S0022-0302(93)77348-8.

Ózsvári, László, and Dorottya Ivanyos. 2022. "The Use of Teat Disinfectants and Milking Machine Cleaning Products in Commercial Holstein-Friesian Farms." *Frontiers in Veterinary Science* 9 (1): 1–15. https://doi.org/10.3389/fvets.2022.956843.

Paiano, R. B., L. Z. Moreno, V. T.M. Gomes, B. M. Parra, M. R. Barbosa, M. I.Z. Sato, J. Bonilla, G. Pugliesi, P. S. Baruselli, and A. M. Moreno. 2022. "Assessment of the Main Pathogens Associated with Clinical and Subclinical Endometritis in Cows by Culture and MALDI-TOF Mass Spectrometry Identification." *Journal of Dairy Science* 105 (4): 3367–76. https://doi.org/10.3168/jds.2021-20642.

Pal, Mahendra, Fikru Gizaw, Gelane Fekadu, Gezahagn Alemayehu, and Venkataramana Kandi. 2017. "Public Health and Economic Importance of Bovine Brucellosis: An Overview." *American Journal of Epidemiology and Infectious Disease* 5 (2): 27–34. https://doi.org/10.12691/ajeid-5-2-2.

Pasca, Claudia, Liviu Alexandru Marghitas, Daniel Severus Dezmirean, Ioana Adriana Matei, Victorita Bonta, Ioan Pasca, Flore Chirila, Adrian Cîmpean, and Nicodim Iosif Fit. 2020. "Efficacy of Natural Formulations in Bovine Mastitis Pathology: Alternative Solution to Antibiotic Treatment." *Journal of Veterinary Research* 64 (4): 523–29. https://doi.org/10.2478/jvetres-2020-0067.

Pascottini, O. Bogado, S. J. Van Schyndel, J. F.W. Spricigo, J. Rousseau, J. S. Weese, and S. J. LeBlanc. 2020. "Dynamics of Uterine Microbiota in Postpartum Dairy Cows with Clinical or Subclinical Endometritis." *Scientific Reports* 10 (1): 1–11. https://doi.org/10.1038/s41598-020-69317-z.

Pérez, Verónica Karen Castro, Geraldo Márcio da Costa, Alessandro Sá Guimarães, Marcos Bryan Heinemann, Andrey Pereira Lage, and Elaine Maria Seles Dorneles. 2020. "Relationship between Virulence Factors and Antimicrobial Resistance in Staphylococcus Aureus from Bovine Mastitis." *Journal of Global Antimicrobial Resistance* 22:792–802. https://doi.org/10.1016/j.jgar.2020.06.010.

PETER, Cristina Mendes, Lariane da Silva Barcelos, Matheus Iuri Frühauf, Yandra Nadalin Botton, João Luiz Zani, and Geferson Fischer. 2021. "Is Propolis a Promising Candidate on Bacterial Bovine Mastitis' Treatment?" *Science and Animal Health* 9 (2): 89–103. https://doi.org/10.15210/sah.v9i2.21598.

Phillips, C J C, and S A Schofield. 1994. "The Effect of Cubicle and Straw Yard Housing on the Behaviour, Production and Hoof Health of Dairy Cows." *Animal Welfare* 3 (1): 37–44. https://doi.org/10.1017/s0962728600016365.

Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. "Rapid Extraction of Bacterial Genomic DNA with Guanidium Thiocyanate." *Letters in Applied Microbiology* 8 (4): 151–56. https://doi.org/10.1111/j.1472-765X.1989.tb00262.x.

Pobiega, Katarzyna, Karolina Kraśniewska, Dorota Derewiaka, and Małgorzata Gniewosz. 2019. "Comparison of the Antimicrobial Activity of Propolis Extracts Obtained by Means of Various Extraction Methods." *Journal of Food Science and Technology* 56 (12): 5386–95. https://doi.org/10.1007/s13197-019-04009-9.

Prestinaci, Francesca, Patrizio Pezzotti, and Annalisa Pantosti. 2015. "Antimicrobial Resistance: A Global Multifaceted Phenomenon." *Pathogens and Global Health* 109 (7): 309–18. https://doi.org/10.1179/2047773215Y.0000000030.

Prieto, Pilar, Manuel Pineda, and Miguel Aguilar. 1999. "Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E." *Analytical Biochemistry* 269 (2): 337–41. https://doi.org/10.1006/abio.1999.4019.

Puppel, Kamila, Marcin Gołębiewski, Grzegorz Grodkowski, Jan Slósarz, Małgorzata Kunowska-Slósarz, Paweł Solarczyk, Monika Łukasiewicz, Marek Balcerak, and Tomasz Przysucha. 2019. "Composition and Factors Affecting Quality of Bovine Colostrum: A Review." *Animals* 9 (12). https://doi.org/10.3390/ani9121070.

Ramos, Juan L., Estrella Duque, María Trinidad Gallegos, Patricia Godoy, María Isabel Ramos-González, Antonia Rojas, Wilson Terán, and Ana Segura. 2002. "Mechanisms of Solvent Tolerance in Gram-Negative Bacteria." *Annual Review of Microbiology* 56:743–68. https://doi.org/10.1146/annurev.micro.56.012302.161038.

Rato, Márcia G., Ricardo Bexiga, Carlos Florindo, Lina M. Cavaco, Cristina L. Vilela, and Ilda Santos-Sanches. 2013. "Antimicrobial Resistance and Molecular Epidemiology of Streptococci from Bovine Mastitis." *Veterinary Microbiology* 161 (3–4): 286–94. https://doi.org/10.1016/j.vetmic.2012.07.043.

Redfern, Emma A., Liam A. Sinclair, and Philip A. Robinson. 2021. "Dairy Cow Health and Management in the Transition Period: The Need to Understand the Human Dimension." *Research in Veterinary Science* 137 (April): 94–101. https://doi.org/10.1016/j.rvsc.2021.04.029.

Ripley, Brian, Bill Venables, Douglas M. Bates, Kurt Hornik, Albrecht Gebhardt, and David Firth. 2025. "MASS: Support Functions and Datasets for Venables and Ripley's MASS." https://doi.org/10.32614/CRAN.package.MASS.

Rodr, Cristina, Carlos Alonso-calleja, Camino Garc, Javier Carballo, and Rosa Capita. 2022. "Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for Twelve Antimicrobials (Biocides and Antibiotics) in Eight Strains of Listeria Monocytogenes." *Biology* 11 (Mic): 46.

Ruegg, Pamela L. 2017. "A 100-Year Review: Mastitis Detection, Management, and Prevention." *Journal of Dairy Science* 100 (12): 10381–97. https://doi.org/10.3168/jds.2017-13023.

Santos, Heidy C. Dos, Dielson S. Vieira, Sandra M. Yamamoto, Mateus M. Costa, Maria C.A. Sá, Eva M.S. Silva, and Tania M.S. Silva. 2019. "Antimicrobial Activity of Propolis Extract Fractions against Staphylococcus Spp. Isolated from Goat Mastitis." *Brazilian Journal of Veterinary Research* 39 (12): 954–60. https://doi.org/10.1590/1678-5150-PVB-5940.

Santos, Laerte M., Maísa S. Fonseca, Ana R. Sokolonski, Kathleen R. Deegan, Roberto P.C. Araújo, Marcelo A. Umsza-Guez, Josiane D.V. Barbosa, Ricardo D. Portela, and Bruna A.S. Machado. 2020. "Propolis: Types, Composition, Biological Activities, and Veterinary Product Patent Prospecting." *Journal of the Science of Food and Agriculture* 100 (4): 1369–82. https://doi.org/10.1002/jsfa.10024.

Santos, Renata Paoli, Fernando Nogueira Souza, Carla Gasparotto Chande Vasconcelos, Adriana Cortez, Dalila Lapinha Silva Oliveira Rosa, Annatachi Botelho Jardim, Adriano França Cunha, Ângela Maria Quintão Lana, Marcos Bryan Heinemann, and Mônica Maria Oliveira Pinho Cerqueira. 2016. "In Vitro Efficacy of Teat Antiseptics against Staphylococcus Aureus Strains Isolated from Bovine Mastitis." *Ciencias Agrarias* 37 (4): 1997–2002. https://doi.org/10.5433/1679-0359.2016v37n4p1997.

Silva, Juliana Rosa da, Glei Dos Anjos De Carvalho Castro, Maysa Serpa Gonçalves, Dircéia Aparecida Da Costa Custódio, Gláucia Frasnelli Mian, and Geraldo Márcio da Costa. 2017. "In Vitro Antimicrobial Susceptibility and Genetic Resistance Determinants of Streptococcus Agalactiae Isolated from Mastitic Cows in Brazilian Dairy Herds." *Semina:Ciencias Agrarias* 38 (4): 2581–94. https://doi.org/10.5433/1679-0359.2017v38n4Supl1p2581.

Simões, Manuel, Richard N. Bennett, and Eduardo A.S. Rosa. 2009. "Understanding Antimicrobial Activities of Phytochemicals against Multidrug Resistant Bacteria and Biofilms." *Natural Product Reports* 26 (6): 746–57. https://doi.org/10.1039/b821648g.

Šuran, Jelena, Jasna Aladrović, Blanka Beer Ljubić, Josipa Vlainić, Marija Mamić, Božo Radić, Goran Bačić, et al. 2020. "The Antioxidant Effect of the Novel Bee-Product Based Intramammary Formulation Apimast® in Dairy Cattle." *Veterinarski Arhiv* 90 (3): 225–33. https://doi.org/10.24099/VET.ARHIV.0865.

Sutherland, S. S. 1980. "Immunology of Bovine Brucellosis." *Veterinary Bulletin* 50 (5): 359–63. http://scioteca.caf.com/bitstream/handle/123456789/1091/RED2017-Eng-8ene.pdf?sequence=12&isAllowed=y%0Ahttp://dx.doi.org/10.1016/j.regsciurbeco.2008.06.005%0Ahttps://www.researchgate.net/publication/305320484_SISTEM_PEMBETUNGAN_TERPUSAT_STRATEGI_MELESTARI.

Tibbs-Cortes, Bienvenido W., Faith M. Rahic-Seggerman, Stephan Schmitz-Esser, Paola M. Boggiatto, Steven Olsen, and Ellie J. Putz. 2024. "Fecal and Vaginal Microbiota of Vaccinated and Non-Vaccinated Pregnant Elk Challenged with Brucella Abortus." *Frontiers in Veterinary Science* 11 (January): 1–16. https://doi.org/10.3389/fvets.2024.1334858.

Tomanić, Dragana, Marko Samardžija, and Zorana Kovačević. 2023. "Alternatives to Antimicrobial Treatment in Bovine Mastitis Therapy: A Review." *Antibiotics* 12 (4). https://doi.org/10.3390/antibiotics12040683.

Tong, Chaoyu, Hong Hu, Gang Chen, Zhengyan Li, Aifeng Li, and Jianye Zhang. 2021. "Chlorine Disinfectants Promote Microbial Resistance in Pseudomonas Sp." *Environmental Research* 199 (May): 111296. https://doi.org/10.1016/j.envres.2021.111296.

Wang, Fei, Hui Liu, Junya Li, Wenwen Zhang, Bin Jiang, and Hongzhuan Xuan. 2021. "Australian Propolis Ethanol Extract Exerts Antibacterial Activity against Methicillin-Resistant Staphylococcus Aureus by Mechanisms of Disrupting Cell Structure, Reversing Resistance, and Resisting Biofilm." *Brazilian Journal of Microbiology* 52 (4): 1651–64. https://doi.org/10.1007/s42770-021-00547-7.

White, D.G., and P.F. McDermott. 2001. "Emergence and Transfer of Antibacterial Resistance." *Journal of Dairy Science* 84:E151–55. https://doi.org/10.3168/jds.s0022-0302(01)70209-3.

Wickham, Hadley. 2016. *Ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Wray, C. 1975. "Survival and Spread of Pathogenic Bacteria of Veterinary Importance within the Environment" 45 (8): 6.

Xiao, Xingning, Li Bai, Sheng Wang, Lisha Liu, Xiaoyun Qu, Jianmin Zhang, Yingping Xiao, et al. 2022. "Chlorine Tolerance and Cross-Resistance to Antibiotics in Poultry-Associated Salmonella Isolates in China." *Frontiers in Microbiology* 12 (February): 1–11. https://doi.org/10.3389/fmicb.2021.833743.

Yan, Linlin. 2021. "Ggvenn: Draw Venn Diagram by 'Ggplot2.'" *R Package Version* 19.

Yanuartono, Alfarisa Nururrozi, Soedarmanto Indarjulianto, Hary Purnamaningsih, and Universitas Gadjah Mada. 2020. "The Benefits of Teat Dipping as Prevention of Mastitis." *Journal of Livestock Science and Production* 4 (1): 231–49. https://doi.org/10.31002/jalspro.v4i1.2796.

Zeinab, Breijyeh, Jubeh Buthaina, and Karaman Rafik. 2023. "Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It." *Molecules* 25 (6): 1–23.

Zhang, Ning, Desheng Huang, Wei Wu, Jing Liu, Feng Liang, Baosen Zhou, and Peng Guan. 2018. "Animal Brucellosis Control or Eradication Programs Worldwide: A Systematic Review of Experiences and Lessons Learned." *Preventive Veterinary Medicine* 160:105–15. https://doi.org/10.1016/j.prevetmed.2018.10.002.

Zigo, František, Milan Vasil', Silvia Ondrašovičová, Jana Výrostková, Jolanta Bujok, and Ewa Pecka-Kielb. 2021. "Maintaining Optimal Mammary Gland Health and Prevention of Mastitis." *Frontiers in Veterinary Science* 8 (February): 1–17. https://doi.org/10.3389/fvets.2021.607311.

Zullkiflee, Nadzirah, Hussein Taha, and Anwar Usman. 2022. "Propolis: Its Role and Efficacy in Human Health and Diseases." *Molecules* 27 (18). https://doi.org/10.3390/molecules27186120.