Universidade de Évora - Instituto de Investigação e Formação Avançada

Programa de Doutoramento em Ciências Veterinárias

Tese de Doutoramento

Staphylococci isolated from caprine and ovine mastitic milk: Virulence Factors and Antimicrobial and Antibiofilm effectiveness of Propolis Extracts

Nara Patrícia Cavalcanti Andrade

Orientador(es) | Maria Cristina Queiroga

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Dedication

To my son Gabriel Cavalcanti Alves and my grandparents

who taught me how to live, Maria Alzira and João Domiciano Cavalcanti.

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List of Abbreviations and Symbols

16S – 16S ribosomal RNA

3D – three dimensional

A – absorbance

AFM – atomic force microscopy

AMP – aromatic and medicinal plants

AMR – antimicrobial resistance

ANOVA –analysis of variance

AST – antimicrobial susceptibility testing

ATCC – American Type Culture Collection

BA – blood agar

Bap – biofilm associated protein

BEH – bridged ethylene hybrid

BHIb – Brain Heart Infusion broth

bp – base pair

BR – Brazil

CAPE – caffeic acid phenolic ester

CE – Ceará

CFU – colony forming unit

CLSI – Clinical and Laboratory Standards Institute

CM – clinical mastitis

CMT – California mastitis test

CNS – coagulase negative *Staphylococcus*

CPS – coagulase positive *Staphylococcus*

df – degrees of freedom

dNTP – deoxyribonucleotide triphosphate

ECC – epicatechin content

eDNA – environmental DNA

EDTA – ethylenediaminetetraacetic acid

EO – essentials oils

ESI – electrospray ionization interface

ε – molar absorbance

FAO – Agriculture Organization of the United Nations

FD – dilution factor

GAE – gallic acid equivalent

GTFs – glycosyltransferases

HSD – honest significant difference

IMI – Intramammary Infection

kV – Kilovolts

Lh – litre per hour

MBC – minimum bactericidal concentration

MCa – MacConkey agar

MCA – multiple correspondence analysis

MDR – multi drug resistant

MG – Minas Gerais

MHA – Muller-Hinton agar

MHB - Mueller-Hinton broth

MIC – minimal inhibitory concentration

MR-CNS –methicillin resistant coagulase-negative staphylococci

MRSA – methicillin resistant *Staphylococcus aureus*

MSCRAMM – microbial surface components recognizing adhesive matrix molecules

MSSA methicillin sensitive *Staphylococcus aureus*

MW – molecular weight

N – North

n.d. – not detectable

NE – Northeast

neg - negative

NET – neutrophil extracellular trap

NMC – National Mastitis Council

NUTs – nomenclature of territorial units

OD – optical density

OIE – World Organisation for Animal Health

PBP – penicillin-binding protein

PCR – Polymerase Chain Reaction

PDA – photo diode array

PIA – polysaccharid intercellular adhesin

PS/A – polysaccharide capsular adhesin

PT – Portugal

QE – quercetin equivalents

QS – quorum sensing

r^2 – R-squared

S – South

SCC – somatic cell count

SCCmec – staphylococcal cassette chromosome mec

SCM – subclinical mastitis

SE – Southeast

SE – staphylococcal enterotoxin

SEM – standard error of the mean

SPE – solid phase extraction

SSR – short sequence repeats

Staph. – *Staphylococcus*

TBE – tris/borate/EDTA buffer

TSBg – tryptone soya broth glucose

TSST – toxic shock syndrome toxin

UPLC-QTOF-MS/MS – ultra-high-pressure liquid chromatography coupled with a hybrid quadrupole orthogonal time-of-flight mass spectrometer

WHO – World Health Organization

Σ – sigma

$\chi 2$ –chi-square

Staphylococci isolated from caprine and ovine mastitic milk: Virulence Factors and Antimicrobial and Antibiofilm effectiveness of Propolis Extracts

Abstract

Small ruminant mastitis is a major problem for milk producers and consumers causing economic losses and public health threats. This disease is mainly caused by *Staphylococcus*. The aim of this work was to study propolis for the control of small ruminant mastitis as an alternative to conventional antimicrobials. A total of 137 *Staphylococcus* belonging to 13 species, recovered from the milk of goats and sheep, were studied. Phenotypic biofilm production and antimicrobial susceptibility were examined. Genes coding for virulence factors were studied: *coa, nuc, bap, icaA, icaD, blaZ, mecA, mecC, tetK* and *tetM*. Propolis is a resinous substance produced by honeybees which has been used as a natural medicine for its antiseptic, antimicrobial, antioxidant, anti-inflammatory, and other immunomodulatory properties. Propolis ethanol extracts (PEE) were prepared using different propolis from both Brazil and Portugal. These PEE were chemically characterised and their antimicrobial and antibiofilm activities were assessed. Results showed an association between biofilm production and mastitis inflammatory response. Beta-lactam resistance was mainly detected and an association between animal species and resistance to some antibiotics was found. All isolates were susceptible to gentamicin and cefazolin. The *nuc* gene was detected in several coagulase negative staphylococci, highlighting its inadequacy for *S. aureus* identification. Regarding the use of propolis for mastitis control, most PEE showed inhibitory activity against all staphylococci isolates. Furthermore, PEE are bactericidal, which is a very important feature of propolis. Moreover, PEE are effective in inhibiting biofilm formation and in destroying the formed biofilm. The presence of individual phenolics enhanced bactericidal activity, whereas triterpenes negatively influenced both antimicrobial and antibiofilm activity. The results of the present study suggest that propolis should be considered for the control of small ruminant mastitis caused by staphylococci. Nevertheless, future studies are needed to identify the individual propolis compounds exhibiting both antimicrobial and antibiofilm activity.

Keywords: mastitis, staphylococci, virulence factors, propolis, antimicrobial, antibiofilm.

Estafilococos isolados de leite mastítico caprino e ovino: Factores de Virulência e eficácia Antimicrobiana e Antibiofilme de Extratos de Própolis

Resumo

A mastite dos pequenos ruminantes é um problema para os produtores e consumidores de leite, causando perdas económicas e ameaças à saúde pública. Esta doença é principalmente causada por *Staphylococcus*. O objetivo deste trabalho foi estudar a própolis para o controlo da mastite dos pequenos ruminantes como alternativa aos antimicrobianos. Estudaram-se 137 *Staphylococcus* de 13 espécies, isolados do leite de cabras e ovelhas. Avaliou-se a produção de biofilme e a suscetibilidade aos antimicrobianos. Analisaram-se alguns genes de virulência: *coa*, *nuc*, *bap*, *icaA*, *icaD*, *blaZ*, *mecA*, *mecC*, *tetK* e *tetM*. A própolis é uma substância resinosa, produzida por abelhas, utilizada como medicamento natural pelas suas propriedades antissépticas, antimicrobianas, antioxidantes, anti-inflamatórias, entre outras. Os extratos etanólicos de própolis (PEE) foram preparados com diferentes própolis do Brasil e de Portugal. Estes PEE foram caraterizados quimicamente e as suas atividades antimicrobiana e antibiofilme avaliadas. Existe uma associação entre a produção de biofilme e a resposta inflamatória à mastite. A resistência aos beta-lactâmicos foi a mais frequente e encontrou-se uma associação entre espécie animal e resistência a alguns antimicrobianos. Todos os isolados foram suscetíveis à gentamicina e à cefazolina. O gene *nuc* foi detetado em estafilococos coagulase-negativos, mostrando-se inadequado para a identificação de *S. aureus*. Quanto ao uso de própolis no controlo da mastite, a maioria dos PEE mostrou atividade inibitória contra todos os estafilococos. Além disso, os PEE são bactericidas, uma importante característica do própolis. Adicionalmente, os PEE inibem eficazmente a formação e destroem o biofilme formado. A presença de determinados fenóis aumenta a atividade bactericida, enquanto que os triterpenos influenciam negativamente as atividades antimicrobiana e antibiofilme. Os resultados obtidos sugerem que a própolis seja usada no controlo da mastite de pequenos ruminantes causada por estafilococos. No entanto, são necessários mais estudos para identificar individualmente os compostos do própolis com atividade antimicrobiana e antibiofilme.

Palavras chave: mastites, estafilococos, fatores de virulência, própolis, antimicrobiano, antibiofilme.

Chapter 1

1. Introduction

Small ruminants breeding is sustainably advantageous for its rusticity, adaptation to the environment, easy husbandry, and reproductive capacity. Goat and sheep milk production is a common activity in several countries, especially in developing countries (Viana et al., 2008; Rahmatalla et al., 2017). This activity is highly related to family farming, with a strong commitment to regional development, whose production is intended for home milk consumption and for manufacturing traditional cheeses (Peixoto et al., 2010a; Schröder et al., 2011). Small ruminants' milk is known for its beneficial and therapeutic effects, suitable for individuals who are allergic to cow's milk and is beneficial for the nutrition and health of young and old people (Ribeiro and Ribeiro, 2010).

In 2016, small ruminant milk production showed a global decline of 4.4% compared to the previous year, following the bovine sector, with a reduction of 4,5% (INE, 2017). The main drawback affecting the milk production is the mammary gland inflammatory process, a disease denominated mastitis, which is common in dairy farms. Besides the reduction in

milk yield and on animal performance, this affection increases mortality rate and consequently economic losses to the dairy producer (Blagitz et al., 2008; Peixoto et al., 2010b). Mastitis negatively affects the composition and physical-chemical milk characteristics, leading to fat and lactose loss and a slightly higher milk-protein content (Gelasakis et al., 2018), impairing cheese manufacturing (Seegers et al., 2003; Halasa et al., 2009). Moreover, mastitis pathogens may produce toxins responsible for food poisoning (Argudín et al., 2010; Vasconcellos and Ito, 2011). Therefore, this disease reflects both in animal health and welfare and in milk and dairy products production, as well as in consumers' safety (Keefe, 2012).

1.1 – Motivation

Mastitis control, both prophylaxis and treatment, is currently mostly dependent on antimicrobial and antiseptics use. However, these exert selection pressure on resistant and multi-resistant bacterial strains (Contreras et al., 2007; Virdis et al., 2010; Martins et al., 2017). Antibiotic resistant bacteria, other than impairing mastitis control, may transfer resistance genes to the indigenous microbiota in the consumer's gut (Lee, 2003), consisting in a big threat to public health.

Some antimicrobials that are critically important for human medicine are currently being used for mastitis control. Several antimicrobial resistance genes were already detected in milk isolates causing sheep (Jamali et al., 2015; Martins et al., 2017) and goats mastitis (Virdis et al., 2010).

Resistant bacteria exist in humans, animals, food and the environment. Antimicrobials use, both in animal and human medicine, was responsible for the increase in resistance, leading to a threat to public health systems in the last 20 years (Queenan et al., 2016; Ferri et al., 2017). Antimicrobial resistance (AMR) is a global problem of complex epidemiology and should be considered in a broad and integrated "One Health" approach (Weese et al., 2013).

Measures to avoid the dissemination of resistance genes and the selection pressure for resistant and multiresistant bacteria, must be implemented. To develop new alternatives to the use of antimicrobials and antiseptics for use in mastitis control is urgent,

to improve milk yield and milk quality without risk to public health. Some natural compounds derived from plants, which have already inhibited bacteria without causing microbial resistance, even after prolonged exposure (Ohno et al., 2003), may be addressed as alternatives.

Propolis is a resinous mass produced by bees *Apis mellifera*. Honeybees collect selectively bioactive resin compounds from different plant parts that they manipulate with their salivary gland secretions forming this glue type mass which they use to close the small openings in the hive to protect it from invaders and providing a favourable environment (Drescher et al., 2019).

Propolis biological properties are related to its chemical composition, which differs in its structure and concentration depending on sources availability for resin harvesting (Toreti et al., 2013). Propolis has been used to treat different pathologies, among others, due to its antibacterial activity (Wojtyczka et al., 2013). Its activity against bacterial biofilms has been reported lately (Wojtyczka et al., 2013; Veloz et al., 2015; Doganli, 2016; De Marco et al., 2017).

1.2 – Objectives and thesis outline

Considering propolis antimicrobial and antibiofilm activities, this work was designed to evaluate the use of propolis for small ruminant mastitis control. With that propose, ten propolis samples (seven from Brazil and three from Portugal) were used to produce propolis ethanol extracts (PEE), which were than analysed for their chemical composition and assayed for their antibacterial and antibiofilm performance against staphylococci isolated from milk samples from small ruminants with mastitis.

Therefore, the specific objectives were:

1 - To identify *Staphylococcus* species isolated from small ruminants' milk samples and investigate the ability to produce biofilm and the antimicrobial susceptibility patterns, both phenotypically and genotypically.

2 – To study propolis composition in order to evaluate the use of propolis ethanol extracts (PEE) for mastitis control purposes.

3 – To evaluate the *in vitro* activity of PEE against staphylococci isolated from the milk of sheep and goats with mastitis.

4 – To investigate the *in vitro* antibiofilm activity of PEE against biofilm produced by staphylococci isolated from mastitis milk from sheep and goats, considering both inhibition of biofilm formation and the ability to disrupt established biofilm.

References

1. Argudín, M.Á., Mendoza, M.C. and Rodicio, M.R. (2010). Food Poisoning and *Staphylococcus aureus* Enterotoxins. *Toxins.* **2**: 1751–1773. doi:10.3390/toxins2071751
2. Blagitz, M.G., Batista, C.F., Souza, F.N., Benites, N.R., Melville, P.A., Stricagnolo, C.R., Ricciardi, M., Gomes, V., Azedo, M.R., Sanches, B.G.S. and Libera, A.M.M.P.D. (2008). Cellular and microbiological profile of Santa Ines ewes in the lactation and the post-weaning period. *Pesqui. Vet. Bras*. **28**: 417–422. doi:10.1590/S0100-736X2008000900004
3. Contreras, A., Sierra, D., Sánchez, A., Corrales, J.C., Marco, J.C., Paape, M.J. and Gonzalo, C. (2007). Mastitis in small ruminants. *Small Rumin. Res*. **68**: 145–153. doi:10.1016/j.smallrumres.2006.09.011
4. De Marco, S., Piccioni, M., Pagiotti, R., Pietrella, D. (2017). Antibiofilm and Antioxidant Activity of Propolis and Bud Poplar Resins versus Pseudomonas aeruginosa. Evidence-Based Complementary and Alternative Medicine. ID 5163575. doi:10.1155/2017/5163575
5. Doganli, G.A. (2016). Phenolic content and antibiofilm activity of propolis against clinical MSSA strains. *Rec Nat Prod*.**10**: 617–627.
6. Drescher, N., Klein, A.M., Schmitt, T. and Leonhardt, S.D. (2019). A clue on bee glue: New insight into the sources and factors driving resin intake in honeybees *(Apis mellifera*). *PLoS One* **14**: 1–20. doi:10.1371/journal.pone.0210594
7. Ferri, M., Ranucci, E., Romagnoli, P. and Giaccone, V. (2017). Antimicrobial resistance: A global emerging threat to public health systems. *Crit. Rev. Food Sci. Nutr.* **57**: 2857–2876. doi:10.1080/10408398.2015.1077192
8. Gelasakis, A.I., Angelidis, A., Giannakou, R. and Arsenos, G. (2018). Bacterial subclinical mastitis and its effect on milk quality traits in low-input dairy goat herds. *Vet. Rec.* **183**: 449. doi:10.1136/vr.104804
9. Halasa, T., Nielen, M., Roos, A.P.W., Hoorne, R.V., Jong, G., Lam, T.J.G.M., Werven, T. and Hogeveen, H. (2009). Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model. *J. Dairy Sci*. **92**: 599–606. doi:10.3168/jds.2008-1564
10. INE, I. P. (2017). *Estatísticas agrícolas 2016*. Instituto Nacional de Estatística, I. P. Lisboa-Portugal. www.ine.pt. ISBN: 978-989-25-0395-0
11. Jamali, H., Paydar, M., Radmehr, B., Ismail, S. and Dadrasnia, A. (2015). Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. *Food Control.* **54**: 383–388. doi:10.1016/j.foodcont.2015.02.013
12. Keefe, G. (2012). Update on control of *Staphylococcus aureus* and *Streptococcus agalactiae* for management of mastitis. *Vet. Clin. North Am. - Food Anim. Pract*. **28**: 203–216. doi:10.1016/j.cvfa.2012.03.010
13. Lee, J.H. (2003). Methicillin (Oxacillin)-Resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl. environ. microbiol*. **69**: 6489–6494. doi: 10.1128/AEM.69.11.6489–6494.2003
14. Martins, K.B., Faccioli, P.Y., Bonesso, M.F., Fernandes, S., Oliveira, A.A., Dantas, A., Zafalon, L.F. and Cunha, M.L.R.S. (2017). Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. *J. Dairy Sci*. **100**: 2184–2195. doi:10.3168/jds.2016-11583
15. Ohno, T., Kita, M., Yamaoka, Y., Imamura, S., Yamamoto, T., Mitsufuji, S., Kodama, T., Kashima, K. and Imanishi, J. (2003). Antimicrobial activity of essential oils against Helicobacter pylori. *Helicobacte.r* **8**: 207–215. doi:10.1046/j.1523-5378.2003.00146.x
16. Peixoto, R.M., França, C.A., Souza Júnior, A.F., Veschi, J.L.A. Costa, M.M. (2010a). Etiologia e perfil de sensibilidade antimicrobiana dos isolados bacterianos da mastite em pequenos ruminantes e concordância de técnicas empregadas no diagnóstico. *Pesqui. Vet. Bras*. **30**: 735–740. doi:10.1590/S0100-736X2010000900005
17. Peixoto, R.M., Mota, R.A. e Costa, M.M. (2010b). Mastite em pequenos ruminantes no Brasil. *Pesqui. Vet. Bras*. **30**: 754–762. doi:10.1590/S0100-736X2010000900008
18. Queenan, K., Häsler, B. and Rushton, J. (2016). A One Health approach to antimicrobial resistance surveillance: is there a business case for it? *Int. J. Antimicrob. Agents* **48**: 422–427. Doi:10.1016/j.ijantimicag.2016.06.014
19. Rahmatalla, S.A., Arends, D., Reissmann, M., Said Ahmed, A., Wimmers, K., Reyer, H. and Brockmann, G.A. (2017). Whole genome population genetics analysis of Sudanese goats identifies regions harboring genes

associated with major traits. *BMC Genet*. **18**: 1–10. Doi:10.1186/s12863-017-0553-z

20. Ribeiro, A.C. and Ribeiro, S.D.A. (2010). Specialty products made from goat milk. *Small Rumin. Res*. **89**: 225–233. Doi:10.1016/j.smallrumres.2009.12.048
21. Schröder, J., Maus, I., Trost, E. and Tauch, A. (2011). Complete genome sequence of Corynebacterium variabile DSM 44702 isolated from the surface of smear-ripened cheeses and insights into cheese ripening and flavor generation. *BMC Genomics* **12**: 545. Doi:10.1186/1471-2164-12-545
22. Seegers, H., Fourichon, C. and Beaudeau, F. (2003). Production effects related to mastitis and mastitis economics in dairy cattle herds. *Vet. Res.* **34**: 475 – 491. Doi:10.1051/vetres:2003027
23. Toreti, V.C., Sato, H.H., Pastore, G.M. and Park, Y.K. (2013). Recent Progress of Propolis for Its Biological and Chemical Compositions and Its Botanical Origin. *Hindawi Journals*. doi: 10.1155/2013/697390
24. Vasconcellos, S.A. e Ito, F.H. (2011). Principais zoonoses transmitidas pelo leite. *Revista de Educação Continuada em Medicina Veterinária*. **9**: 32–37. doi:10.36440/recmvz.v9i1.388
25. Veloz, J.J., Saavedra, N., Lillo, A., Alvear, M., Barrientos, L. and Salazar, L.A. (2015). Antibiofilm Activity of Chilean Propolis on *Streptococcus mutans* Is Influenced by the Year of Collection. *Biomed Res. Int*. doi:10.1155/2015/291351
26. Viana, J.G.A., Garibaldi, J. and Viana, A., 2008. Panorama Geral da Ovinocultura no Mundo e no Brasil. *Rev. Ovinos.* **4**: 1–9.
27. Virdis, S., Scarano, C., Cossu, F., Spanu, V., Spanu, C. and Santis, E.P.L. (2010). Antibiotic resistance in *Staphylococcus aureus* and coagulase negative staphylococci isolated from goats with subclinical mastitis. *Vet. Med. Int*. doi:10.4061/2010/517060
28. Weese, J.S., Page, S.W. and Prescott, J.F. (2013). Antimicrobial Stewardship in Animals, in: *Antimicrobial Therapy in Veterinary Medicine*. John Wiley & Sons, pp. 117–132. doi:10.1002/9781118675014.ch7
29. Wojtyczka, R.D., Kępa, M., Idzik, D., Kubina, R., Kabała-Dzik, A., Dziedzic, A. and Wąsik, T.J. (2013). *In Vitro* Antimicrobial Activity of Ethanolic Extract of Polish Propolis against Biofilm Forming *Staphylococcus epidermidis* Strains. *Evidence-Based Complement*. *Altern. Med*. **2013**: 1–11. doi:10.1155/2013/590703

Chapter 2

2. Literature review

2.1 – Mastitis

The word Mastitis (Greek “mastos”, breast; + “itis", inflammation) was defined in the mid-19th century as the inflammation reaction of the mammary gland. Mastitis occurs worldwide, being influenced by factors related to the animal and its environmental conditions (Menzies and Ramanoon, 2001; Bergonier et al., 2003; Mendonça et al., 2012).

Mastitis cause may be of physical, chemical, or infectious origin, the latter being the most frequent (Markey et al., 2013). Intramammary infection (IMI) caused by bacteria is the most prevalent in dairy herds (Hussein et al 2020; Puggioni et al 2020), and staphylococci are the main pathogens found in small ruminants mastitis (Peixoto et al., 2010; Queiroga, 2017a).

Mastitis may be clinical (CM), when it involves visible changes in the milk and/or clinical signs, or subclinical if no changes may be observed. For the diagnosis of subclinical mastitis (SCM), it is necessary to assess milk features such as elevated somatic cell count

(SCC), chemical changes and/or bacteriological condition. For SCC in small ruminants, much caution is required not to lead to the wrong diagnosis, due to differences among species, age, breed, parity by number, lactation stage and number, prolificacy, seasonality, farming system and facilities (Jimenez-Granado et al., 2014; Kumar et al., 2016; Mishra et al., 2018).

Milk yield and quality are decreased due to mastitis (Cuccuru et al., 2011). This condition was associated with a loss of 4.1 to 12% milk in sheep and 0.8 to 2.3% in goats (Leitner et al., 2008). Furthermore, milk fat, protein, casein and curd yield levels are also reduced (Silanikove et al., 2014).

2.1.1 – Aetiology

Bacteria of the genus *Staphylococcus* are the most isolated pathogens from mastitic milk samples from small ruminants (Contreras et al., 1999; Queiroga, 2017). Research has suggested that coagulase-negative staphylococci (CNS) are most commonly accountable for sheep (Queiroga et al., 2019) and goats mastitis (Peixoto et al., 2010). Coagulase negative staphylococci are also the most prevalent microorganisms found in goats and sheep udder skin and teat sphincter microbiota (Moroni et al., 2005).

Coagulase negative *Staphylococcus epidermidis* is a pathogen that causes persistent infections in sheep mammary gland (Bergonier et al., 2003) and has been reported as the main SCM etiological agent in small ruminants (Contreras et al., 1999; Queiroga, 2017). *Staphylococcus aureus* has also been isolated from the milk of animals with SCM. However, it is one of the main pathogens found in milk samples from small ruminants with CM, where they cause severe symptoms in them (Constable et al., 2016).

A wide variety of bacteria have been reported as mastitis pathogens in ewes as *Escherichia col*i, *Streptococcus* spp. and *Mannheimia haemolytica*, the latter being a peracute and gangrenous mastitis cause. In addition, mastitis in sheep may be associated with *Clostridium perfringens* A, *Pseudomonas* spp. or *Corynebacterium pseudotuberculosis* in a low percentage (Queiroga et al, 2019). In the inflammation of the goats mammary gland, bacteria such as *Trueperella pyogenes*, *Bacillus coagulans*, *Bacillus licheniformis*, *Klebsiella pneumoniae* and *Actinobacillus equuli* are found in a small percentage (Constable et al., 2016).

2.1.2 – Mastitis control

Mammary gland health depends on careful management and on adequate facilities (Mota, 2008). Hygienic milking, for both hand and machine milking, is an extremely valuable issue since it can prevent or minimize the dissemination of microorganisms from one animal to another (Bergonier et al., 2003). The lack of hygiene in manual milking can lead to bacteria spread from infected animals to the healthy ones through the hand of the milker (Langoni et al., 2017). Failure to periodically review milking equipment can cause vacuum fluctuation and backflow in addition to bacterial contamination. Before milking, routine inspection of the udder and careful milk secretion observation must be performed to prevent milking equipment contamination and further infection dissemination (Mota, 2008).

Primiparous and/or young healthy animals should be milked first, followed by subclinical infections animals, and lastly, the ones showing udder changes and the ones under treatment, whose milk cannot be marketed (Bergonier et al., 2003; Mota, 2008).

Post milking teat dipping procedure has been considered as an effective method to prevent further IMI cases (Contreras et al., 2007; Kamal and Bayoumi, 2015). The main dipping purpose is to eliminate pathogens of the external opening of the teat canal (Rainard, 2017). However, it should be used with caution because mastitis sporadic outbreaks have been related to disinfectants (Contreras and Rodríguez, 2011; Kelly and Wilson, 2016). Some solutions for teat dipping are not suitable for organic agriculture, such as iodine or chlorine-based (Contreras et al., 2007). However, new broad-spectrum antiseptic agents are being tested (Pedrini and Margatho, 2003; Reyes-Jara et al., 2016; Martins et al., 2017b).

In many farms, small ruminants are raised to produce different products such as milk, meat, skin, and wool. Antimicrobials are administered to control different diseases. Antimicrobial use is also the main strategy for mastitis control, both for therapeutic and prophylactic purposes (Queiroga, 2007; Constable et al., 2016). However, licensed antibiotic preparations for intramammary application for sheep and goats are scarce and, occasionally, the estimated dosage and withdrawal time has not been established (Saad and Ahmed, 2018). These must be strictly followed because the incorrect use can lead to residues in the milk (Beltrán et al., 2015) and exert a selection pressure for resistant

bacteria that can cause problems in milk and dairy products consumers (Lee, 2003; Gomes and Henriques, 2016). Antimicrobial residues in milk, water, food and the environment, due to lack of good agriculture practices, lead to antimicrobial entry in the food chain (Lee, 2003; Beltrán et al., 2015; Berruga et al., 2016). Adequate mastitis control at the dairy farm is considered a crucial process to ensure animal health and milk quality (Kamal and Bayoumi, 2015). Information on antimicrobial susceptibility of the most prevalent pathogens in a herd is essential to determine which drug can achieve the best clinical outcome and limit the resistant strain selection.

To reduce the antimicrobial use for mastitis control is a goal for many researchers. Gomes and Henriques (2016) reviewed alternatives to antimicrobials for bovine mastitis control such as bacteriophage therapy, nanoparticles, vaccines, and natural compounds, among others. Regarding bacteriophages therapy, Mishra et al. (2014) provided information for lytic bacteriophages uses against multiresistant S. *aureus* isolated from goat mastitis. Silver nanoparticles were tested by Yuan et al. (2017) in *S. aureus* and *Pseudomonas aeruginosa*, isolated from goats milk samples, and their results showed antibacterial effects, depending on the dose and inhibition time.

Studies on the natural substances used in veterinary medicine include essential oils and vegetables, such as *Punica granatum*, *Hymenaea martiana* and *Ocimum sanctum*, which showed effective results against intramammary inflammation in small ruminants, both *in vitro* and *in vivo* (Jassim and Abdullah, 2014; Peixoto et al., 2016; Kelly and Wilson, 2016; Dash et al., 2016; Abdalhamed et al., 2018); *Schinus terebinthifolius* (Muhs et al., 2017) and *Mimosa tenuiflora*, *Stryphnodendron adstringens* and *Eugenia uniflora* that inhibited the *S. aureus* development, CNS and *Escherichia coli*, among other species (Gonçalves et al., 2005).

Honey and propolis have also been studied. Natural components such as terpenes and phenolic compounds, as tannins, flavonoids, and their pigments like anthocyanins, may potentiate the antimicrobial action of these products (Cisowska et al., 2011; Peixoto et al., 2016; Abdalhamed et al., 2018). Propolis nanoparticles have also been tested against *S. aureus* showing a promising nanocarrier for propolis extract high concentrations in a stable aqueous medium, showing antimicrobial activity with moderate cytotoxicity for mammary alveolar cells *in vitro* (Pinheiro Machado et al., 2019).

The use of vaccines for mastitis prevention depends on their efficacy to the specific etiologic agents (Contreras and Rodríguez, 2011; Constable et al., 2016). For goats and sheep, autovaccines can be produced for the pathogens causing the intramammary infection (IMI) in the flock (Bergonier et al., 2003). Experiments showed that, after vaccination, the infection rate was lower, and the spontaneous cure rate was higher in comparison to the control groups in goats (Kautz et al., 2014) and dairy sheep (Tollersrud et al., 2002; Amorena et al., 1994). Although, vaccines for IMI prevention by *S. aureus* have existed for decades, their efficacy has been limited (Bannerman and Wall, 2005). Considering that there are different strains present in the herd, or even within a single animal, it is difficult to find a vaccine that is effective for the eradication of this disease (Bergonier et al., 2003; Bannerman and Wall, 2005).

2.1.2.1 – Antimicrobial therapy

As previously mentioned, mastitis control is highly dependent on antimicrobial therapy. Different classes of antimicrobials have been used for mastitis treatment and prevention. β-lactams and tetracyclines are the most suitable antimicrobials for the treatment of *Staphylococcus* mastitis and should be chosen over more modern molecules that should only be used in case of resistance, since they must be kept for use in human medicine.

Penicillin and their derivatives are β-lactam antimicrobial acting on cell wall synthesis inhibition. Penicillin-binding proteins (PBPs) are an enzyme group required for bacterial cell wall biosynthesis, which catalyses transpeptidation for the peptidoglycan chain formation. β-lactams bind to PBPs and inhibit peptidoglycan crosslink formation, thus lysing the bacteria (Paterson et al., 2014). Penicillin G, produced by the *Penicillium chrysogenum* fermentation show bactericidal action and is available in drugs for intramammary application.

Tetracyclines are broad-spectrum antibiotics and are also found as synthetic or semi-synthetic form. These molecules inhibit the bacterial cells protein synthesis by preventing the association of aminoacyl-tRNA with the 30S subunit of the ribosome. This association is reversible, explaining its bacteriostatic effect. Most bacteria actively uptake tetracyclines,

so their intracellular concentration gets 50 times higher than the outside concentration ensuring its specific activity (Chopra and Roberts, 2001).

2.2 – Staphylococcus

2.2.1 – Identification

Staphylococci belong to the family *Staphylococcaceae*, which comes from the Greek *staphyle*, which means cluster, and *coccus*, meaning grain. These are Gram-positive bacteria that grow in a characteristic pattern that resembles the grapes cluster. Most of these cocci have diameters of 0.5 to 1.5 µm. They are capable of growing under high osmotic pressure conditions and low humidity, under aerobic and anaerobic atmospheric conditions, even in the presence of 10% sodium chloride concentration, and temperature ranging between 18 °C and 40 °C (Murray et al., 2017; Tortora et al., 2017).

Conventional methods are used for the phenotypic characters determination for staphylococci initial identification, through morphological, dyeing, and biochemical characteristics (Quinn et al., 2005). However, it may require days before the result and depend on several factors to be reliable. Other methods such as quick identification kits or automated systems require only a few hours to conclusion demonstrating between 70% and 90% accuracy (Schleifer and Bell, 2009; Fetsch et al., 2018).

Nowadays, staphylococci may be identified through genotypic analysis, which show higher accuracy, comparing with conventional and automated methods (Schleifer and Bell, 2009; Fetsch et al., 2018).

According to the List of Prokaryotic names with Standing in Nomenclature (LPSN), the *Staphylococcus* genus currently includes 55 species (Parte et al., 2020). Some *Staphylococcus* produce the enzyme coagulase, and are called coagulase-positive *Staphylococcus* (CPS), while those that do not exhibit this feature are collectively referred to as coagulase-negative *Staphylococcus* (Murray et al., 2017; Tortora et al., 2017).

2.2.2 – *Staphylococcus* in small ruminant mastitis

As stated above, bacteria of the genus *Staphylococcus* are the most frequently found as small ruminant mastitis aetiology. Coagulase negative staphylococci are more abundant and less virulent, causing mainly SCM (Murray et al., 2017; Tortora et al., 2017), than *S. aureus*, which causes most cases of CM and shows to be more virulent (Queiroga, 2007; Bergonier et al., 2014; Tortora et al., 2017).

Staphylococcus aureus produces coagulase, thus being a CPS. Its name stands for its yellow-gold colonies, because of the carotenoid pigments expression (Liu and Nizet, 2009). This species can cause gangrenous mastitis in goats together with *Clostridium perfringens* and *Escherichia coli* (Ribeiro et al., 2007). Subclinical mastitis caused by *S. aureus* can also result in a 20% to 30% reduction in milk production. In addition, the sheep mortality rate with *S. aureus* CM varies between 25% and 50%, and the affected mammary glands of survivors generally become non-productive (Constable et al., 2016).

Among CNS species, *S. epidermidis* is commonly cited in the literature, usually associated with the high SCC values in dairy ewes (Contreras and Rodríguez, 2011; Queiroga, 2017). This species together with *S. simulans* and *S. warneri* showed major occurrence in sheep, before and after antimicrobial treatment at drying-off for two consecutive milking seasons (Zafalon et al., 2017).

In goat's milk samples, *S. caprae* species is commonly isolated (Bergonier and Berthelot, 2003; Peixoto et al., 2010), besides *S. epidermidis*, *S. xylosus*, *S. chromogenes*, *S. simulans* and *S. haemolyticus* species (Deinhofer and Pernthaner, 1995; Moroni et al., 2005; Vasiľ, 2007). However, *S. caprae* is also rarely isolated from sheep and cow milk (Vanderhaeghen et al., 2014; Condas et al., 2017).

2.2.3 – Virulence factors

Staphylococcus species hold several virulence factors conferring them the ability to damage the host tissues and to resist defence mechanisms, thus impairing mastitis control (Vasiľ, 2007; Contreras and Rodríguez, 2011; Ferreira et al., 2014a; Ferreira et al., 2014b; Bertelloni et al., 2015; Martins et al., 2017a).

Toxin production are considered the main virulence factors. Staphylocci produce thermostable enterotoxins through metabolic activity that can remain in milk, even after being pasteurized at 100 °C for 30 minutes. When produced by staphylococci causing mastitis in small ruminants, these may be responsible for food poisoning in contaminated milk and dairy products (Argudín et al., 2010; Vasconcellos and Ito, 2011). Moreover, staphylococcal enterotoxin genes have been detected in *S. aureus* isolated from cheeses and raw milk from goats and sheep (Carfora et al., 2015), as well as in *S. aureus* isolated from a human after consuming goat cheese (Johler et al., 2015). In milk and dairy products from small ruminants, the staphylococcal enterotoxins and toxic shock syndrome toxins are often commonly found in *S. aureus* strains and more seldom in CNS isolates (Scherrer et al., 2004; Salaberry et al., 2015; Azara et al., 2017; Martins et al., 2017a; Vitale et al., 2018).

Other virulence factors are bacterial strategies to overcome host defence mechanisms and antimicrobial actions. The ability to produce biofilms and to get ways to resist antimicrobials are some of these strategies (Murray et al., 2017).

Different virulence factors may play divergent roles in SCM caused by *Staphylococcus* (Salaberry et al., 2015). These factors increase the damage to the mammary gland and hinder the mastitis treatment (Azara et al., 2017).

2.2.3.1 – Enzymes

Staphylococci produce a wide variety of enzymes in order to invade the body and evade immune mechanisms.

Coagulase reacts with prothrombin in the blood producing staphylothrombin, which then convert fibrinogen to fibrin, resulting in the formation of clots. These clots protect the bacterium from phagocytosis and other host defence elements (Constable et al., 2016). Coagulase can be present in the cell wall or be freely secreted by the bacterium (Babu et al., 2014). The detection of coagulase is performed on the laboratory to differentiate CPS from CNS, generally using a tube coagulase test. There are different methods to identify this enzyme, the molecular ones being the most effective. This enzyme is encoded by the *coa* gene, which can be detected by polymerase chain reaction (PCR) (Babu et al., 2014).

The thermostable nuclease enzyme is also a virulence factor present in some staphylococci, which hydrolyzes DNA and RNA in host cells, causing tissue destruction and spreading of staphylococci (Foster, 2005). It may promote their evasion from neutrophil extracellular traps (NETs), which are DNA strands networks with antimicrobial proteins which allow neutrophils to lyse the micro-organisms in the extracellular environment, without phagocytizing them (Berends et al., 2010; Kenny et al., 2017). This enzyme also degrades environmental DNA (eDNA) and has been associated with biofilm regulation (Mann et al., 2009; Kiedrowski et al., 2011). It is encoded by the nuc gene, which was considered the gold standard for the identification of S. *aureus* (Kateete et al., 2010) and is still used for that purpose, although it is present in other staphylococci species (Schleifer and Bell, 2009; Hirotaki et al., 2011) and some S. *aureus* not carrying this gene have been described (van Leeuwen et al., 2008; Xu et al., 2015).

2.2.3.2 – Biofilm production

Biofilms are bacterial multi-layered cluster embedded in an extracellular matrix composed by exopolysaccharide, proteins, and environmental DNA (eDNA) (Büttner et al., 2015). Thereby, the sessile bacterium is protected against cellular and humoral defence mechanisms of the host, also getting higher resistance to antimicrobial agents used for the mastitis treatment (Barrio et al., 2000; Cucarella et al., 2004). According to Melchior et al. (2006), biofilm fixation and production are important factors also to emphasize other *Staphylococcus* virulence mechanisms.

According to Cramton et al. (1999), two sequential steps are involved in biofilm formation, cells adhesion to a solid substrate followed by cell-cell adhesion. For the first step, a polysaccharide capsular adhesin (PS/A) and a polysaccharide intercellular adhesin (PIA) may be involved (Laverty et al., 2013). Bacterial cells aggregation is expressed by a cationic glucosamine-based lipopolysaccharide. In some cases, proteins may function as alternative aggregating substances. Surfactant peptides were recognized as key factors involved in generating the 3D structure of a staphylococcal biofilm between cell disruptive forces, which casually may lead to the detachment of entire cell clusters (Otto, 2008). Inside the biofilm, the quorum sensing (QS) is the process through which bacteria produce and detect signalling molecules and, thus, coordinate the population's behaviour in biofilm. This

phenomenon plays a crucial role using self-inducing signalling molecules, which when accumulate to a threshold concentration activate a transcriptional regulator, which in turn regulate various genes expression, affecting the bacterial virulence (Kong et al., 2006; Rutherford and Bassler, 2012).

The PS/A and PIA are encoded by the *ica*ADBC genes of the intercellular adhesion operon *ica* (Laverty et al., 2013). In *Staphylococcus* species, this operon is also suggested to participate in cell-cell adhesion (Cramton et al., 1999; O'Toole et al., 2000) and has been found in both *S. aureus* and *S. epidermidis*, suggesting that the initial stages in biofilm formation would be similar between these two species (Cramton et al., 1999). For *S. epidermidis*, the ability to produce an exopolysaccharide matrix has been considered the defining virulence factor (Fey and Olson, 2010).

The *icaA* and *icaD* genes have a significant role in biofilm formation in *S. aureus* (Vasudevan et al., 2003). These genes have been considered essential factors for intercellular adhesion (Nourbakhsh and Namvar, 2016). However, in the absence of these genes a biofilm-associated protein (bap), encoded by *bap* gene, or biofilm homologous protein, encoded by *bhp* gene, may alternatively be responsible for biofilm production (Cucarella et al., 2004; Martins et al., 2017a). The *bap* gene was first identified in *S. aureus* isolated from mastitis (Cucarella et al., 2001). The biofilm-associated protein is a member of the surface proteins group that share several important structural and functional characteristics in the biofilm formation, besides having a role in bacterial infectious processes (Lasa and Penadés, 2006).

According to Cucarella et al. (2004) *S. aureus* isolates, which showed stronger biofilm production, had *ica* operon together with the *bap* gene. However, Tormo et al. (2005) found only the bap gene in staphylococci with strong biofilm formation. Szweda et al. (2012) reported that *S. aureus* carried only *ica*A and *icaD* genes, but not the *bap* gene, while Martins et al. (2017a) detected only the *bap* gene in some CNS. however, the same authors described 61.6% CNS biofilm producers isolated from sheep milk harbouring any of the *bap*, *bhp* and *ica* operon genes.

2.2.3.3 – Antimicrobial resistance

During bacterial growing, genetic mutations occur naturally in bacteria and some may develop strains resistant to antibiotics (Srivastava and Dutt, 2013). Bacteria may also get resistance genes though horizontal gene transfer: conjugation, when a bacterium carrying resistant genes transfer these genes to other bacteria; transformation, when a DNA fragment from the environment enters a recipient bacterium and transduction when a bacteriophage transfer a DNA fragment from one bacterium to another (Kaiser, 2020).

In a bacterial population, different cells may have different resistance levels. When an antimicrobial is introduced in this population environment, those susceptible to the drug are inactivated and thus only resistant bacteria survive. So, the antimicrobial exerts a selection pressure for resistant strains (U.S. Congress, 1995).

The product of genetic recombination can decrease antibiotic access to cell targets, modify antimicrobial targets or inactivate the molecule, a so inducing bacterial resistance to antimicrobials (Williams, 1999).

β-lactam antimicrobials are inactivated by β-lactamase enzymes, which catalyse the hydrolysis of the β-lactam ring cutting the amide bond (Williams, 1999). In Gram-negative bacteria, these enzymes may be present mainly in periplasmic space and in Gram-positive in the extracellular environment (Bush, 1988). In staphylococci these enzymes are encoded by *blaZ* gene and when encoded from chromosomes, are transferred to the same species, but when encoded by plasmids they are transferable between different species of bacteria.

MRSA resistance is due to the Penicillin-binding proteins mutant, additional PBPs (PBP2a), which encoded by *mec*A gene, as well as its *mec*B and *mec*C homolog (Paterson et al., 2014; Nasution et al., 2018). Recently, a study showed that the acquisition of methicillin resistance by the *mecA* gene was possible by transducing SCCmec type IV and SCCmec type I, by bacteriophages 80α and 29 for *S. aureus* receptor strains (Scharn et al., 2013).

Many pathogenic bacteria are resistant to tetracyclines due to proteins associated with the membrane that favours efflux, thus taking tetracycline out of the cell. These proteins are encoding by *tet* genes. These genes can be transported for other bacteria by transformation or conjugation. *Tet*M genes can be found on the bacterial chromosome and

carried mainly by conjugative transposons. The *tet*K gene is generally carried by small plasmids and can be inserted in SCCmec type III (Ito et al., 2003).

Staphylococcal mastitis isolates from small ruminants have been reported to be resistant to β-lactams with prevalence between 41% and 88% (Agnol et al., 2013; Jamali et al., 2015; Kürekci, 2016; Salaberry et al., 2016; Chu et al., 2017; Ayis and Fadlalla, 2017; Obaidat et al., 2018; Hristov, 2018). Moreover, amoxicillin, a penicillin derivative, is more effective against β-lactamases-producing microorganisms, when used together with clavulanic acid, which inactivates β-lactamases (DGAV, 2013). Obaidat et al. (2018) showed better combined action because only 20% of the *S. aureus*, from small and large ruminants milk, resisted their joint action, while 50% were resistant to individual penicillin. Regarding tetracyclines, 26.3% to 73.7% of staphylococci isolated from small ruminant milk samples also showed resistance (Ayis and Fadlalla, 2017; Obaidat et al., 2018).

2.3 – Propolis

2.3.1 – Origin and properties

The Greeks called propolis at the gates of a city, a word taken by the prefix 'pro' and 'polis' for city (Ghisalberti, 1979). Propolis is a mass produced by honeybees with various resinous substances, from plant sources (Alvarez-Suarez, 2017). Resins are sticky and aromatic plants exudates, secreted in the initial flower development and leaf buds. After removing the resins with their jaws, *Apis mellifera* bees triturate and soften them with the aid of 10-hydroxydocenoic acid, a substance produced in their salivary glands, which they then mix with wax, pollen and microelements, resulting in a glue type, used to fill gaps, both in the breeding wells and in the hive wall, allowing better thermal insulation and preventing the entry of undesirable visitors (Ghisalberti, 1979; Costa and Oliveira, 2005). In addition, bees also use it to completely cover and immobilize small animals and invaders insects, embalming them and preventing their decomposition creating a sterile environment. Propolis was also used by Egyptians to embalm corpses due to its anti-putrefactive properties (Ghisalberti, 1979; Bankova et al., 1996; Castaldo and Capasso, 2002).

This resinous paste has been used for centuries to treat different human diseases. It was recognized for its medicinal properties by Greek and Roman physicians, such as Aristotle, Dioscorides, Pliny and Galen, by Arab doctors in the Middle Age, by the Incas of the Old-World civilizations, and by the London pharmacopeia in the 17th century, who listed propolis as official medicine. Between the 17th and 20th centuries, propolis became very popular in Europe due to its antibacterial activity (Castaldo and Capasso, 2002).

Propolis compounds can exhibit different actions, such as antioxidant, anti-inflammatory (Yildiz et al. 2014), antiulcerogenic (Barros et al., 2008), antitumoral (Frozza et al., 2013), antidiabetogenic (Pacheco et al., 2011), antiatherogenic and anti-angiogenic (Daleprane et al., 2011), immunomodulatory (Castaldo and Capasso, 2002), antifungal (Agüero et al., 2011; Mendonça et al., 2015), antiviral (Nolkemper et al., 2010) namely for human immunodeficiency virus (anti-HIV) (Gekker et al., 2005). Propolis also showed action versus some bacterial virulence factors, such as anti-biofilm (Wojtyczka et al., 2013; Doganli, 2016), anti-quorum sensing (Kasote et al., 2015) and antimotility (Mirzoeva et al., 1997; Josenhans and Suerbaum, 2002).

2.3.2 – Composition

Propolis chemical composition and colour are variable and dependent on the vegetation around the hive (Ghisalberti, 1979; Koru et al., 2007). Moreover, chemical composition variation generates differences in their pharmacological properties (Bueno-Silva et al., 2017; Afrouzan et al., 2018).

Propolis generally consists of 30 to 40% waxes, 5 to 10% volatile oils and aromatic acids, 50 to 60% resins and balsams, and 5% pollen grains. These latter are a source of essential elements, such as magnesium, nickel, calcium, iron, zinc, aluminium, strontium, copper, manganese and small amounts of vitamins B1, B2, B6, C and E, and sugars such as arabinose, fructose, glucose, sucrose and maltose (Ghisalberti, 1979; Bonvehí et al., 1994; Castaldo and Capasso, 2002; Park et al., 2002).

The main pharmacologically active compounds in propolis are: bioflavonoids and derivatives (pinocembrin, pinostrobin, galangin, isalpinine, pinobanksina, isosakuranetin, canferida, quercetin, naringenin, apigenin, chrysin, formononetin, vestitol, neovestitol,

isoliquiritigenin, anthocyanins and medicarpin); other phenolic compounds (terpenes, caffeine and its esters, artepillin C, and its derivatives, ferulic, trans-cinnamic, prenylated p-coumaric); phenolic aldehydes; ketones (chalcone); tannins and fatty acids (Marcucci et al., 2001; Castaldo and Capasso, 2002; Koo et al., 2002; Uzel et al., 2005; Funari and Ferro, 2006; Inui et al., 2014; Bueno-Silva et al., 2017; Afrouzan et al., 2018). Some authors reported that in propolis, phenolic acids are more abundant than flavonoids (Woisky and Salatino, 1998). Several compounds already identified are present in all samples, but other depends on the seasonality and flora where the samples are collected (Castaldo and Capasso, 2002; Vargas et al., 2004; Alencar et al., 2007).

2.3.3 – Components extraction and pharmacological activity

Several factors may affect the antimicrobial action of each propolis extract. Solvent type, propolis concentration and the methodology used for extraction determine the components to be extracted from the original sample (Park and Ikegaki, 1998; Pinto et al., 2001; Castaldo and Capasso, 2002; Silva et al., 2003).

Pinto et al. (2001) studied the Gram-positive bacteria susceptibility, isolated from milk, to propolis extracts produced with different methodologies and solvents, such as water, ethanol, methanol, ethyl acetate and chloroform at a concentration of 100 mg/ml. Ethanolic extract showed the best action followed by the methanolic extract. However, extracts produced with water, ethyl acetate and chloroform did not show any bacterial activity as the pure ethanol and methanol used as controls.

Regarding ethanol extracts, although some antimicrobial activity was observed in extracts produced with 30% to 50% ethanol, the best performance was obtained with concentrations between 60% and 80%. In these highest ethanol concentrations occurs the extraction of the highest percentages of bioactive components, such as phenolics, that are less soluble in water (Park and Ikegaki, 1998; Pinto et al., 2001). However, a proper PEE is safe when its preparation is standardized (Castaldo and Capasso, 2002; Silva et al., 2003).

2.3.4 – Antimicrobial activity

The antibacterial action of propolis is more marked against Gram-positive bacteria than for Gram-negative organisms (Campos et al., 2017).

For *Streptococcus agalactiae*, Takaisi-kikuni and Schilcher (1994) described that propolis components caused cell lysis by disrupting the cell wall, cytoplasmic membrane and cytoplasm, and bacterial inhibition was caused by protein synthesis inhibition and DNA replication impairment. Scazzocchio et al. (2006) described *S. aureus* inhibition of lipase and coagulase enzymes by PEE activity. More recently, Campos et al. (2017) showed by atomic force microscopy that the components present in the propolis extract, interacted with *S. aureus* and *E. coli*, generating an increase in the cell volume, indicating that the action on the bacteria cell wall is the main mechanism that leads to bacterial inhibition. These authors reported that Gram-negative bacteria required a higher EEP concentration for cell wall disruption. Mirzoeva et al. (1997) stated that the caffeic acid component of the propolis extract inhibited motility and increased the membrane ionic permeability of *Bacillus subtilis*.

The cell wall of Gram-negative bacteria is of greater chemical complexity, lower flexibility, and higher lipid content, conferring a higher resistance to propolis activity compared to Gram-positive (Vargas et al., 2004; Campos et al., 2017). Studies show that to overcome the structure of the Gram-negative bacteria membrane, a double propolis extract concentration must be used comparing to Gram-positive (Mirzoeva et al., 1997).

2.3.5 – Propolis synergy with antibiotics

Propolis extracts can be used alone or in combination with antibiotics (Afrouzan et al., 2018). This association produces synergy potentiating the antibiotics antimicrobial effect on various bacteria.

Subinhibitory concentrations of propolis extracts together with antibiotics were analysed to observe their inhibitory action on multidrug resistant microorganisms. The synergistic action demonstrated the propolis potential to improve the action of certain antibiotics, which had been previously undetected (Stepanović et al., 2003).

Other authors also observed good results when PEE sub-inhibitory concentrations were added to ampicillin, gentamicin, and streptomycin against *S. aureus* (Scazzocchio et al., 2006). Ampicillin together with PEE was active against *Listeria monocytogenes* for the treatment of goats and sheep with listeriosis. Its synergy showed more efficiency than antibiotics combination (Ismael et al., 2009). However, in another study, this association showed no action against *S. aureus* (Król et al., 1993).

When PEE was added to chloramphenicol, ceftriaxone and vancomycin showed moderate activity and when together with erythromycin did not show antimicrobial action (Scazzocchio et al., 2006).

Differences in PEE composition could explain divergences in PEE synergies with the same antibiotic, for the same bacterial species, due to the activity of the different compounds present in each propolis sample. Fernandes et al. (2005) observed that the better synergistic results detected were for the PEE together with antibiotics that cause interference in bacterial protein synthesis.

2.3.6 – Propolis components with antimicrobial action

In 1994, Takaisi-Kikuni and Schilcher (1994) showed that propolis antimicrobial activity is due to the synergism between various components, such as phenolic acids, flavonoids, and sugars. Likewise, Mirzoeva et al. (1997), observed the efficacy of some propolis components, such as caffeic acid phenolic ester (CAPE), quercentin, naringerin and caffeic acid against Gram-positive and some Gram-negative bacteria. This synergy has the greatest effect on bacterial cells when they are actively growing.

Other authors refer the main propolis compounds commonly found with potential antimicrobial action, both for Gram-positive and Gram-negative bacteria, are flavonoids (pinocembrin, pinostrobin chalcone, isalpinine, pinobanksina, quercetin, naringenin, galangin and chrysin), phenolic and terpene compounds (Castaldo and Capasso, 2002; Uzel et al., 2005; Afrouzan et al., 2018).

Several studies show different compounds are characteristic of specific regions of resin collection by bees. Red coloured propolis has biologically active compounds that have not been reported in other propolis types (Trusheva et al., 2006; Alencar et al., 2007). Isoflavonoid medicarpin compound found in red propolis, by Inui et al. (2014), showed

better antimicrobial activity against *S. aureus*, *B. subtilis* and *P. aeruginosa* than other compounds.

Green PEE displayed better antimicrobial activities against *S. aureus* resistant and sensitive to methicillin (MRSA and MSSA) compared to *Baccharis dracuculifolia* extracts (vegetable source for green propolis production) and artepillin C (compound found in Green propolis and *B. dracunculifolia*). The better EEP activity may be related to the synergy between these constituents or between these and others present in the sample (Veiga et al., 2017). This research confirms the results from the late 20th century by Król et al. (1993) and Bankova et al. (1996), which related a synergism among propolis constituents showing better antimicrobial activity altogether, when compared to the isolated compounds. This synergistic potential was also confirmed by Bittencourt et al. (2015).

2.3.7 – Propolis components with antibiofilm action

Glycosyltransferases (GTFs) are enzymes important for biofilm development (Islam et al., 2008). These enzymes are involved in the synthesis of the glucan polysaccharide, which can provide binding sites for bacteria (Schilling and Bowen, 1992). Potent inhibitors of GTF activity have been found in propolis extracts such as flavone and flavonol, with apigenin being the most effective constituent for this action (Koo et al., 2002).

On the other hand, substances with QS inhibitory functions were proposed by Brackman and Coenye (2015), as promising antimicrobial agents. Quorum Sensing process develops following different systems such as autoinducing peptide (AIP) in Gram-positive bacteria, acyl-homoserine lactone (AHL) in Gram-negative bacteria and the autoinducer-2 (AI-2) existing in both bacteria types. Savka et al. (2015) observed that the components found in propolis inhibited the QS system AHL of Gram-negative bacteria, emphasizing that the extracts that showed higher amounts of flavanones and dihydroflavonols, flavones and flavonols, cinnamic acid derivatives and fatty acids showed the best results in inhibiting this system and, consequently, inhibiting biofilm.

Polyphenol tannins (condensed and hydrolysable), often found in propolis, are also denoted as possible antibiofilm compounds for Gram-negative bacteria, but in higher concentrations (Trentin et al., 2013) compared to Gram-positive bacteria for which a

concentration as low as 0.2 μg / mL was effective to inhibit the biofilm formation. Antibiofilm activity may also be due to flavonoids (kaempferol, myricetin, pinocembrin, apigenin, genistein and quercetin), which were present in greater amount in the studied sample (Veloz et al., 2015).

2.3.8 – *In vitro* antimicrobial activity of propolis against staphylococci from small ruminants milk

Aqueous and ethanolic green propolis extracts were evaluated for staphylococci susceptibility on isolates from goat's milk. Both extracts were efficient, however, the ethanol extract showed greater activity (Santos Neto et al., 2009). Likewise, Silva et al. (2012), showed satisfactory antibacterial results of brown propolis extract for *Staphylococcus* sp. Propolis antimicrobial studies against goat and sheep mastitis pathogens are scarce.

2.3.9 – *In vivo* antimicrobial activity of propolis in small ruminants

Studies on the use of propolis to improve small ruminants performance are on the rise and showing positive results. In 1983, Havsteen (1983) described that flavonoids administered orally are metabolized and expelled in the urine. Thus, the propolis addition to sheep rations (Silva et al., 2015), goats (Lana et al., 2007) and cows (Aguiar et al., 2014) did not show a negative effect on the nutrients digestibility and could be used as a reliable antimicrobial compound as a food complement for ruminants not affecting normal rumen parameters.

Selem (2012) described that red PEE added to a Tifton hay substrate base (50:50) significantly decreased SCC while increasing fat, protein and lactose together with milk production, improving sheep health during breeding season and performance of lambs. This author also stated that the isoflavonoids were the components responsible for the propolis biological activity. Morsy et al. (2016) administrated red propolis extracts, orally, (3g/sheep/day) for 21 days before the expected lambing date. These authors also observed that the isoflavonoids present in the propolis extract decreased SCC and significantly increased total protein and sheep energy status thus improving milk production. The

authors concluded that propolis extract prepartum administration was positively supported in the transition from pregnancy to lactation, with health benefits for the ewes and the lambs.

A propolis based intramammary formulation was assessed for tolerance and efficacy on 100 goats in intensive milk production. Samples were analysed for milk composition, SCC, and microbiological culture. The formulation was applied three times and the antimicrobial, immunostimulatory and anti-inflammatory activity was observed in 1% to 3% propolis concentrations. The authors suggest that the intramammary formulation has the potential to gradually replace antibiotics, with the advantage that propolis does not require a withdrawal period and repeated use does not cause antimicrobial resistance (Bačić et al., 2016).

References

1. Abdalhamed, A.M., Zeedan, G.S.G. and Zeina, H.A.A.A. (2018). Isolation and identification of bacteria causing mastitis in small ruminants and their susceptibility to antibiotics, honey, essential oils, and plant extracts. *Vet. World.* **11**: 355–362. doi:10.14202/vetworld.2018.355-362
2. Afrouzan, H., Tahghighi, A., Zakeri, S. and Es-Haghi, A. (2018). Chemical composition and antimicrobial activities of Iranian Propolis. *Iran. Biomed. J.* **22**: 50–65. doi:10.22034/ibj.22.1.50
3. Agnol, A.M.D., Cavalcante, M.B., França, C.A.., Krewer, C.C., Queiros, A.A., Costa, M.M., Bragança, J.F.M. e Girardini, L.K. (2013). Caracterização fenotípica e molecular de isolados de *Staphylococcus* spp. obtidos de leite de ovelhas do Município de Chapecó-SC. *Semin. Agrar*. **34**: 311–322. doi:10.5433/1679-0359.2013v34n1p311
4. Agüero, M.B., Svetaz, L., Sánchez, M., Luna, L., Lima, B., López, M.L., Zacchino, S., Palermo, J., Wunderlin, D., Feresin, G.E. and Tapia, A. (2011). Argentinean Andean propolis associated with the medicinal plant *Larrea nitida* Cav. (Zygophyllaceae). HPLC-MS and GC-MS characterization and antifungal activity. *Food Chem. Toxicol*. **49**: 1970–1978. doi:10.1016/j.fct.2011.05.008
5. Aguiar, S.C., Paula, E.M., Yoshimura, E.H., Santos, W.B.R., Machado, E., Valero, M.V., Santos, G.T. and Zeoula, L.M. (2014). Effects of phenolic compounds in propolis on digestive and ruminal parameters in dairy cows. *Rev. Bras. Zootec*. **43**: 197–206. doi:10.1590/S1516-35982014000400006
6. Alencar, S.M., Oldoni, T.L.C., Castro, M.L., Cabral, I.S.R., Costa-Neto, C.M., Cury, J., Rosalen, P.L. and Ikegaki, M. (2007). Chemical composition and biological activity of a new type of Brazilian propolis: red propolis. *J. Ethnopharmacoly* **113**: 278–83. doi:10.1016/j.jep.2007.06.005
7. Alvarez-Suarez, J.M. (2017). *Bee Products - Chemical and Biological Properties*. Chapter 6, by Miguel, M.G. and Figueiredo, A.C., Springer International Publishing AG doi: 10.1007/978-3-319-59689-1_6
8. Amorena, B., Baselga, R., Albizu, I. (1994). Use of liposome-immunopotentiated exopolysaccharide as a component of an ovine mastitis staphylococcal vaccine. *Vaccine* **12**: 243–249. doi:10.1016/0264-410X(94)90201-1
9. Argudín, M.Á., Mendoza, M.C. and Rodicio, M.R. (2010). Food Poisoning and *Staphylococcus aureus* Enterotoxins. *Toxins.* **2**: 1751–1773. doi:10.3390/toxins2071751
10. Ayis, A.A. El and Fadlalla, E. (2017). Antibiotic Susceptibility of Major Bacteria Cause Ovine Mastitis in River Nile State, Sudan. *Imp. J. Interdiscip. Res*. **3**: 2454–1362. ISSN: 2454-1362, http://www.onlinejournal.in
11. Azara, E., Longheu, C., Sanna, G. and Tola, S. (2017). Biofilm formation and virulence factor analysis of *Staphylococcus aureus* isolates collected from ovine mastitis. *J. Appl. Microbiol*. **123**: 372–379. doi:10.1111/jam.13502
12. Babu, N.G.R., Shree, G.B.B., Rekha, L. and Karthiga, P. (2014). Molecular analysis of Coagulase (*coa*) gene polymorphism in clinical isolates of *Staphylococcus aureus* by PCR-RFLP. *IJIRSET*. **3**: 8163–8168.
13. Bačić, G., Mačešić, N., Radin, L., Aladrović, J., Matanović, K., Mašek, T., Brozić, D., Benić, M., Radić, B., Bačić, I. and Šuran, J. (2016). Intramammary Propolis Formulation for Prevention and Treatment of Mastitis in Dairy Ruminants (RC.2.2.08-0003). *J. Anim. Res.* **6**: 47–49. doi:10.5958/2277-940X.2015.00165.5
14. Bankova, V., Marcucci, M.C., Simova, S., Nikolova, N., Kujumgiev, A. and Popov, S. (1996). Antibacterial diterpenic acids from Brazilian propolis. *Z Naturforsch C J Biosci*. **51**: 277–280. doi: 10.1515/znc-1996-5-602
15. Bannerman, D.D. and Wall, R.J. (2005). A Novel Strategy for the Prevention of *Staphylococcus aureus*-Induced Mastitis in Dairy Cows. *ISB News Report*, May 2005.
16. Barrio, B., Vangroenweghe, F., Dosogne, H. and Burvenich, C. (2000). Decreased neutrophil bactericidal activity during phagocytosis of a slime-producing *Staphylococcus aureus* strain. *Vet. Res*. **31**: 603–609. doi:10.1051/vetres:2000143
17. Barros, M.P., Lemos, M., Maistro, E.L., Leite, M.F., Sousa, J.P.B., Bastos, J.K. and Andrade, S.F. (2008). Evaluation of antiulcer activity of the main phenolic acids found in Brazilian Green Propolis. *J. Ethnopharmacol*. **120**: 372–377. doi:10.1016/j.jep.2008.09.015
18. Beltrán, M.C., Althaus, R.L., Molina, A., Berruga, M.I. and Molina, M.P. (2015). Analytical strategy for the detection of antibiotic residues in sheep and goat's milk. *Spanish J. Agric. Res*. **13**: 1–9. doi:10.5424/sjar/2015131-6522

19. Berends, E.T.M., Horswill, A.R., Haste, N.M., Monestier, M., Nizet, V. and Von Köckritz-Blickwede, M. (2010). Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J. Innate Immun*. **2**: 576–586. doi:10.1159/000319909
20. Bergonier, D. and Berthelot, X. (2003). New advances in epizootiology and control of ewe mastitis. *Livest. Prod. Sci*. **79**: 1–16. doi:10.1016/S0301-6226(02)00145-8
21. Bergonier, D., Sobral, D., Feßler, A.T., Jacquet, E., Gilbert, F.B., Schwarz, S., Treilles, M., Bouloc, P., Pourcel, C. and Vergnaud, G. (2014). *Staphylococcus aureus* from 152 cases of bovine, ovine and caprine mastitis investigated by Multiple-locus variable number of tandem repeat analysis (MLVA). *Vet. Res*. **45**: 1–8. doi:10.1186/s13567-014-0097-4
22. Berruga, M.I., Molina, A., Althaus, R.L. and Molina, M.P. (2016). Control and prevention of antibiotic residues and contaminants in sheep and goat's milk. *Small Rumin. Res*. **142**: 38–43. doi:10.1016/j.smallrumres.2016.02.023
23. Bertelloni, F., Fratini, F., Ebani, V.V., Galiero, A., Turchi, B. and Cerri, D. (2015). Detection of genes encoding for enterotoxins, TSST-1, and biofilm production in coagulase-negative staphylococci from bovine bulk tank milk. *Dairy Sci. Technol*. **95**: 341–352. doi:10.1007/s13594-015-0214-9
24. Bittencourt, M.L.F., Ribeiro, P.R., Franco, R.L.P., Hilhorst, H.W.M., Castro, R.D. and Fernandez, L.G. (2015). Metabolite profiling, antioxidant and antibacterial activities of Brazilian propolis: Use of correlation and multivariate analyses to identify potential bioactive compounds. Food *Res. Int*. **76**: 449–457. doi:10.1016/j.foodres.2015.07.008
25. Bonvehí, J.S., Coll, F.V. and Jordà, R.E. (1994). The composition, active components and bacteriostatic activity of propolis in dietetics. *J. Am. Oil Chem. Soc*. **71**: 529–532. doi:10.1007/BF02540666
26. Brackman, G. and Coenye, T. (2015). Quorum Sensing Inhibitors as Anti-Biofilm Agents. *Curr. Pharm. Des*. **21**: 5–11. doi:10.2174/1381612820666140905114627
27. Bueno-Silva, B., Marsola, A., Ikegaki, M., Alencar, S.M. and Rosalen, P.L. (2017). The effect of seasons on Brazilian red propolis and its botanical source: chemical composition and antibacterial activity. *Nat. Prod. Res*. **31**: 1318–1324. doi:10.1080/14786419.2016.1239088
28. Bush, K. (1988). Beta-lactamase inhibitors from laboratory to clinic. *Clin. Microbiol. Rev*. **1**: 109–123. doi:10.1128/CMR.1.1.109
29. Büttner, H., Mack, D., Rohde, H. (2015). Structural basis of *Staphylococcus epidermidis* biofilm formation: Mechanisms and molecular interactions. *Front. Cell. Infect. Microbiol*. **5**: 1–15. doi:10.3389/fcimb.2015.00014
30. Campos, J.V., Benedito, O., Assis, G. and Filho, R.B. (2017). Análise por AFM da ação do extratos de própolis verde sobre as morfologias de bactérias *Staphylococcus aureus* e *Escherichia coli*. *IX Workshop de Nanotecnologia Aplicada ao Agronegócio.* Embrapa Instrumentação, São Carlos/SP, 21 a 22 Novembro 2017. pp. 327–330.
31. Carfora, V., Caprioli, A., Marri, N., Sagrafoli, D., Boselli, C., Giacinti, G., Giangolini, G., Sorbara, L., Dottarelli, S., Battisti, A. and Amatiste, S. (2015). Enterotoxin genes, enterotoxin production, and methicillin resistance in *Staphylococcus aureus* isolated from milk and dairy products in Central Italy. *Int. Dairy J*. **42**: 12–15. doi:10.1016/j.idairyj.2014.10.009
32. Castaldo, S. and Capasso, F. (2002). Propolis, an old remedy used in modern medicine. *Fitoterapia* **73**: 1–6. doi:10.1016/S0367-326X(02)00185-5
33. Chopra, I., Roberts, M. (2001). Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiol. Mol. Biol.* **65**: 232–260. doi:10.1128/mmbr.65.2.232-260.2001
34. Chu, C.S., Huang, H.H., Chiang, S.H., Chou, C.C., Lai, J.M., Shih, W.L., Changchien, C.H., Lin, H.C., Chuang, S.T. and Su, Y.C. (2017). Investigation into antimicrobial resistance, enterotoxin and cassette chromosome gene of *Staphylococcus aureus* isolates from humans, cows and goats in Taiwan. *Thai J. Vet. Med.* **47**: 481–492.
35. Cisowska, A., Wojnicz, D. and Hendrich, A.B. (2011). Anthocyanins as antimicrobial agents of natural plant origin. *Nat. Prod. Commun*. **6**: 149–156. doi: 10.1177/1934578X1100600136
36. Condas, L.A.Z., De Buck, J., Nobrega, D.B., Carson, D.A., Roy, J.-P., Keefe, G.P., DeVries, T.J., Middleton, J.R., Dufour, S. and Barkema, H.W. (2017). Distribution of non- *aureus* staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. *J. Dairy Sci.* **100**:5592–5612 doi:10.3168/jds.2016-12479
37. Constable, P., Hinchcliff, K.W., Done, S.and Gruenberg, W. (2016). *Veterinary Medicine*, *A textbook of the diseases of cattle, horses, sheep, pigs and goats*. 11ª Edition. Hardcover ISBN: 9780702052460. eBook ISBN: 9780702070587.

38. Contreras, A., Paape, M.J. and Miller, R.H. (1999). Prevalence of subclinical intramammary infection caused by *Staphylococcus epidermidis* in a commercial dairy goat herd. *Small Rumin. Res*. **31**: 203–208. doi:10.1016/S0921-4488(98)00147-3
39. Contreras, A., Sierra, D., Sánchez, A., Corrales, J.C., Marco, J.C., Paape, M.J. and Gonzalo, C. (2007). Mastitis in small ruminants. *Small Rumin. Res*. **68**: 145–153. doi:10.1016/j.smallrumres.2006.09.011
40. Contreras, G.A. and Rodríguez, J.M. (2011). Mastitis: Comparative etiology and epidemiology. *J. Mammary Gland Biol. Neoplasia* **16**: 339–356. doi:10.1007/s10911-011-9234-0
41. Costa, P.S.C. e Oliveira, J.S. (2005). *Manual prático de criação de abelhas*. 1ª edição, Viçosa-MG.
42. Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W. and Gotz, F. (1999). The Intercellular Adhesion (*ica*) Locus Is Present in *Staphylococcus aureus* and Is Required for Biofilm Formation. *American Society for Microbiology.* **67**, 5427–5433. doi: 10.1128/IAI.67.10.5427-5433.1999
43. Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I. and Penades, J.R. (2001). Bap, a *Staphylococcus aureus* Surface Protein Involved in Biofilm Formation. *Microbiology* **183**: 2888–2896. doi:10.1128/JB.183.9.2888
44. Cucarella, C., Tormo, M.Á., Úbeda, C., Trotonda, M.P., Monzón, M., Peris, C., Amorena, B., Lasa, Í. and Penadés, J.R. (2004). Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*. *Infect. Immun*. **72**: 2177–2185. doi:10.1128/IAI.72.4.2177-2185.2004
45. Cuccuru, C., Meloni, M., Sala, E., Scaccabarozzi, L., Locatelli, C., Moroni, P. and Bronzo, V. (2011). Effects of intramammary infections on somatic cell score and milk yield in Sarda sheep. *N. Z. Vet. J.* **59**: 128–131. doi:10.1080/00480169.2011.562862
46. Daleprane, J.B., da Silva Freitas, V., Pacheco, A., Rudnicki, M., Faine, L.A., Dörr, F.A., Ikegaki, M., Salazar, L.A., Ong, T.P. and Abdalla, D.S.P. (2011). Anti-atherogenic and anti-angiogenic activities of polyphenols from propolis. *J. Nutr. Biochem*. **23**: 557–566. doi:10.1016/j.jnutbio.2011.02.012
47. Dash, J.R., Sar, T.K., Samanta, I. and Mandal, T.K. (2016). Effects of herbal extract of *Ocimum sanctum* as supportive therapy with intravenous ceftriaxone in experimentally induced staphylococcal chronic mastitis in goat. *Small Rumin. Res.* **137**: 1–8. doi:10.1016/j.smallrumres.2016.02.013
48. Deinhofer, M. and Pernthaner, A. (1995). *Staphylococcus* spp. as mastitis-related pathogens in goat milk. *Vet. Microbiol*. **43**: 161–166. doi:10.1016/0378-1135(95)92532-G
49. DGAV. (2013). Direção Geral de Alimentação e Veterinária - DSMDS. Anexo I - Resumo das Características do Medicamento Entyvio (Vedolizumab). *Eur. Med. Agency.* 1–41. https://silo.tips/download/anexo-i-resumo-das-caracteristicas-do-medicamento-219 (accessed 11/06/2020).
50. Doganli, G.A. (2016). "Phenolic Content and Antibiofilm Activity of Propolis against Clinical MSSA Strains." *Records of Natural Products*. **10**: 617–27.
51. Fernandes, A., Balestrin, E.C., Betoni, J.E.C., Orsi, R.O., Cunha, M.L.R.S. and Montelli, A.C. (2005). Propolis: Anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. *Mem. Inst. Oswaldo Cruz* **100**: 563–566. doi:10.1590/S0074-02762005000500018
52. Ferreira, A.A., Tette, P.A.S., Mendonça, R.C.S., Soares, A.S. and Carvalho, M.M. (2014a). Detection of exopolysaccharide production and biofilm-related genes in *Staphylococcus* spp. isolated from a poultry processing plant. *Food Sci. Technol*. **34**: 710–716. doi:10.1590/1678-457X.6446
53. Ferreira, D.H., Carvalho, M.G.X., Nardelli, M.J., Sousa, F.G.C. and Oliveira, C.J.B. (2014b). Occurrence of enterotoxin-encoding genes in *Staphylococcus aureus* causing mastitis in lactating goats. *Pesqui. Vet. Bras*. **34**: 633–636. doi:10.1590/S0100-736X2014000700004
54. Fetsch, A. (2018). *Staphylococcus aureus*; Academic Press: Cambridge, MA, USA, ISBN: 978-0-12-809671-0
55. Fey, P.D. and Olson, M.E. (2010). Current concepts in biofilm formation of *Staphylococcus epidermidis*. *Futur. Microbiol.* **5**: 917–933. doi:10.2217/fmb.10.56
56. Foster, T.J. (2005). Immune evasion by staphylococci. *Nature Reviews Microbiology.* **3**: 948- 958.
57. Frozza, C.O., Garcia, C.S.C., Gambato, G., Souza, M.D.O., Salvador, M., Moura, S., Padilha, F.F., Seixas, F.K., Collares, T., Borsuk, S., Dellagostin, O.A., Henriques, J.A.P. and Roesch-Ely, M. (2013). Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. *Food Chem. Toxicol*. **52**: 137–142. doi:10.1016/j.fct.2012.11.013
58. Funari, C.S. and Ferro, V.O. (2006). Análise de própolis. *Cienc. Tecnol. Aliment*. *Campinas*. **26**: 171–178. doi:10.1590/S0101-20612006000100028
59. Gekker, G., Hu, S., Spivak, M., Lokensgard, J.R. and Peterson, P.K. (2005). Anti-HIV-1 activity of propolis in CD4+ lymphocyte and microglial cell cultures. *J. Ethnopharmacol*. **102**: 158–163. doi:10.1016/j.jep.2005.05.045

60. Ghisalberti, E.L. (1979). Propolis: A Review. *J. Bee World.* **60**: 59–84. doi:10.1080/0005772X.1979.11097738
61. Gomes, F. and Henriques, M. (2016). Control of Bovine Mastitis: Old and Recent Therapeutic Approaches. *Curr. Microbiol*. **72**: 377–382. doi:10.1007/s00284-015-0958-8
62. Gonçalves, A.I., Filho, A.A. and Menezes, H. (2005). Estudo comparativo da atividade antimicrobiana de Extratos de algumas árvores nativas. *Arq. Inst. Biol.* **72**: 353–358.
63. Havsteen, B. (1983). Flavonoids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol*. **32**: 1141–1148. doi:10.1016/0006-2952(83)90262-9
64. Hirotaki, S., Sasaki, T., Kuwahara-Arai, K., Hiramatsu, K., 2011. Rapid and accurate identification of human-associated staphylococci by use of multiplex PCR. J. Clin. Microbiol. 49, 3627–3631. doi:10.1128/JCM.00488-11
65. Hristov, K. (2018). Antimicrobial sensitivity of pathogens causing subclinical mastitis in goats in Bulgaria. *Indian J. Anim. Res.* **52**: 296–300. doi:10.18805/ijar.v0iOF.8497
66. Hussein, H.A., Fouad, M.T., Abd El-Razik, K.A., Abo El-Maaty, A.M., D'Ambrosio, C., Scaloni, A., Gomaa, A.M. (2020). Study on prevalence and bacterial etiology of mastitis, and effects of subclinical mastitis and stage of lactation on SCC in dairy goats in Egypt. *Trop. Anim. Health Prod*. doi:10.1007/s11250-020-02331-5
67. Inui, S., Hatano, A., Yoshino, M., Hosoya, T., Shimamura, Y., Masuda, S., Ahn, M.R., Tazawa, S., Araki, Y. and Kumazawa, S. (2014). Identification of the phenolic compounds contributing to antibacterial activity in ethanol extracts of Brazilian red propolis. *Nat. Prod. Res*. **28**: 1293–1296. doi:10.1080/14786419.2014.898146
68. Islam, B., Khan, S.N., Haque, I., Alam, M., Mushfiq, M. and Khan, A.U. (2008). Novel anti-adherence activity of mulberry leaves: Inhibition of *Streptococcus mutans* biofilm by 1-deoxynojirimycin isolated from Morus alba. *J. Antimicrob. Chemother*. **62**: 751–757. doi:10.1093/jac/dkn253
69. Ismael, A.B., Masoud, E.E. and El-Nabtity, S.M. (2009). Potential clinical role of propolis in treatment of clinical ovine and caprine listeriosis. *Vet. Med. J.,* **57**: 723–736. https://www.researchgate.net/publication/283328858
70. Ito, T., Okuma, K., Ma, X.X., Yuzawa, H., Hiramatsu, K. (2003). Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: Genomic island SCC. *Drug Resist. Updat*. **6**: 41–52. doi:10.1016/S1368-7646(03)00003-7
71. Jamali, H., Paydar, M., Radmehr, B., Ismail, S. and Dadrasnia, A. (2015). Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. *Food Control.* **54**: 383–388. doi:10.1016/j.foodcont.2015.02.013
72. Jassim, H. and Abdullah, A. (2014). Study the Inhibitory Effect of Aqueous Extract of *Punica granatum* L. on Resistant *Staphylococcus aureus* Isolate from Mastitic milk. *Journal For Veterinary Medical Sciences.* **5**: 1–8. doi: 10.13140/RG.2.2.32574.72006
73. Jimenez-Granado, R., Sanchez-Rodriguez, M., Arce, C. and Rodriguez-Estevez, V. (2014). Factors affecting somatic cell count in dairy goats: A review. *Spanish J. Agric*. Res. **12**:133–150. doi:10.5424/sjar/2014121-3803
74. Johler, S., Giannini, P., Jermini, M., Hummerjohann, J., Baumgartner, A. and Stephan, R. (2015). Further evidence for staphylococcal food poisoining outbreaks caused by egc-encoded enterotoxins. *Toxins.* **7**: 997–1004. doi: 10.3390/toxins7030997
75. Josenhans, C. and Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *Int. J. Med. Microbiol.* **291**: 605–614. doi:10.1078/1438-4221-00173
76. Kaiser, G. (2020). Horizontal Gene Transfer in Bacteria. https://bio.libretexts.org/@go/page/3150
77. Kamal, R.M. and Bayoumi, M.A. (2015). Efficacy of premilking and postmilking teat dipping as a control of subclinical mastitis in Egyptian Dairy cattle. *Int. Food Res. J.* **22**: 1037–1042.
78. Kasote, D., Ahmad, A., Chen, W., Combrinck, S. and Viljoen, A. (2015). HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis. *Phytochem. Lett*. **11**: 326–331. doi:10.1016/j.phytol.2014.08.017
79. Kateete, D.P., Kimani, C.N., Katabazi, F.A., Okeng, A., Okee, M.S., Nanteza, A., Joloba, M.L. and Najjuka, F.C. (2010). Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Ann. *Clin. Microbiol. Antimicrob*. **9**: 1–7. doi:10.1186/1476-0711-9-23
80. Kautz, F.M., Nickerson, S.C. and Ely, L.O. (2014). Use of a staphylococcal vaccine to reduce prevalence of mastitis and lower somatic cell counts in a registered saanen dairy goat herd. *Res. Vet. Sci.* **97**: 18–19. doi:10.1016/j.rvsc.2014.04.013
81. Kelly, E.J. and Wilson, D.J. (2016). *Pseudomonas aeruginosa* mastitis in two goats associated with an

essential oil–based teat dip. *J. Vet. Diagnostic Investig*. **28**: 760–762. doi:10.1177/1040638716672255
82. Kenny, E.F., Herzig, A., Krüger, R., Muth, A., Mondal, S., Thompson, P.R., Brinkmann, V., Bernuth, H. and Zychlinsky, A. (2017). Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife* **6**. e24437. doi:10.7554/eLife.24437
83. Kiedrowski, M.R., Kavanaugh, J.S., Malone, C.L., Mootz, J.M., Voyich, J.M., Smeltzer, M.S., Bayles, K.W. and Horswill, A.R. (2011). Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. *PLoS One* **6**: e26714. doi:10.1371/journal.pone.0026714
84. Kong, K.F., Vuong, C. and Otto, M. (2006). *Staphylococcus* quorum sensing in biofilm formation and infection. *Int J Med Microbiol*. **296**:133–139. doi: 10.1016/j.ijmm.2006.01.042. Epub 2006 Feb 17
85. Koo, H., Rosalen, P.L., Cury, J.A., Park, Y.K. and Bowen, W.H. (2002). Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. *Antimicrob. Agents Chemother.* **46**: 1302–1309. doi:10.1128/AAC.46.5.1302-1309.2002
86. Koru, O., Toksoy, F., Acikel, C.H., Tunca, Y.M., Baysallar, M., Guclu, A.U., Akca, E., Tuylu, A.O., Sorkun, K., Tanyuksel, M. and Salih, B. (2007). *In vitro* antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens Anaerobe **13**: 140–145. doi: 10.1016/j.anaerobe.2007.02.001
87. Król, W., Scheller, S., Shani, J., Pietsz, G. and Czuba, Z. (1993). Synergistic effect of ethanol extract of propolis and antibiotics on the growth of *Staphylococcus aureus*. *Arzneimittel Forschung*. **43**: 607–609. PMID: 8329008
88. Kumar, R., Gupta, D.K., Bansal, B.K., Singh, S., Sharma, S., Kumar, A. and Uppal, S.K. (2016). Prevalence, Current Antibiogram and Risk Factors Associated With Mastitis in Dairy Goats in Punjab **5**: 4580–4593.
89. Kürekci, C. (2016). Short communication: Prevalence, antimicrobial resistance, and resistant traits of coagulase-negative staphylococci isolated from cheese samples in Turkey. *J. Dairy Sci*. **99**: 2675–2679. doi:10.3168/jds.2015-10725
90. Lana, R.D.P., Camardelli, M.M.L., Rodrigues, M.T., Eifert, E.D.C., Oliveira, M.V.M., Stradiotti, D. and Oliveira, J.S. (2007). Óleo de soja e própolis na alimentação de cabras leiteiras: Consumo de matéria seca e de nutrientes e parâmetros de fermentação ruminal. *Rev. Bras. Zootec.* **36**: 191–197. doi:10.1590/S1516-35982007000100023
91. Langoni, H., Salina, A., Oliveira, G.C., Junqueira, N.B., Menozzi, B.D. and Joaquim, S.F. (2017). Considerações sobre o tratamento das mastites. *Pesqui. Vet. Bras.* **37**: 1261–1269. doi:10.1590/s0100-736x2017001100011
92. Lasa, I. and Penadés, J.R. (2006). Bap: A family of surface proteins involved in biofilm formation. *Res. Microbiol*. **157**: 99–107. doi:10.1016/j.resmic.2005.11.003
93. Laverty, G., Gorman, S.P. and Gilmore, B.F. (2013). Biomolecular mechanisms of staphylococcal biofilm formation. *Future Microbiol*. **8**: 509–524. doi:10.2217/fmb.13.7
94. Lee, J.H. (2003). Methicillin (Oxacillin)-Resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl. environ. microbiol*. **69**: 6489–6494. doi: 10.1128/AEM.69.11.6489–6494.2003
95. Leitner, G., Silanikove, N. and Merin, U. (2008). Estimate of milk and curd yield loss of sheep and goats with intrammamary infection and its relation to somatic cell count. *Small Rumin. Res*. **74**: 221–225. doi:10.1016/j.smallrumres.2007.02.009
96. Liu, G.Y. and Nizet, V. (2009). Color me bad: microbial pigments as virulence factors. *Trends Microbiol*. **17**: 406–413. doi:10.1016/j.tim.2009.06.006
97. Mann, E.E., Rice, K.C., Boles, B.R., Endres, J.L., Ranjit, D., Chandramohan, L., Tsang, L.H., Smeltzer, M.S., Horswill, A.R., Bayles, K.W., 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One 4. doi:10.1371/journal.pone.0005822
98. Marcucci, M.C., Ferreres, F., Garcı, C. (2001). Phenolic compounds from Brazilian propolis with pharmacological activities. Journal of Etnopharmacology. **74**: 105–112.
99. Markey, B., Leonard, F., Archambault, M., Cullinane, A. and Maguire, D. (2013). *Clinical Veterinary Microbiology*. 2ª Edition. eBook ISBN: 9780702055881
100. Martins, C.M.M.R., Pinheiro, E.S.C., Gentilini, M., Benavides, M.L. and Santos, M.V. (2017b). Corrigendum to "Efficacy of a high free iodine barrier teat disinfectant for the prevention of naturally occurring new intramammary infections and clinical mastitis in dairy cows" (J. Dairy Sci. 100:3930–3939). *J. Dairy Sci*. **100**: 8630. doi:10.3168/jds.2017-100-10-8630
101. Martins, K.B., Faccioli, P.Y., Bonesso, M.F., Fernandes, S., Oliveira, A.A., Dantas, A., Zafalon, L.F. and Cunha, M.L.R.S. (2017a). Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical

mastitis. *J. Dairy Sci*. **100**: 2184–2195. doi:10.3168/jds.2016-11583
102. Melchior, M.B., Fink-Gremmels, J. and Gaastra, W. (2006). Comparative assessment of the antimicrobial susceptibility of *Staphylococcus aureus* isolates from bovine mastitis in biofilm versus planktonic culture. *J. Vet. Med. Ser. B Infect. Dis. Vet. Public Heal*. **53**: 326–332. doi:10.1111/j.1439-0450.2006.00962.x
103. Mendonça, E.C.L., Marques, V.F., Melo, D.A., Alencar, T.A., Coelho, I.S., Coelho, S.M.O. and Souza, M.M.S. (2012). Caracterização fenogenotípica da resistência antimicrobiana em *Staphylococcus* spp. isolados de mastite bovina. *Pesqui. Vet. Bras.* **32**: 859–864. doi:10.1590/S0100-736X2012000900008
104. Mendonça, L.S., Mendonça, F.M.R., Araújo, Y.L.F.M., Araújo, E.D., Ramalho, S.A., Narain, N., Jain, S., Orellana, S.C., Padilha, F.F. and Cardoso, J.C. (2015). Chemical markers and antifungal activity of red propolis from Sergipe, Brazil. *Food Sci. Technol*. **35**: 291–298. doi:10.1590/1678-457X.6554
105. Menzies, P.I. and Ramanoon, S.Z. (2001). Mastitis of sheep and goats. *Vet. Clin. North Am. Food Anim. Pract*. **17**: 333–358. doi:10.1016/S0749-0720(15)30032-3
106. Mirzoeva, O.K., Grishanin, R.N. and Calder, P.C. (1997). Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. *Microbiological Research.* **152**: 239-246. doi: 10.1016/S0944-5013(97)80034-1
107. Mishra, A.K., Sharma, N., Kumar, A., Kumar, N., Gundallahalli Bayyappa, M.R., Kumar, S. and Kumar, N. (2014). Isolation, characterization and therapeutic potential assessment of bacteriophages virulent to *Staphylococcus aureus* associated with goat mastitis. *Iran. J. Vet. Res.* **15**: 320–325. doi:10.22099/ijvr.2014.2585
108. Mishra, A.K., Sharma, N., Singh, D.D., Gururaj, K., Abhishek, Kumar, V. and Sharma, D.K. (2018). Prevalence and bacterial etiology of subclinical mastitis in goats reared in organized farms. *Vet. World.* **11**: 20–24. doi:10.14202/vetworld.2018.20-24
109. Moroni, P., Pisoni, G., Antonini, M., Ruffo, G., Carli, S., Varisco, G. and Boettcher, P. (2005). Subclinical Mastitis and Antimicrobial Susceptibility of *Staphylococcus caprae* and *Staphylococcus epidermidis* Isolated from Two Italian Goat Herds. *J. Dairy Sci.* **88**: 1694–1704. doi:10.3168/jds.S0022-0302(05)72841-1
110. Morsy, A.S., Soltan, Y.A., Sallam, S.M.A., Alencar, S.M. and Abdalla, A.L. (2016). Impact of Brazilian red propolis extract on blood metabolites, milk production, and lamb performance of Santa Inês ewes. *Trop. Anim. Health Prod*. **48**: 1043–1050. doi:10.1007/s11250-016-1054-1
111. Mota, R.A. (2008). Aspectos epidemiológicos, diagnóstico e controle das mastites em caprinos e ovinos. *Tecnol. e Ciên. Agropec.* **2**: 57–61.
112. Muhs, A., Lyles, J.T., Parlet, C.P., Nelson, K., Kavanaugh, J.S., Horswill, A.R. and Quave, C.L. (2017). Virulence Inhibitors from Brazilian Peppertree Block Quorum Sensing and Abate Dermonecrosis in Skin Infection Models. *Sci. Rep.* **7**: 1–15. doi:10.1038/srep42275
113. Murray, P.R., Rosenthal, K.S. and Pfaller, M.A. (2017). *Medical microbiology*. Elsevier. Philadelphia. 8ª edition. ISBN 978-0-323-29956-5
114. Nasution, G.S., Suryanto, D., Kusumawati, R.L. (2018). Detection of *mec*A gene from methicillin resistant *Staphylococcus aureus* isolates of north sumatera. *IOP Conf. Ser. Earth Environ. Sci*. 130, 012026. doi:10.1088/1755-1315/130/1/012026
115. Nolkemper, S., Reichling, J., Sensch, K.H. and Schnitzler, P. (2010). Mechanism of herpes simplex virus type 2 suppression by propolis extracts. *Phytomedicine* **17**: 132–138. doi:10.1016/j.phymed.2009.07.006
116. Nourbakhsh, F. and Namvar, A.E. (2016). Detection of genes involved in biofilm formation in *Staphylococcus aureus* isolates. *GMS Hyg. Infect. Control*. **11**: Doc07. doi:10.3205/dgkh000267
117. Obaidat, M.M., Roess, A.A., Mahasneh,A.A. and Al-Hakimi, R.A. (2018). Antibiotic-resistance, enterotoxin gene profiles and farm-level prevalence of *Staphylococcus aureus* in cow, sheep and goat bulk tank milk in Jordan. *International Dairy Journal*. **81**: 28–34. Doi: 10.1016/j.idairyj.2018.02.001
118. O'Toole, G., Kaplan, H.B. and Kolter, R. (2000). Biofilm formation as microbial development. *Annu Rev Microbiol.* **54**: 49–79. doi:10.1146/annurev.micro.54.1.49
119. Otto, M. (2008). Staphylococcal Biofilms. *Curr Top Microbiol Immunol*. **322**: 207–228. PMID: 18453278
120. Pacheco, A., Daleprane, J.B., Freitas, V.S., Ferderbar, S., Hirabara, S., Cuevas, A., Saavedra, N., Curi, R., Abdalla, D.S.P. and Pacheco, L.A.S. (2011). Efecto del Propóleos Chileno sobre el Metabolismo de Glucosa en Ratones Diabéticos. *J. Morphol.* **29**: 754–761. Doi:10.4067/S0717-95022011000300015
121. Park, Y.K. and Ikegaki, M. (1998). Preparation of Water and Ethanolic Extracts of Propolis and Evaluation of the Preparations. *Biosci. Biotechnol. Biochem*. **62**: 2230–2232. doi:10.1271/bbb.62.2230
122. Park, Y.K., Alencar, M.S., Scamparini, A.R.P. and Aguiar, C.L. (2002). Propolis produced in south Brazil, Argentine and Uruguay: phytochemical evidence for the plant origin. *Red Rev. Científicas América Lat*.

32: 997–1003. doi:10.1590/S0103-84782002000600013
123. Parte, A.C., Sardà Carbasse, J., Meier-Kolthoff, J.P., Reimer, L.C. and Göker, M. (2020). List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *International Journal of Systematic and Evolutionary Microbiology*, accepted for publication; doi: 10.1099/ijsem.0.004332
124. Paterson, G.K., Harrison, E.M., Holmes, M.A. (2014). The emergence of *mec*C methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. **22**: 42–47. doi:10.1016/j.tim.2013.11.003
125. Pedrini, S.C.B. e Margatho, L.F.F. (2003). Sensibilidade de microrganismos patogênicos isolados de casos de mastite clínica em bovinos frente a diferentes tipos de desinfetantes. *Arq. Inst. Biol.* **70**: 391–395.
126. Peixoto, R.M., França, C.A., Souza Júnior, A.F., Veschi, J.L.A. Costa, M.M. (2010). Etiologia e perfil de sensibilidade antimicrobiana dos isolados bacterianos da mastite em pequenos ruminantes e concordância de técnicas empregadas no diagnóstico. *Pesqui. Vet. Bras*. **30**: 735–740. doi:10.1590/S0100-736X2010000900005
127. Peixoto, R.M., Araújo, RM.P., Peixoto, L.J.S., Rocha, W.S., Sá, M.C.A. and Costa, M.M. (2016). Milk composition of saanen goats experimentally Infected by *Staphylococcus aureus* and subjected to two Treatment protocols. *Cienc. Anim. Bras*. **17**: 449–458. doi:10.1590/1089-6891v17i334876
128. Pinheiro Machado, G.T., Veleirinho, M.B., Mazzarino, L., Machado Filho, L.C.P., Maraschin, M., Cerri, R.L.A. and Kuhnen, S. (2019). Development of propolis nanoparticles for the treatment of bovine mastitis: In vitro studies on antimicrobial and cytotoxic activities. *Can. J. Anim. Sci*. **99**: 713–723. doi:10.1139/cjas-2018-0173
129. Pinto, M.S., FariaA, J.E., Message, D., Cassini, S.T.A., Pereira, C.S. and Gioso, M.M. (2001). Effect of green propolis extracts on patogenic bacteria isolated from milk of cows with mastitis. *Braz. J. vet. Res. anim. Sci.* **38**: 278–283. Doi:10.1590/S1413-95962001000600006
130. Puggioni, G.M.G., Tedde, V., Uzzau, S., Dore, S., Liciardi, M., Cannas, E.A., Pollera, C., Moroni, P., Bronzo, V., Addis, M.F. (2020). Relationship of late lactation milk somatic cell count and cathelicidin with intramammary infection in small ruminants. *Pathogens*. **9**. doi:10.3390/pathogens9010037
131. Queiroga, C.M. (2007). Mastites ovinas: Epidemiologia, factores de virulência e antigénios imunorrelevantes de agentes etiológicos. Tese doutoramento em Medicina Veterinária. Universidade de Évora. Évora. 328 pp. Repositório Universidade de Évora. http://dspace.uevora.pt/rdpc/handle/10174/4240
132. Queiroga, M.C. (2017). Prevalence and aetiology of sheep mastitis in Alentejo region of Portugal. *Small Rumin. Res*. **153**: 123–130. Doi:10.1016/j.smallrumres.2017.06.003
133. Queiroga, M.C., Duarte, E. and Laranjo, M. (2019). "*Mastitis in sheep: a disease to be taken seriously*", Chapter 1, pp. 1-91. In "Sheep Diseases: Signs, Symptoms and Prevention." Edited by Michael Papst, Nova Science Publishers, Inc., New York, USA. ISBN: 978-1-53615-987-5. https://novapublishers.com/shop/sheep-diseases-signs-symptoms-and-prevention/
134. Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W.J. and Leonard E.C. (2005). *Veterinary Microbiology and Microbial Disease*. Ed. Artmed. ISBN: 9788536304861
135. Rainard, P. (2017). Mammary microbiota of dairy ruminants: Fact or fiction? *Vet. Res*. **48**: 1–10. Doi:10.1186/s13567-017-0429-2
136. Reyes-Jara, A., Cordero, N., Aguirre, J., Troncoso, M. and Figueroa, G. (2016). Antibacterial effect of copper on microorganisms isolated from bovine mastitis. *Front. Microbiol*. **7**: 1–10. Doi:10.3389/fmicb.2016.00626
137. Ribeiro, M.G., Lara, G.H.B., Bicudo, S.D., Souza, A.V.G., Salerno, T., Siqueira, A.K. and Geraldo, J.S. (2007). An unusual gangrenous goat mastitis caused by *Staphylococcus aureus*, *Clostridium perfringens* and *Escherichia coli* co-infection. *Arq. Bras. Med. Vet. E Zootec*. **59**: 810–812. Doi:10.1590/S0102-09352007000300037
138. Rutherford, S.T. and Bassler, B.L. (2012). Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med*. **2**: 1–25. Doi: 10.1101/cshperspect.a012427
139. Saad, M.M.E.D. and Ahmed, M.B.M. (2018). Necessary Usage of Antibiotics in Animals. Antibiot. *Use Anim*. Doi:10.5772/intechopen.71257
140. Salaberry, S.R.S., Saidenberg, A.B.S., Zuniga, E., Melville, P.A., Santos, F.G.B., Guimarães, E.C., Gregori, F. and Benites, N.R. (2015). Virulence factors genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis. *Microb. Pathog*. **85**: 35–39. Doi:10.1016/j.micpath.2015.05.007
141. Santos Neto, T., Mota, R., Silva, L., Viana, D., Lima-Filho, J., Sarubbo, L., Converti, A., Porto, A. (2009). Susceptibility of *Staphylococcus* spp. Isolated from Milk of Goats with Mastitis to Antibiotics and Green Propolis Extracts. *Lett. Drug Des. Discov*. **6**: 63–68. Doi:10.2174/157018009787158599
142. Savka, M.A., Dailey, L., Popova, M., Mihaylova, R., Merritt, B., Masek, M., Le, P., Nor, S.R.M., Ahmad, M.,

Hudson, A.O. and Bankova, V. (2015). Chemical composition and disruption of quorum sensing signaling in geographically diverse United States propolis. *Evidence-based Complement. Altern. Med*. Article ID 472593. **2015**: 10 p. Doi:10.1155/2015/472593

143. Scazzocchio, F., D'Auria, F.D., Alessandrini, D. and Pantanella, F. (2006). Multifactorial aspects of antimicrobial activity of propolis. *Microbiological Research*. **161**: 327-333. Doi: 10.1016/j.micres.2005.12.003
144. Scharn, C.R., Tenover, F.C., Goering, R. V. (2013). Transduction of Staphylococcal Cassette Chromosome mec Elements between Strains of *Staphylococcus aureus*. Antimicrob*. Agents Chemother*. **57**: 5233–5238. doi:10.1128/AAC.01058-13
145. Scherrer, D., Corti, S., Muehlherr, J.E., Zweifel, C. and Stephan, R. (2004). Phenotypic and genotypic characteristics of *Staphylococcus aureus* isolates from raw bulk-tank milk samples of goats and sheep. *Vet. Microbiol*. **101**: 101–107. Doi:10.1016/j.vetmic.2004.03.016
146. Schilling, K.M. and Bowen, W.H. (1992). Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infect. Immun*. **60**: 284–295. Doi: 10.1128/IAI.60.1.284-295.1992
147. Schleifer, K-H., and Bell, J.A. (2009). Family VIII. Staphylococcaceae fam. Nov. By De Vos P., Garrity G.M., Jones D., et al., In: *Bergey's manual of systematic bacteriology*, 2nd ed, vol 3. The Firmicutes. Springer Dordrecht Heidelberg London New York. Pp.392-426. ISBN: 978-0-387-95041-9 e-ISBN: 978-0-387-68489-5. Doi: 10.1007/b92997
148. Selem, A.S.M.A. (2012). *Effect of propolis on ruminal fermentation, reproductive and productive performance of Santa Ines ewes*. Tese de doutoramento em ciências. Área de Concentração: Energia Nuclear na Agricultura e no Ambiente. Univ. de São Paulo, Piracicaba, Brazil. 121 pp. doi: 10.11606/T.64.2012.tde-22042013-164545
149. Silanikove, N., Merin, U. and Leitner, G. (2014). On effects of subclinical mastitis and stage of lactation on milk quality in goats. *Small Rumin. Res.* **122**: 76–82. doi:10.1016/j.smallrumres.2014.07.018
150. Silva, C.S.R., Villaça, C.L.P.B., Peixoto, R.M., Mota, R.A., Ribeiro, M.F. and Costa, M.M. (2012). Antibacterial effect of Brazilian brown propolis in different solvents against *Staphylococcus* spp. isolated from caprine mastitis. *Cienc. Anim. Bras*. **13**: 247–251. doi:10.5216/cab.v13i2.15121
151. Silva, E.C.A. (2003). Preparo do extrato de própolis legal. Revista Mensagem Doce – *APACAME*. Available from: http://www.apacame.org.br/mensagemdoce/70/artigo2.htm
152. Silva, F.G.B., Yamamoto, S.M., Silva, E.M.S., Queiroz, M.A.Á., Gordiano, L.A. and Formiga, M.A. (2015). Extrato de própolis e monensina sódica sobre os parâmetros de fermentação ruminal e hematológicos de ovinos. *Acta Sc–. - Anim. Sci*. **37**: 273–280. doi:10.4025/actascianimsci.v37i3.25725
153. Srivastava, S. and Dutt, S. (2013). Imatinib mesylate resistance and mutations: An Indian experience. *Indian J. Med. Paediatr. Oncol*. **34**: 213–220. doi:10.4103/0971-5851.123748
154. Stepanović, S., Antić, N., Dakić, I. and Švabić-Vlahović, M. (2003). *In vitro* antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. *Microbiol. Res*. **158**: 353–357. doi:10.1078/0944-5013-00215
155. Szweda, P., Schielmann, M., Milewski, S., Frankowska, A. and Jakubczak, A. (2012). Biofilm production and presence of ica and bap genes in *Staphylococcus aureus* strains isolated from cows with mastitis in the Eastern Poland. *Polish J. Microbiol*. **61**: 65–69. doi:10.33073/pjm-2012-009
156. Takaisi-Kikuni, N.B. and Schilcher, H. (1994). Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. *Planta Med*. **60**: 222–227. doi:10.1055/s-2006-959463
157. Tollersrud, T., Nørstebø, P.E., Engvik, J.P., Andersen, S.R., Reitan, L.J. and Lund, A. (2002). Antibody responses in sheep vaccinated against *Staphylococcus aureus* mastitis: A comparison of two experimental vaccines containing different adjuvants. *Vet. Res. Commun*. **26**: 587–600. doi:10.1023/A:1020960402112
158. Tormo, M.Á., Knecht, E., Götz, F., Lasa, I., Penadés, J.R. (2005). Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: Evidence of horizontal gene transfer? Microbiology **151**: 2465–2475. doi:10.1099/mic.0.27865-0
159. Tortora, G.J., Case, CL. and Funke, B.R. (2017). *Microbiologia* - 12ª edition., Porto Alegre. Artmed. P.964. ISBN 9780321929150
160. Trentin, D.S., Silva, D.B., Amaral, M.W., Zimmer, K.R., Silva, M. V., Lopes, N.P., Giordani, R.B. and Macedo, A.J. (2013). Tannins Possessing Bacteriostatic Effect Impair *Pseudomonas aeruginosa* Adhesion and Biofilm Formation. *PLoS One* **8**. e66257. doi:10.1371/journal.pone.0066257
161. Trusheva, B., Popova, M., Bankova, V., Simova, S., Marcucci, M.C., Miorin, P.L., Pasin, F.D.R. and

Tsvetkova, I. (2006). Bioactive constituents of Brazilian red propolis. Evidence-based Complement. *Altern. Med*. **3**: 249–254. doi:10.1093/ecam/nel006

162. U.S. Congress, Office of Technology Assessment. (1995). Impacts of Antibiotic-Resistant Bacteria, OTA-H-629 (Washington, DC: U.S. Government Printing Office, September).
163. Uzel, A., Sorkun, K., Onçag, O., Cogulu, D., Gençay, Omur. and Salih, B. (2005). Chemical compositions and antimicrobial activities of four different Anatolian propolis samples. *Microbiology Research*. **160**: 189-195.
164. Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F. and Vliegher, S. (2014). Invited review: Effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. *J. Dairy Sci*. **97**: 5275–5293. doi:10.3168/jds.2013-7775
165. Van Leeuwen, W., Roorda, L., Hendriks, W., Francois, P. and Schrenzel, J., (2008). A *nuc*-deficient meticillin-resistant *Staphylococcus aureus* strain. FEMS Immunol. Med. Microbiol. **54**: 157. doi:10.1111/j.1574-695X.2008.00478.x
166. Vargas, A.C., Loguercio, A.P., Witt, N.M. e Viana, L.R. (2004). Atividade antimicrobiana "in vitro" de extrato alcóolico de própolis. *Ciência Rural, Santa Maria*. **34**: 159–163. ISSN 0103-8478
167. Vasconcellos, S.A. e Ito, F.H. (2011). Principais zoonoses transmitidas pelo leite. *Revista de Educação Continuada em Medicina Veterinária*. **9**: 32–37. doi:10.36440/recmvz.v9i1.388
168. Vasiľ, M. (2007). Aetiology of mastites and enterotoxin production by *Staphylococcus* spp. isolated from milk of two sheep herds. *Slovak J. Anim. Sci.* **40**: 189–195.
169. Vasudevan, P., Nair, M.K.M., Annamalai, T. and Venkitanarayanan, K.S. (2003). Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. *Vet. Microbiol*. **92**: 179–185. doi:10.1016/S0378-1135(02)00360-7
170. Veiga, R.S., Mendonça, S., Mendes, P.B., Paulino, N., Mimica, M.J., Lagareiro Netto, A.A., Lira, I.S., López, B.G.C., Negrão, V. and Marcucci, M.C. (2017). Artepillin C and phenolic compounds responsible for antimicrobial and antioxidant activity of green propolis and *Baccharis dracunculifolia* DC. *J. Appl. Microbiol*. **122**: 911–920. doi:10.1111/jam.13400
171. Veloz, J.J., Saavedra, N., Lillo, A., Alvear, M., Barrientos, L. and Salazar, L.A. (2015). Antibiofilm Activity of Chilean Propolis on *Streptococcus mutans* Is Influenced by the Year of Collection. *Biomed Res. Int*. doi:10.1155/2015/291351
172. Vitale, M., Gaglio, S., Galluzzo, P., Cascone, G., Piraino, C., Di Marco Lo Presti, V. Alduina, R. (2018). Antibiotic Resistance Profiling, Analysis of Virulence Aspects and Molecular Genotyping of *Staphylococcus aureus* Isolated in Sicily, Italy. *Foodborne Pathog*. *Dis.* **15**: 177–185. doi:10.1089/fpd.2017.2338
173. Williams, J.D. (1999). β-Lactamases and β-lactamase inhibitors. **1**: 7–9.
174. Woisky, R.G. and Salatino, A. (1998). Analysis of propolis: Some parameters and procedures for chemical quality control. *J. Apic. Res*. **37**: 99–105. doi:10.1080/00218839.1998.11100961
175. Wojtyczka, R.D., Kępa, M., Idzik, D., Kubina, R., Kabała-Dzik, A., Dziedzic, A. and Wąsik, T.J. (2013). *In Vitro* Antimicrobial Activity of Ethanolic Extract of Polish Propolis against Biofilm Forming *Staphylococcus epidermidis* Strains. *Evidence-Based Complement*. *Altern. Med*. **2013**: 1–11. doi:10.1155/2013/590703
176. Xu, J., Tan, X., Zhang, X., Xia, X. and Sun, H. (2015). The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. *Microb. Pathog*. **88**: 29–38. doi:10.1016/j.micpath.2015.08.004
177. Yildiz, O., Karahalil, F., Can, Z., Sahin, H. and Kolayli, S. (2014). Total monoamine oxidase (MAO) inhibition by chestnut honey, pollen and propolis. *Journal of Enzyme Inhibition and Medicinal Chemistry*, **29**: 690-694. doi:10.3109/14756366.2013.843171
178. Yuan, Y.G., Peng, Q.L. and Gurunathan, S. (2017). Effects of silver nanoparticles on multiple drug-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from mastitis-infected goats: An alternative approach for antimicrobial therapy. *Int. J. Mol. Sci*. **18**: 569. doi:10.3390/ijms18030569
179. Zafalon, L.F., Cunha, M.L.R.S., Riboli, D.F.M. e Pilon, L.E. (2017). Persistência de *Staphylococcus* Coagulase-Negativos em Glândulas Mamárias de Ovelhas com Mastite Subclínica Após o Tratamento Antimicrobiano à Secagem. *Ciência Anim. Bras*. **18**: 1–11. doi:10.1590/1089-6891v18e-44328

Chapter 3

3. Virulence Factors in *Staphylococcus* Associated with Small Ruminant Mastitis: Biofilm Production and Antimicrobial Resistance Genes

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Virulence Factors in *Staphylococcus* Associated with Small Ruminant Mastitis: Biofilm Production and Antimicrobial Resistance Genes

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Abstract

Small ruminant mastitis is a serious problem, mainly caused by *Staphylococcus* spp. Different virulence factors affect mastitis pathogenesis. The aim of this study was to investigate virulence factors genes for biofilm production and antimicrobial resistance to β-lactams and tetracyclines in 137 staphylococcal isolates from goats (86) and sheep (51). The presence *of coa, nuc, bap, icaA, icaD, blaZ, mecA, mecC, tetK,* and *tetM* genes was investigated. The *nuc* gene was detected in all *S. aureus* isolates and in some coagulase-negative staphylococci (CNS). None of the *S. aureus* isolates carried the *bap* gene, while 8 out of 18 CNS harbored this gene. The *icaA* gene was detected in *S. aureus* and *S. warneri*, while *icaD* only in *S. aureus*. None of the isolates carrying the *bap* gene harbored the *ica* genes. None of the biofilm-associated genes were detected in 14 isolates (six *S. aureus* and eight CNS). An association was found between *Staphylococcus* species and resistance to some antibiotics and between antimicrobial resistance and animal species. Nine penicillin-susceptible isolates exhibited the *blaZ* gene, questioning the reliability of susceptibility testing. Most *S. aureus* isolates were susceptible to tetracycline, and no cefazolin or gentamycin resistance was detected. These should replace other currently used antimicrobials.

Keywords: mastitis; staphylococci; virulence factors; genes; biofilm; antimicrobial resistance

1. Introduction

Mastitis is the inflammation of the mammary gland, mainly due to intramammary infection (IMI). In small ruminants, this disease is considered a serious economic issue due to the mortality of lactating females, cost of treatment, reduced milk yield and quality [1,2], as well as a public health concern associated with risk of consumer food poisoning [3,4].

Several pathogens can cause mastitis in small ruminants; however, species of staphylococci are the most frequently isolated microorganisms from goat and sheep milk [2,5–8]. *Staphylococcus aureus* is one of the main pathogens associated with mastitis in small ruminants [9]. Incidence of clinical mastitis in sheep due to this bacterium may reach 20% with a mortality rate between 25% and 50%, and the affected mammary halves in surviving animals are frequently destroyed. Chronic mastitis may cause a 25 to 30% reduction in milk yield from the affected udder [10].

Coagulase negative staphylococci (CNS), although not as virulent as *S. aureus*, often cause subclinical mastitis in small ruminants [5,11–13]. This type of infection, most times not detected by the farmer, clearly reduces milk production, also changing milk composition, indirectly impairing the milk product's properties [14]. CNS are the most prevalent pathogens of the mammary gland in goats and sheep with subclinical mastitis, affecting 60% to 80.7% in goats and 45% to 48% in sheep [1]. Other authors have reported as much as 70.1% of subclinical mastitis in sheep is caused by CNS [5].

Virulence factors are bacterial molecules that enhance their capacity to establish and to survive within the host and, thus, contribute to bring damage to the host. Staphylococci possess a wide array of virulence factors [15]. Coagulase enzyme acts on plasma fibrinogen to form fibrin clots that protect the microorganisms from phagocytosis and shelter them from other cellular and soluble host defence mechanisms. This enzyme, encoded by the *coa* gene, is commonly used to distinguish coagulase positive staphylococci (CPS), namely *S. aureus*, *S. intermedius*, and *S. pseudintermedius*, from CNS species [16]. Nevertheless, this gene has been found also in species known as CNS such as *S. epidermidis*, *S. chromogenes*, and *S. hominis* [17]. The coa gene has also recently been associated with biofilm production [18].

The staphylococcal nuclease is a thermostable nuclease encoded by the *nuc* gene [19], which hydrolyzes DNA and RNA in host cells, causing tissue destruction and spreading of staphylococci [20], also promoting the escape of microorganisms when retained by neutrophil extracellular traps (NETs), allowing the bacteria to evade this host defence mechanism [21,22]. For decades, the *nuc* gene has been considered the golden standard for *Staphylococcus aureus* identification and is still used presently [23–25]. However, the *nuc* gene has been detected in staphylococci of animal origin other than *S. aureus* [26]. Moreover, the *nuc* encoded staphylococcal thermonuclease is a biofilm inhibitor that degrades the environmental DNA (eDNA) associated with biofilm [27,28].

The production of biofilm is considered a major virulence factor that, besides protecting from host defence mechanisms, also shields bacteria against antimicrobial agents [29]. Furthermore, the persistence of biofilm-producing isolates in the dairy environment enhances the dispersal of virulence factors though the transfer of genetic material to other bacteria [30]. Biofilm major components are an exopolysaccharide matrix, proteins, and eDNA, along with the bacterial cells [31]. The exopolysaccharide, polysaccharide intercellular adhesin (PIA), is also a non-protein adhesin [32] assisting in bacterial adhesion to different surfaces, comprising the first critical event in the establishment of an infection [33]. Staphylococcal PIA is encoded by the *ica* operon [34], and biofilm-associated protein (Bap) is a surface protein connected to the cell wall encoded by the *bap* gene [35].

Antimicrobial resistance (AMR) is a major problem hampering the treatment of an ever ncreasing range of infections caused by bacteria [36]. Staphylococci resistance has been reported for different antimicrobials used for mastitis control in small ruminants [7,36–38]. Genes often described in *Staphylococcus* spp. isolated from the milk of small ruminants are *blaZ* and *mecA*, responsible for β-lactam resistance and *tetK* and *tetM*, accounting for tetracycline resistance [39–41]. The presence of resistant bacteria in contaminated food products may lead to the transfer of resistance genes to the indigenous microbiota in the human gut [42].

The aim of this study was to identify *Staphylococcus* species isolated from small ruminants' milk samples and investigate the presence of genes encoding virulence factors

associated with both biofilm (*coa, nuc, bap, icaA,* and *icaD*) and antimicrobial resistance to β-lactams (*blaZ, mecA,* and *mecC*) and tetracyclines (*tetK* and *tetM*).

2. Results and Discussion

2.1. Bacteriological Results

From the 646 milk samples collected from goats (508) and sheep (138), bacteriological cultures resulted positive in 191 samples: 131 goat milk and 60 sheep milk. A total of 137 staphylococcal isolates were recovered, of which 86 were isolated from goat and 51 from sheep milk samples.

2.2. Staphylococci Identification

Excellent (96 to 99% probability) and very good (93 to 95% probability) identification was observed for most *Staphylococcus*. Unidentified isolates and isolates with low discrimination results were confirmed by 16S rRNA gene sequencing. Concerning goat milk samples, four *S. aureus*, one *Staphylococcus* sp., and 12 different CNS species were found: *S. caprae* (25), *S. chromogenes* (10), *S. epidermidis* (11), *S. simulans* (8), *S. warneri* (7), *S. capitis* (4), *S. lentus* (4), *S. hominis* (4), *S. hyicus* (3), *S. auricularis* (2), *S. haemolyticus* (2), and *S. equorum* (1). On the other hand, 31 *S. aureus* and seven different CNS species were recovered from sheep milk samples: *S. chromogenes* (9), *S. epidermidis* (3), *S. auricularis* (2), *S. haemolyticus* (2), *S. simulans* (2), *S. lentus* (1), and *S. rostri* (1). *Staphylococcus rostri* has only been seldom isolated from the milk of a sheep with subclinical mastitis [43,44]. In the CNS group, *S. caprae* was the most found species and was isolated only from goat's milk samples. It is a commensal organism that prevails in the skin of the goat udder [19] This species is most commonly found in cases of goat mastitis [37,45–47], but it was also isolated from sheep [5,48], buffalo [17], and cow's milk [49]. In this study, other *Staphylococcus* species were only isolated from goats: *S. warneri*, *S. capitis*, *S. hominis*, *S hyicus*, and *S. equorum*. This was probably because the sheep sampling was smaller, since all these species have been isolated before from sheep milk by several other authors [44].

2.3. Biofilm Production

Of the 137 *Staphylococcus* isolates analyzed, 103 were biofilm producers (75%). Biofilm forming isolates belong to the following species: *S. aureus* (29/35), *S. caprae* (22/25), *S. chromogenes* (12/19), *S. epidermidis* (11/14), *S. warneri* (7/7), *S. simulans*(6/10), *S. auricularis* (4/4), *S. capitis* (3/4), *S. lentus* (3/5), *S. haemolyticus* (2/4), *S. hominis* (2/4), *S. equorum* (1/1), and *Staphylococcus* sp. (1/1). All *S. epidermidis* goat isolates were found to produce biofilm in the present study, in accordance with the findings of others authors that reported *S. epidermidis* as the most commonly found species in biofilm-associated human infections [50]. However, none of the sheep *S. epidermidis* isolates were biofilm producers. In fact, other studies had already reported only 8% of biofilm-producing isolates among sheep mastitis *S. epidermidis* [51].

2.4. Genes Associated to Biofilm

We investigated the presence of *coa* and *nuc* genes in all 137 staphylococcal isolates, mainly for identification purposes and due to historical reasons. In fact, the ability of a strain to produce coagulase, encoded by the *coa* gene, is the basis of the primary classification of staphylococci in coagulase-positive or coagulase-negative [16]. All *S. aureus* isolates (35) harbored the *coa* gene, as well as isolate B200E1, not identified to the species level. Based on this result, this isolate was probable also *S. aureus*. Therefore, the 101 *Staphylococcus* isolates not carrying the *coa* gene were confirmed as CNS. Furthermore, in the present study, different amplicons of the *coa* gene with band sizes ranging from 400 to 900 bp were detected (Figure 3.1), as already reported by others [52–55]. In fact, the *coa* gene also has a discriminatory power between isolates because of the heterogeneity of its 3' variable region containing 81-bp tandem short sequence repeats (SSR) [56–58].

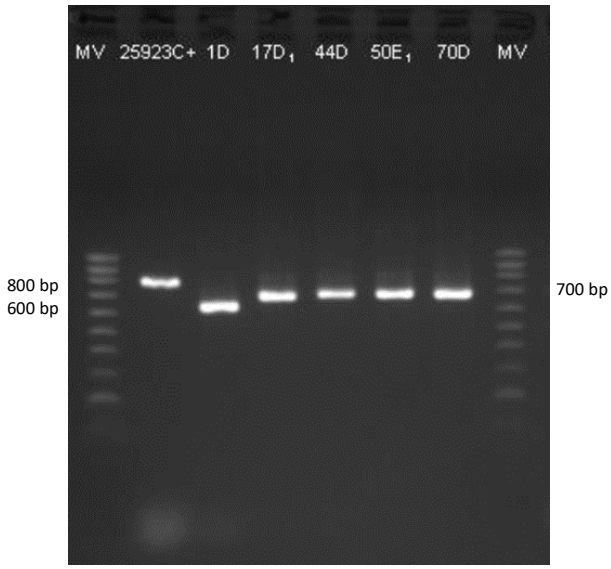

Figure 3.1. Agarose gel electrophoresis of *S.aureus coa* gene PCR products. NZYDNA Ladder V (200-1000 bp) (NZYTech, Lisbon, Portugal).

The *nuc* gene was detected in 67 out of 137 isolates (48.9%), of which only 35 are *S. aureus*. The other *nuc* positive isolates include: *S. chromogenes* (8), *S. warneri* (4), *S. auricularis* (3), *S. caprae* (3), *S. hyicus* (3), *S. lentus* (3), *S. epidermidis* (2), *S. simulans* (2), *S. capitis* (1), *S. haemolyticus* (1), *S. hominis* (1), and *Staphylococcus* sp. (1). Furthermore, an association was found between the *Staphylococcus* species and the presence of the *nuc* gene ($\chi 2=70.968$, df=14, $P<0.001$). In fact, all *S. aureus* harbour the *nuc* gene, while most CNS (70/101) do not. However, the *nuc* gene was also detected in more than 50% of the isolates in some CNS species: *S. warneri* (4/7), *S. lentus* (3/5), *S. auricularis* (3/4), and *S. hyicus* (3/3).

The presence of the *nuc* gene was used in the past to identify *S. aureus* [23,25]. The *nuc* gene is present in most *S. aureus* isolates, however some isolates not carrying this gene have been described [59,60]. Moreover, the *nuc* gene has also been detected in other species of *Staphylococcus*, both CPS and CNS [61,62].

For the detection of the biofilm production genes, *bap*, *icaA* and *icaD*, the 44 *nuc*-positive biofilm-producing isolates were selected. *nuc*-positive biofilm-producing staphylococci and biofilm-associated genes are shown in Table 3.1.

Table 3.1. *nuc*-positive biofilm-producing staphylococci isolates and biofilm-associated genes.

Isolate	Origin	Animal	Bacterial species	*coa*	*nuc*	*bap*	*ica*A	*ica*D
1D	PT	goat	*S. aureus*	+	+	-	+	+
13D1	PT	goat	*S. warneri*	-	+	-	-	-
17D1	PT	goat	*S. aureus*	+	+	-	-	+
44D	PT	goat	*S. aureus*	+	+	-	+	+
47D2	PT	goat	*S. chromogenes*	-	+	+	-	-
50E1	PT	goat	*S. aureus*	+	+	-	+	+
54E1	PT	goat	*S. warneri*	-	+	+	-	-
54E2	PT	goat	*S. warneri*	-	+	-	+	-
55D1	PT	goat	*S. capitis*	-	+	-	-	-
60D2	PT	goat	*S. chromogenes*	-	+	+	-	-
65D	PT	goat	*S. caprae*	-	+	-	-	-
70D	PT	sheep	*S. aureus*	+	+	-	-	+
71E	PT	sheep	*S. aureus*	+	+	-	-	-
72D	PT	sheep	*S. aureus*	+	+	-	-	+
72E	PT	sheep	*S. aureus*	+	+	-	-	+
83D	PT	sheep	*S. aureus*	+	+	-	-	-
B51E	BR	goat	*S. chromogenes*	-	+	-	-	-
B64	BR	goat	*S. chromogenes*	-	+	-	-	-
B76E	BR	goat	*S. chromogenes*	-	+	+	-	-
B101	BR	goat	*S. warneri*	-	+	-	+	-
B159D	BR	goat	*S. chromogenes*	-	+	+	-	-
B159E	BR	goat	*S. chromogenes*	-	+	+	-	-
B190D	BR	goat	*S. auricularis*	-	+	-	-	-
B209D2	BR	goat	*S. simulans*	-	+	+	-	-
B209E	BR	goat	*S. simulans*	-	+	-	-	-
B219D3	BR	sheep	*S. auricularis*	-	+	-	-	-
B219D5	BR	sheep	*S. aureus*	+	+	-	-	-
B223D	BR	sheep	*S. aureus*	+	+	-	-	-
B250D	BR	sheep	*S. auricularis*	-	+	+	-	-
CQ152E1	PT	sheep	*S. aureus*	+	+	-	+	+
CQ185D1	PT	sheep	*S. aureus*	+	+	-	+	+
CQ196E	PT	sheep	*S. aureus*	+	+	-	-	+
CQ201E	PT	sheep	*S. aureus*	+	+	-	-	+
CQ268D1	PT	sheep	*S. aureus*	+	+	-	-	+
CQ270E1	PT	sheep	*S. aureus*	+	+	-	-	-
CQ285D	PT	sheep	*S. aureus*	+	+	-	-	+
CQ286D	PT	sheep	*S. aureus*	+	+	-	-	+
CQ290D1	PT	sheep	*S. aureus*	+	+	-	-	+
CQ290D2	PT	sheep	*S. aureus*	+	+	-	-	+
CQ296D	PT	sheep	*S. aureus*	+	+	-	-	+
CQ335E	PT	sheep	*S. aureus*	+	+	-	-	-
CQ336E2	PT	sheep	*S. aureus*	+	+	-	-	+
CQ349D	PT	sheep	*S. aureus*	+	+	-	-	-
CQ354D	PT	sheep	*S. aureus*	+	+	-	-	+

PT-Portugal; BR-Brazil.

The *bap* gene was amplified in eight isolates: *S. chromogenes* (5), *S. auricularis* (1), *S. simulans* (1), and *S. warneri* (1). None of the *S. aureus nuc*-positive biofilm-producing isolates carries the *bap* gene. In fact, the *bap* gene has been reported mainly in *S. aureus* strains isolated from cattle [24,63,64]. However, Martins et al. [65] have detected the *bap* gene in four sheep milk *S. aureus* isolates. In our study, 8 out of 18 CNS *nuc*-positive biofilm producing isolates harbored the *bap* gene. The *bap* gene encodes a cell wall associated protein named Bap (for biofilm associated protein), which enhances biofilm formation as it mediates bacterial primary attachment to abiotic surfaces and intercellular adherence [35]. Other studies have reported the presence of the bap gene in several CNS isolates [66].

The presence of the *icaA* gene was detected in seven isolates: *S. aureus* (5) and *S. warneri* (2). On the other hand, the *icaD* gene was present in 19 *S. aureus* isolates. Furthermore, five *S. aureus* isolates carried both *icaA* and *icaD* genes simultaneously. Xu, Tan, Zhang, Xia, and Sun [59] detected the *icaD* gene in 20 out of 28 *S. aureus* bovine mastitis isolates, while it was not detected in any of the 76 CNS analyzed. The same authors reported the absence of the *icaA* gene in all analyzed staphylococcal isolates [59]. No isolate carrying the *bap* gene harbored the *ica* operon genes, as reported before by other authors [67]. However, Marques et al. [68] found one single bovine mastitis *S. aureus* isolate (out of 20) that simultaneously carried *bap, icaA,* and *icaD*.

None of the three biofilm-associated genes were detected in 14 of the *nuc*-positive biofilm-producing isolates: *S. aureus* (6) and CNS (8). Other authors have also reported the absence of *bap, icaA,* and *icaD* genes in biofilm-producing *S. aureus* [24,69,70]. Despite no association being found between the presence of the *nuc* gene and biofilm production, most biofilm-producing isolates harbored the *nuc* gene (53.4%), while it was only detected in about 35% of the non-producers. Nevertheless, Kiedrowski, Kavanaugh, Malone, Mootz, Voyich, Smeltzer, Bayles, and Horswill [28] described an inverse correlation between Nuc thermonuclease activity and biofilm formation and confirmed the important role for eDNA in the *S. aureus* biofilm matrix.

Apparently, CNS produce biofilm mainly via Bap, as already suggested by Zuniga et al. [71], who found the *bap* gene to be more frequently present in CNS when compared to CPS.

Meanwhile, most *S. aureus* seem to form biofilm through PIA since they harbor the *icaA* and *icaD* genes. Other authors have reported that a low prevalence of the *bap* gene in *S. aureus* indicates that the *ica* operon-dependent mechanism may be the main responsible for the adhesion and biofilm formation in this species [68]. Notwithstanding, it has been reported that biofilm synthesis in *S. aureus* can also be encoded by the *bap* gene [72].

Other biofilm formation mechanisms in staphylococci not harboring the classical biofilm-production genes, *bap, icaA,* and *icaD*, need to be explored. Furthermore, some of the isolates not carrying *bap, icaA,* and *icaD* also did not harbor the *coa* gene, which has been reported as associated with biofilm formation [18]. However, the nuc gene might be an important factor to consider since all 44 isolates were biofilm producers and harbored the *nuc* gene, although Nuc has been referred to as a biofilm inhibitor [27,28].

2.5. Antimicrobial Resistance

Out of 137 staphylococcal isolates analyzed for antimicrobial susceptibility, 15 were multidrug resistant, 36 were non-susceptible to two antimicrobial categories, and 61 to one antimicrobial category, according to the classification proposed by Magiorakos et al. [73]. Moreover, no antimicrobial resistances were detected in 24 staphylococcal isolates. Staphylococci isolated from milk from small ruminants with mastitis are known for their multiresistance [74]. In this work, the multidrug resistant (MDR) isolates belonged to the following species: *S. aureus* (8), *S. lentus* (3), *S. chromogenes* (2), *S. caprae* (1), and *S. warneri* (1). Contrarily, Taponen and Pyorala [75] reported that multiresistance was more common in CNS than in *S. aureus* from bovine mastitis. Susceptibility patterns of CPS and CNS isolates are shown in Figure 3.2. For most antimicrobials tested, a higher percentage of resistant isolates was observed among CNS when compared to CPS. Vasileiou et al. [76] also reported more resistant CNS isolates than *S. aureus*. However, mastitis caused by CNS responds much better to antimicrobial treatment than *S. aureus* mastitis [75]. Staphylococcal isolates were mainly non-susceptible to streptomycin (50/137), penicillin (38/137), ampicillin (34/137), lincomycin (33/137), oxacillin (22/137), cloxacillin (21/137), and tetracycline (17/137), as previously reported [77] (Figure 3.2). Moreover, most CPS isolates were non-susceptible to streptomycin and lincomycin. On the other hand, CNS isolates were mostly non-susceptible to the β-lactams and tetracyclines. In addition, an

association was found between *Staphylococcus* species and antimicrobial resistance to penicillin (χ 2 = 45.981, df = 14, p < 0.001), ampicillin (χ 2 = 48.327, df = 14, p < 0.001), streptomycin (χ 2 = 137.705, df = 28, p < 0.001), lincomycin (χ 2 = 156.536, df = 28, p < 0.001), cephalexin (χ 2 = 57.219, df = 28, p < 0.05), and tetracycline (χ 2 = 51.626, df = 28, p < 0.05).

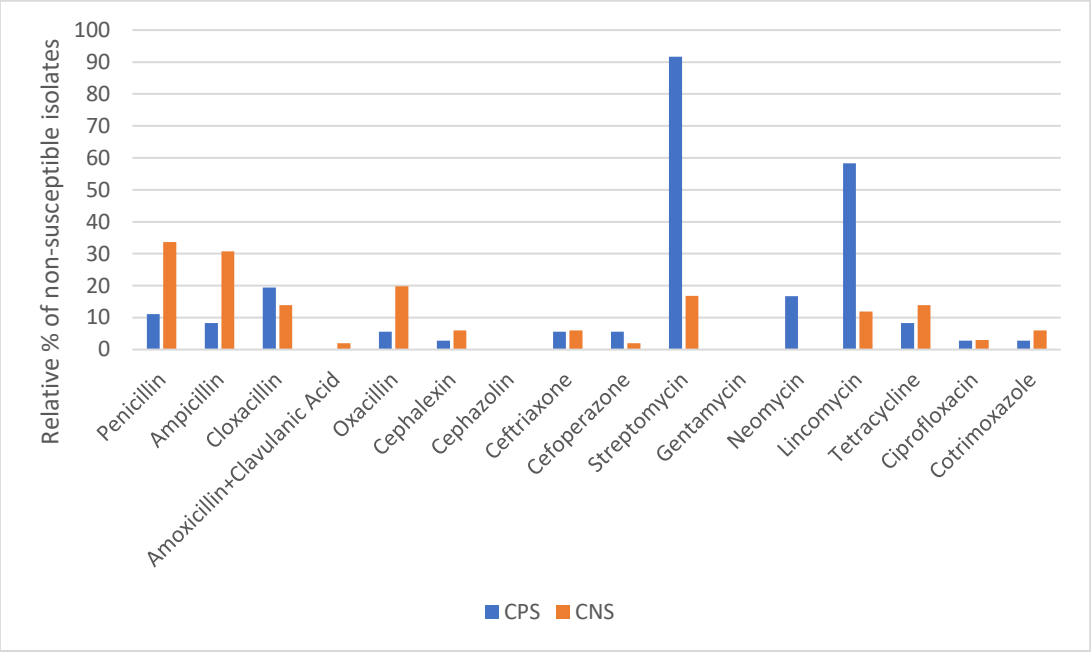

Figure 3.2. Susceptibility patterns of CPS (n=36) and CNS (n=101) isolates to antimicrobials.

Regarding the results shown by the correspondence analysis, most *S. caprae* and *S. capitis* isolates were resistant to penicillin and ampicillin, while all other staphylococci were mostly susceptible to these antimicrobials (Figure 3.3). Most *S. aureus* isolates exhibited an intermediate susceptibility pattern to streptomycin and lincomycin [78]. Additionally, all *S. hyicus* isolates were resistant to streptomycin, while *S. lentus* and *S. rostri* were resistant to lincomycin (Figure 3.3).

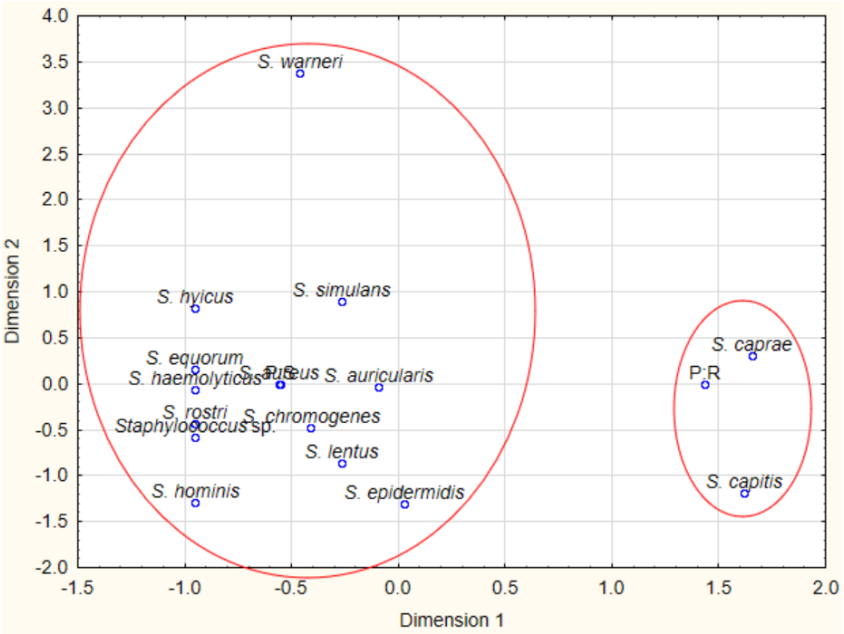

Figure 3.3. CA biplots of the relationship between bacterial species and tolerance to the antimicrobials penicillin (P), ampicillin (AMP), streptomycin (S), and lincomycin (MY).

No staphylococci resistant to cefazolin and gentamycin were identified. Moreover, no non-susceptible *S. aureus* isolates were found to amoxicillin + clavulanic acid. A number of CNS isolates, although resistant to penicillinase-labile penicillins, were susceptible to amoxicillin + clavulanic acid, which was expected due to the inhibitory action of clavulanic acid against β-lactamases [79]. Regarding CNS isolates, none were found to be resistant to neomycin.

One *S. aureus* and one CPS *Staphylococcus* sp. were found to be resistant to oxacillin, while CNS oxacillin resistant isolates belonged to eight species: *S. chromogenes* (5), *S. caprae* (4), *S. lentus* (3), *S. simulans* (3), *S. epidermidis* (2), *S. auricularis* (1), *S. hominis* (1), and *S. warneri* (1). Other authors previously reported the presence of methicillin resistant coagulase-negative staphylococci (MR-CNS) [80,81].

Regarding tetracycline, most *S. aureus* isolates (32/35) were susceptible, while nonsusceptible isolates belonged to the following CNS species: *S. caprae* (4), *S. haemolyticus* (3), *S. lentus* (2), *S. capitis* (1), *S. hominis* (2), *S. rostri* (1), and *S. warneri* (1). Tetracycline has been widely used in veterinary medicine, and other studies have reported a higher percentage of resistant isolates: 42.8% [82] and 28.9% [45]. On the contrary, our results show a relatively low percentage of non-susceptible isolates (12.4%). In recent

years, there has been an abusive use of more recent antimicrobial molecules, such as cephalosporins and quinolones, that may justify the observed reversal in the patterns of resistance to tetracyclines. To avoid the use of critically important antimicrobials for human medicine, tetracyclines, gentamycin, or cefazolin, a first-generation cephalosporin, may be an option for the control of mastitis in small ruminants. However, there should be a tight control over the development of antimicrobial resistances.

Interestingly, an association between resistance to some antibiotics and animal species was found: penicillin (_2 = 26.931, df = 1, p < 0.001), ampicillin (_2 = 26.818, df = 1, p < 0.001), oxacillin (_2 = 6.241, df = 1, p < 0.05), streptomycin (_2 = 26.231, df = 2, p < 0.001), and lincomycin (_2 = 20.831, df = 2, p < 0.001). For example, isolates from goats (G) were more resistant than sheep (S) isolates to β-lactams, penicillin (G-43%; S-2%), ampicillin (G-39%; S- = 0%), and oxacillin (G-22%; S-6%). These differences might be due to different management systems, as suggested by Barrero-Domínguez et al. [45], who reported sheep and goat staphylococcal isolates with the same pulsotypes to exhibit distinct resistance patterns.

2.6. Antimicrobial Resistance Genes

The 44 biofilm producing isolates were selected for the detection of antimicrobial resistance genes involved in the resistance to β-lactams and tetracyclines, namely, *blaZ, mecA, mecC, tetK,* and *tetM*. Table 3.2 shows the antimicrobial genes detected in each isolate, along with its antimicrobial resistance profile.

The *blaZ* gene was detected in 15 staphylococcal isolates belonging to the following species: *S. chromogenes* (7), *S. aureus* (3), *S. warneri* (2), *S. auricularis* (1), *S. caprae* (1), and *S. simulans* (1). Unexpectedly, nine penicillin-susceptible isolates harbor the *blaZ* gene, namely *S. chromogenes* (5), *S. warneri* (2), *S. auricularis* (1), and *S. simulans* (1). El Feghaly et al. [83] also reported penicillin-susceptible isolates harboring the *blaZ* gene and concluded that conventional methods for susceptibility testing such as Kirby-Bauer penicillin disk diffusion may not be reliable. According to CLSI [78], there may be rare isolates of staphylococci containing β-lactamase genes, which may result negative in

phenotypic β-lactamase detection. Additionally, all isolates resistant to penicillin must be considered resistant to all penicillinase-labile penicillins [78].

No staphylococcal isolates harboring the *mecA* or *mecC* genes were detected, although two isolates were found to be non-susceptible to oxacillin and cloxacillin simultaneously, one only to oxacillin and seven to cloxacillin alone. According to the CLSI (2016), oxacillin disk diffusion testing is not reliable for detecting methicillin resistance, at least in *S. aureus*, and cefoxitin should be used for disk diffusion testing. However, Barrero-Domínguez, Luque, Galán-Relaño, Vega-Pla, Huerta, Román, and Astorga [45] also did not detect the *mecA* gene in a cefoxitin-resistant MRSA strain. Thus, other resistance mechanisms cannot be excluded, namely, overproduction of β-lactamase, modified penicillin-binding proteins, distinct SCCmec elements, as well as putative *mecA* mutations [84,85]. Furthermore, Becker et al. [86] have recently reported the presence of a *mecB* gene in a MRSA strain, negative for both *mecA* and *mecC* genes. However, concerning *mecC* detection in our study, we cannot conclude that the isolates with a negative PCR result did not harbor the *mecC* gene, since no positive control strain was available.

An association was found between the resistance to penicillin ($2 = 11.650$, $df = 1$, $p < 0.05$) and ampicillin ($2 = 15.828$, $df = 1$, $p < 0.001$) and the presence of the antimicrobial resistance gene *blaZ*. The association between resistance to penicillin and ampicillin and the presence of the antimicrobial resistance gene *blaZ* has been reported before by other authors [87,88]. However, no association was detected between the resistance to oxacillin and cloxacillin and the presence of the antimicrobial resistance gene *mecA* for this subgroup of 44 isolates.

Only one *S. aureus* isolate carrying the *tetK* and another one carrying the *tetM* gene were identified. Both showed resistance to tetracycline. A *S. warneri* tetracycline-resistant isolate did not harbor either *tetK* or *tetM* (Table 3.3). El-Razik, Arafa, Hedia, and Ibrahim [82] found a *S. intermedius* isolate showing intermediate resistance to tetracycline, not harboring *tetK, tetL, tetM,* and *tetO* genes.

Table 3.2. *nuc*-positive biofilm-producing staphylococcal isolates, phenotypic resistance to selected antimicrobials and their associated antimicrobial resistance genes.

Isolate	Origin	Animal	Bacterial species	P	AMP	OB	AMC	OXA	TET	*blaZ*	*mecA*	*mecC*	*tetK*	*tetM*
1D	PT	goat	*S. aureus*	R	R	R	S	S	S	+	-		-	-
13D1	PT	goat	*S. warneri*	S	S	S	S	S	S	-	-		-	-
17D1	PT	goat	*S. aureus*	R	R	R	S	S	R	+	-		-	+
44D	PT	goat	*S. aureus*	R	R	S	S	S	S	+	-		-	-
47D2	PT	goat	*S. chromogenes*	R	R	R	R	R	S	+	-	-	-	-
50E1	PT	goat	*S. aureus*	S	S	S	S	S	S	-	-		-	-
54E1	PT	goat	*S. warneri*	S	S	R	S	S	S	+	-		-	-
54E2	PT	goat	*S. warneri*	S	S	R	S	R	S	-	-	-	-	-
55D1	PT	goat	*S. capitis*	S	S	S	S	S	S	-	-		-	-
60D2	PT	goat	*S. chromogenes*	R	R	S	S	S	S	+	-		-	-
65D	PT	goat	*S. caprae*	R	R	S	S	S	S	+	-		-	-
70D	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
71E	PT	sheep	*S. aureus*	S	S	R	S	S	S	-	-		-	-
72D	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
72E	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
83D	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
B51E	BR	goat	*S. chromogenes*	S	S	S	S	S	S	+	-		-	-
B64	BR	goat	*S. chromogenes*	S	S	S	S	S	S	+	-		-	-
B76E	BR	goat	*S. chromogenes*	S	S	S	S	S	S	+	-		-	-
B101	BR	goat	*S. warneri*	S	S	S	S	S	R	+	-		-	-
B159D	BR	goat	*S. chromogenes*	S	S	S	S	S	S	+	-		-	-
B159E	BR	goat	*S. chromogenes*	S	S	S	S	S	S	+	-		-	-
B190D	BR	goat	*S. auricularis*	R	S	S	S	S	S	-	-		-	-
B209D2	BR	goat	*S. simulans*	S	S	S	S	S	S	-	-		-	-
B209E	BR	goat	*S. simulans*	S	S	S	S	S	S	+	-		-	-
B219D3	BR	sheep	*S. auricularis*	S	S	S	S	S	S	-	-		-	-
B219D5	BR	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
B223D	BR	sheep	*S. aureus*	S	S	S	S	R	S	-	-	-	-	-
B250D	BR	sheep	*S. auricularis*	S	S	S	S	S	S	+	-		-	-
CQ152E1	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ185D1	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ196E	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ201E	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ268D1	PT	sheep	*S. aureus*	S	S	R	S	S	S	-	-		-	-
CQ270E1	PT	sheep	*S. aureus*	S	S	R	S	S	S	-	-		-	-
CQ285D	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ286D	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ290D1	PT	sheep	*S. aureus*	S	S	R	S	S	S	-	-		-	-
CQ290D2	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ296D	PT	sheep	*S. aureus*	S	S	S	S	S	R	-	-		+	-
CQ335E	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ336E2	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ349D	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-
CQ354D	PT	sheep	*S. aureus*	S	S	S	S	S	S	-	-		-	-

Penicillin (P), ampicillin (AMP), cloxacillin (OB), amoxicillin + clavulanic acid (AMC), oxacillin (OXA), tetracyclines-tetracycline (TET).

3. Materials and Methods

3.1. Milk Samples Collection and Bacteriological Analyses

A total of 328 small ruminants (258 goats and 70 sheep), belonging to 23 both traditional and industrial dairy farms in Portugal and Brazil, were used to collect 646 half-udder milk samples (508 from goats and 138 from sheep).

Milk samples were aseptically collected in a sterile bottle after the teat was carefully disinfected with 70% ethanol and the first flush was rejected. The samples were kept refrigerated and transported to the laboratory. Ten microliters of each milk sample were plated onto MacConkey agar (Oxoid, Hampshire, UK, CM0007) and onto blood agar (BA) (Oxoid, Hampshire, UK; CM0271 with 5% sheep blood) and incubated at 37 ºC for 24 h to 48 h.

Colonies from BA were transferred to brain heart infusion agar (BHI) (Oxoid, Hampshire, UK, CM1136) and again incubated at 37 ºC for 24h for primary identification of the *Staphylococcus* genus through morphological and biochemical characteristics, namely, colony morphology, Gram staining, and catalase reaction, according to Markey et al. [89].

Identification of the species level of all isolates was performed by automated compact system VITEK 2 (bioMérieux, Marcy l'Etoile, France) using GP ID cards following the manufacturer's instructions. Biochemical identification was confirmed by 16S rRNA gene sequencing whenever necessary, using the primers described previously [90].

3.2. Phenotypic Characterisation of Staphylococcal Isolates

3.2.1. Biofilm Detection

Biofilm production was evaluated following the procedures described by Merino et al. [91] with some modifications. In brief, isolates were grown overnight in trypticase soy broth (TSB) at 37 ºC. This overnight culture was diluted 1:40 in TSB supplemented with 0.25% glucose, and 200 mL of this cell suspension was used to inoculate microplates. After 24 h of incubation at 37 ºC, the microplates were washed three times with 200 µL H2O, dried in an inverted position, and stained with 100 µL of 0.25% crystal violet for 2 to 3 min at room temperature. Afterwards, the microplates were rinsed again three times with H2O,

dried, the dye dissolved in 200 µL ethanol-acetone (80:20), and the absorbance measured at 620 nm. Each assay was performed in triplicate and repeated three times. *Staphylococcus epidermidis* ATCC 12,228 and ATCC 35,984 were used as negative and positive controls, respectively. A blank control was also used.

3.2.2. Antimicrobial Sensitivity Test

The antimicrobial sensitivity test (AST) was performed as described before [77] following the performance standard M02-A11 [92]. Resistance to 16 antimicrobials, belonging to six antimicrobial categories, according to Magiorakos et al. [73], was evaluated: (1) β-lactams-penicillin (P), ampicillin (AMP), cloxacillin (OB), amoxicillin + clavulanic acid (AMC), oxacillin (OXA), cephalexin (CL), cefazolin (KZ), ceftriaxone (CRO), cefoperazone (CFP); (2) aminoglycosides-streptomycin (S), gentamycin (CN), neomycin (N); (3) lincosamides-lincomycin (MY); (4) tetracyclines-tetracycline (TET); (5) fluoroquinolones-ciprofloxacin (CIP); and (6) folate pathway inhibitors-cotrimoxazole (sulfamides + trimethoprim) (STX).

For the interpretation of AST results, the CLSI clinical breakpoints M100-S25 were used [78]. Isolates showing intermediate resistance, now called “susceptible increased exposure” [93], were considered non-susceptible. Moreover, isolates resistant to three or more antimicrobial categories were considered multidrug resistant [73].

3.3. Molecular Characterisation of Staphylococcal Isolates

The presence of *coa* and *nuc* genes was investigated in all staphylococcal isolates. *Nuc*-positive biofilm-producing isolates were selected for the detection of the biofilm production genes, *bap, icaA,* and *icaD*, and the antimicrobial resistance genes *blaZ, mecA, tetK*, and *tetM*. The presence of the *mecC* gene was investigated only for *nuc*-positive biofilm-producing isolates, which were simultaneously resistant to oxacillin and cloxacillin and did not harbor the *mecA* gene.

3.3.1. Rapid DNA Extraction

Total DNA was extracted as described previously [94]. Bacterial cultures were grown for 24 h in BHI (Oxoid, Hampshire, UK, CM1136). After this period, they were transferred to microtubes with 200 µL of ultrapure water and centrifuged at 12,000x *g* for two minutes. Two hundred microliters of sterile saline solution (8.5%) were added to the pellet and centrifuged again at 12,000x *g* for two minutes. Subsequently, 100 µL of 0.05 M NaOH was added to the pellet and boiled for four minutes, then placed immediately on ice. Afterwards, 600 µL of ultrapure water was added to the microtubes and centrifuged at 4000x *g* for three minutes. Subsequently, 400 µL were transferred to a new microtube and stored at -20 ºC until use.

3.3.2. PCR Amplification

All amplifications were done in a PTC1148C-MJ Mini thermocycler (BioRad, Hercules, CA, USA).

Amplified DNA fragments were stained with 1X Red Gel (Biotium, Fremont, CA, USA) and run on 1.5% (w/v) agarose gels with 0.5X TBE (Tris-borate-EDTA) buffer. Different NZYDNA Ladders (NZYtech, Lisbon, Portugal) were used as molecular weight markers, depending on the size of the PCR products.

Agarose gels were photographed under ultraviolet light using the Gel Doc XR+ Gel Documentation System (BioRad Universal Hood II, Philadelphia, PA, USA).

For all PCR amplifications, 50 µL PCR reactions were prepared with 5 µL of DNA template, 1 U GoTaq DNA polymerase (Promega, Madison, WI, USA), 1X Green Go Taq Flexi buffer (Promega, WI, USA), 1.5 mM MgCl2 (Promega, WI, USA), 0.2 mM each dNTP (VWR, part of Avantor, Radnor, PA, USA), and 15 pmol each primer (STAB VIDA, Caparica, Portugal). Specific and individual modifications or optimizations were done whenever necessary.

The primers used for amplification of the different genes are listed in Table 3.3.

Table 3.3. Primer sequences for amplification of the different genes.

Gene	Primer	Sequence	Reference
coa	coa-F	5’ - ATA GAG ATG CTG GTA CAG G - 3’	[55]
	coa-R	5’ - GCT TCC GAT TGT TCG ATG C - 3’	
coa	coa2-F	5’ – ATA CTC AAC CGA CGA CAC CG - 3’	[54]
	coa2-R	5’ – GAT TTT GGA TGA AGC GGA TT - 3’	
nuc	nuc-F	5’ – GCG ATT GAT GGT GAT ACG GTT - 3’	[95]
	nuc-R	5’ – AGC CAA GCC TTG ACG AAC TAA AGC - 3’	
bap	bap-F	5’ – CCC TAT ATC GAA GGT GTA GAA TTG CAC - 3’	[35]
	bap-R	5’ – GCT GTT GAA GTT AAT ACT GTA CCT GC - 3’	
*ica*A	icaA-F	5’ – CCT AAC TAA CGA AAG GTA G - 3’	[96]
	icaA-R	5’ – AAG ATA TAG CGA TAA GTG C - 3’	
*ica*D	icaD-F	5’ – AAA CGT AAG AGA GGT GG - 3’	[96]
	icaD-R	5’ – GGC AAT ATG ATC AAG ATA C - 3’	
*bla*Z	blaZ-F	5’ – AAG AGA TTT GCC TAT GCT TC - 3’	[97]
	blaZ-R	5’ – GCT TGA CCA CTT TTA TCA GC - 3’	
*mec*A	mecA-F	5’ – AAA ATC GAT GGT AAA GGT TGG C - 3’	[98]
	mecA-R	5’ – AGT TCT GCA GTA CCG GAT TTG C - 3’	
*mec*C	mecC-F	5’ – GAA AAA AAG GCT TAG AAC GCC TC – 3’	[99]
	mecC-R	5’ – GAA GAT CTT TTC CGT TTT CAG C – 3’	
*tet*K	tetK-F	5’GTAGCGACAATAGGTAATAGT3’	[59]
	tetK-R	5’ GTAGTGACAATAAACCTCCTA 3’	
*tet*M	tetM-F	5’AGTGGAGCGATTACAGAA3’	[59]
	tetM-R	5’CATATGTCCTGGCGTGTCTA3’	

For the detection of the *coa* gene, different primer sequences were used. *Staphylococcus aureus* ATCC 25923 was used as positive control. The first pair of primers, coa-F and coa-R, amplified a 676 bp fragment [55]. The amplification program was as follows: 3 min at 95 ºC, and 35 cycles of 30 s at 94 ºC, 30 s at 55 ºC, 30 s at 72 ºC, and finally, 5 min at 72 ºC. The second pair of primers, coa2-F and coa2-R, amplified a fragment of 1517 bp [54]. The amplification program comprised an initial denaturation of 45 s at 94 ºC, followed by 29 cycles at 94 ºC for 20 s, 55 ºC for 1 min, and 72 ºC for 90 s, and a final extension step of 2 min at 72 ºC.

For the amplification of the *nuc* gene, primers nuc-F and nuc-R, amplifying a 267 bp DNA fragment, were used [95]. *S. aureus* ATCC 25923 was used as positive control and *S. epidermidis* ATCC 12228 as negative control. The amplification program was the following: 5 min at 94 ºC, followed by 37 cycles, consisting of 94 ºC for 1 min, 55 ºC for 30 s, and 72 ºC for 30 s, ending with a final extension step at 72 ºC for 7 min.

For detecting the *bap* gene, primers bap-F and bap-R were used for the amplification of a 971 bp fragment [35]. No positive control strain was available. The amplification program was as follows: 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 45 s, 56.5 ºC for 45 s, and 72 ºC for 50 s, and finally, 72 ºC for 5 min.

Primers icaA-F and icaA-R were used for the amplification of a 1315 bp fragment of the *icaA* gene [96]. *S. epidermidis* ATCC 35984 was used as positive control. The following amplification program was used: 92 ºC for 5 min, followed by 30 cycles of 92 ºC for 45 s, 49 ºC for 45 s, and 72 ºC for 1 min, and a final extension step of 7 min at 72 ºC.

For the *icaD* gene, primers icaD-F and icaD-R were used to amplify a 381 bp fragment [96]. *S. epidermidis* ATCC 35984 was used as positive control. The same amplification program as for *icaA* was used, except for the extension step within the cycles, which was 72 ºC for 30 s.

The presence of the *blaZ* gene was detected using primers blaZ-F and blaZ-R, which amplified a 517 bp fragment [97]. *Staphylococcus aureus* ATCC 29213 was used as positive control and *S. aureus* ATCC 25923 as negative control [100]. The amplification program was as follows: 94 ºC for 4 min, followed by 37 cycles of 94 ºC for 1 min, 50.5 ºC for 30 s, and 72 ºC for 30 s, and finally, 72 ºC for 5 min [97].

To detect the *mecA* gene, primers mecA-F and mecA-R were used to amplify a fragment of 532 bp [98]. *Staphylococcus epidermidis* ATCC 35984 was used as positive control [101] and *S. aureus* ATCC 25923 as negative control [102]. The following amplification program was used: 94 ºC for 2 min, followed by 29 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, and a final extension of 5 min at 72 ºC.

Primers mecC-F and mecC-R were used to amplify a 138 bp fragment [99]. No positive control strain was available. The following amplification program was used: 95 ºC for 2 min, followed by 30 cycles of 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 30 s, and a final extension of 10 min at 72 ºC.

Primers tetK-F and tetK-R were used to amplify a 360 bp fragment of the *tetK* gene [59]. No positive control strain was available. For the amplification of the *tetM* gene, tetM-F and tetM-R were used to amplify a fragment of 158 bp [59]. No positive control strain was available. The amplification program for both *tet* genes was: 94 ºC for 2 min, followed

by 29 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, with a final step of 5 min at 72 ºC.

3.4. Data Analysis

The chi-square test of association was used: to assess the relationship between the presence of the *nuc* gene with *Staphylococcus* species; to investigate if the presence of the *nuc* gene was associated with biofilm production; to check if the resistance to antimicrobials was associated with bacterial species and with the animal species from which these were isolated. For the abovementioned analyses, all 137 isolates were considered.

For the subgroup of 44 *nuc*-positive biofilm-producing isolates, the chi-square test of association was performed to evaluate the putative relationship between phenotypic resistance to antimicrobials and the presence of four resistance genes.

Multiple correspondence analysis (MCA) was used as an exploratory data analysis technique to detect a structure in the relationships between bacterial species and resistance to selected antimicrobials, divided either into two (susceptible and resistant) or three classes (susceptible, intermediate, and resistant), depending on the antimicrobial.

All statistical analyses were performed using the software STATISTICA Version 12 (StatSoft, Inc., Tulsa, OK, USA).

4. Conclusions

Mastitis aetiology showed to be diverse in the two small ruminant species studied. The most abundant species was *S. caprae*, which, however, was only present in goats.

The *nuc* gene was detected in 67 isolates, of which only 35 were *S. aureus*. Most CNS did not harbor this gene; however, it was detected in more than 50% of *S. warneri*, *S. lentus*, *S. auricularis*, and *S. hyicus*. Although many studies still consider the *nuc* gene as the sole character to identify *S. aureus*, our results have clearly demonstrated that this gene is insufficient, because it is present in numerous staphylococcal isolates other than *S. aureus*.

Most staphylococci were biofilm producers. The *bap* gene was only detected in CNS, while *ica* operon genes were mainly detected in *S. aureus* isolates, suggesting that CNS produce biofilm mainly via Bap, and most *S. aureus* form biofilm through PIA. Furthermore, biofilm-producing staphylococcal isolates not harboring the classical biofilm-production genes *bap*, *icaA*, and *icaD* carry the *nuc* gene. Therefore, the role of the Nuc thermonuclease in staphylococci biofilm formation needs to be further investigated.

Antimicrobial resistance seems to be a growing concern in the treatment of sheep and goat mastitis, with only a low number of isolates (18%) not showing any antimicrobial resistances. Furthermore, CNS were generally more resistant to antimicrobials than CPS. Additionally, an association between animal species and resistance to some antimicrobials was found, suggesting different managing systems for the two species.

All staphylococcal isolates were susceptible to cefazolin and gentamycin. Furthermore, all *S. aureus* isolates were shown to be susceptible to amoxicillin + clavulanic acid and most (32/35) to tetracycline. The use of these antimicrobials to control mastitis may be encouraged to avoid the use of others critically important for human medicine that are currently being used, such as third generation cephalosporins and quinolones. Nevertheless, antimicrobial susceptibility tests cannot be neglected, as the development of resistant strains is always a problem.

Regarding antimicrobial resistance genes, nine penicillin-susceptible isolates exhibited the *blaZ* gene, highlighting the poor reliability of conventional methods for susceptibility testing. Moreover, no staphylococcal isolates harboring the *mecA* or *mecC* genes were detected among those found to be non-susceptible to oxacillin. Hence, other methicillin resistance mechanisms need to be explored.

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References

1. Olechnowicz, J.; Ja´skowski, J.M. Mastitis in small ruminants. *Med. Weter*. **2014**, 70, 67–72.
2. Gelasakis, A.I.; Mavrogianni, V.S.; Petridis, I.G.; Vasileiou, N.G.C.; Fthenakis, G.C. Mastitis in sheep—The last 10 years and the future of research. *Vet. Microbiol*. **2015**, 181, 136–146. [CrossRef]
3. De Buyser, M.-L.; Dufour, B.; Maire, M.; Lafarge, V. Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *Int. J. Food Microbiol*. **2001**, 67, 1–17. [CrossRef]
4. Argudín, M.Á.; Mendoza, M.C.; Rodicio, M.R. Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins* **2010**, 2, 1751–1773. [CrossRef] [PubMed]
5. Queiroga, M.C. Prevalence and aetiology of sheep mastitis in Alentejo region of Portugal. *Small Rumin. Res*. **2017**, 153, 123–130. [CrossRef]
6. Abdalhamed, A.M.; Zeedan, G.S.G.; Abou Zeina, H.A.A. Isolation and identification of bacteria causing mastitis in small ruminants and their susceptibility to antibiotics, honey, essential oils, and plant extracts. *Vet. World* **2018**, 11, 355–362. [CrossRef] [PubMed]
7. Ayis, A.A.E.; Fadlalla, E. Antibiotic Susceptibility of Major Bacteria Cause Ovine Mastitis in River Nile State, Sudan. *Imp. J. Interdiscip. Res*. **2017**, 3, 908–916.
8. Salaberry, S.R.S.; Saidenberg, A.B.S.; Zuniga, E.; Gonsales, F.F.; Melville, P.A.; Benites, N.R. Microbiological analysis and sensitivity profile of *Staphylococcus* spp. in subclinical mastitis of dairy goats. *Arq. Bras. Med. Veterinária Zootec*. **2016**, 68, 336–344. [CrossRef]
9. Bergonier, D.; Sobral, D.; Feßler, A.T.; Jacquet, E.; Gilbert, F.B.; Schwarz, S.; Treilles, M.; Bouloc, P.; Pourcel, C.; Vergnaud, G. *Staphylococcus aureus* from 152 cases of bovine, ovine and caprine mastitis investigated by Multiple-locus variable number of tandem repeat analysis (MLVA). *Vet. Res.* **2014**, 45, 97. [CrossRef] [PubMed]
10. Constable, P.D.; Hinchcliff, K.W.; Done, S.H.; Grünberg, W. Veterinary Medicine: *A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats*, 11th ed.; Elsevier: St. Louis, MO, USA, 2017; pp. 1904–2001. ISBN 9780-7020-5246-8.

11. Dore, S.; Liciardi, M.; Amatiste, S.; Bergagna, S.; Bolzoni, G.; Caligiuri, V.; Cerrone, A.; Farina, G.; Montagna, C.O.; Saletti, M.A.; et al. Survey on small ruminant bacterial mastitis in Italy, 2013–2014. *Small Rumin. Res*. **2016**, 141, 91–93. [CrossRef]

12. Mishra, A.K.; Sharma, N.; Singh, D.D.; Gururaj, K.; Abhishek; Kumar, V.; Sharma, D.K. Prevalence and bacterial etiology of subclinical mastitis in goats reared in organized farms. *Vet. World* **2018**, 11, 20–24. [CrossRef] [PubMed]

13. Ruiz Romero, R.A. Identification of and antimicrobial resistance in bacteria causing caprine mastitis in three states and a city in Central Mexico under manual and mechanical milking conditions. *J. Dairyvet. Anim. Res*. **2018**, 7. [CrossRef]

14. Leitner, G.; Rovai, M.; Merin, U. Clinical and subclinical intrammamay infection caused by coagulase negative staphylococci negatively affect milk yield and its quality in dairy sheep. *Small Rumin. Res.* **2019**, 180, 74–78. [CrossRef]

15. Madigan, M.T.; Stahl, D.A.; Sattley, W.M.; Buckley, D.H.; Bender, K.S. *Brock Biology of Microorganisms*, Global Edition; Pearson Education Limited: London, UK, 2018.

16. Murray, P.R.; Rosenthal, K.S.; Pfaller, M.A. *Medical Microbiology*, 9th ed.; Elsevier: Amsterdam, The Netherlands, 2020.

17. Pizauro, L.J.L.; de Almeida, C.C.; Soltes, G.A.; Slavic, D.; de Ávila, F.A.; Zafalon, L.F.; MacInnes, J.I. Short communication: Detection of antibiotic resistance, *mecA*, and virulence genes in coagulase-negative *Staphylococcus* spp. from buffalo milk and the milking environment. *J. Dairy Sci*. **2019**, 102, 11459–11464. [CrossRef]

18. Zapotoczna, M.; McCarthy, H.; Rudkin, J.K.; O'Gara, J.P.; O'Neill, E. An Essential Role for Coagulase in *Staphylococcus aureus* Biofilm Development Reveals New Therapeutic Possibilities for Device-Related Infections. *J. Infect. Dis*. **2015**, 212, 1883–1893. [CrossRef] [PubMed]

19. Schleifer, K.-H.; Bell, J.A. Family VIII-Staphylococcaceae fam. nov. In *Bergey's Manual of Systematic Bacteriology*, 2nd ed.; Vos, P.D., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig,W., Rainey, F.A., Schleifer, K.-H., Whitman,W.B., Eds.; Springer: New York, NY, USA, 2009; Volume 3, pp. 392–421.

20. Hu, Y.; Meng, J.; Shi, C.; Hervin, K.; Fratamico, P.M.; Shi, X. Characterization and comparative analysis of a second thermonuclease from *Staphylococcus aureus*. *Microbiol. Res*. **2013**, 168, 174–182. [CrossRef] [PubMed]

21. Berends, E.T.M.; Horswill, A.R.; Haste, N.M.; Monestier, M.; Nizet, V.; Von Köckritz-Blickwede, M. Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J. Innate Immun*. **2010**, 2, 576–586. [CrossRef] [PubMed]

22. Kenny, E.F.; Herzig, A.; Krüger, R.; Muth, A.; Mondal, S.; Thompson, P.R.; Brinkmann, V.; Bernuth, H.V.; Zychlinsky, A. Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife* **2017**, 6. [CrossRef]

23. Kateete, D.P.; Kimani, C.N.; Katabazi, F.A.; Okeng, A.; Okee, M.S.; Nanteza, A.; Joloba, M.L.; Najjuka, F.C. Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Ann. Clin. Microbiol. Antimicrob*. **2010**, 9, 23. [CrossRef]

24. Torres, G.; Vargas, K.; Sánchez-Jiménez, M.; Reyes-Velez, J.; Olivera-Angel, M. Genotypic and phenotypic characterization of biofilm production by *Staphylococcus aureus* strains isolated from bovine intramammary infections in Colombian dairy farms. *Heliyon* **2019**, 5, e02535. [CrossRef]

25. McClure, J.A.; Zaal DeLongchamp, J.; Conly, J.M.; Zhang, K. Novel Multiplex PCR Assay for Detection of Chlorhexidine-Quaternary Ammonium, Mupirocin, and Methicillin Resistance Genes, with Simultaneous Discrimination of *Staphylococcus aureus* from Coagulase-Negative Staphylococci. *J. Clin. Microbiol*. **2017**, 55, 1857–1864. [CrossRef] [PubMed]

26. Gudding, R. Differentiation of staphylococci on the basis of nuclease properties. *J. Clin. Microbiol*. **1983**, 18, 1098–1101. [CrossRef]

27. Mann, E.E.; Rice, K.C.; Boles, B.R.; Endres, J.L.; Ranjit, D.; Chandramohan, L.; Tsang, L.H.; Smeltzer, M.S.; Horswill, A.R.; Bayles, K.W. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. *PLoS ONE* **2009**, 4, e5822. [CrossRef] [PubMed]

28. Kiedrowski, M.R.; Kavanaugh, J.S.; Malone, C.L.; Mootz, J.M.; Voyich, J.M.; Smeltzer, M.S.; Bayles, K.W.; Horswill, A.R. Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. *PLoS ONE* **2011**, 6, e26714. [CrossRef] [PubMed]

29. Flemming, H.-C.; Wingender, J.; Szewzyk, U.; Steinberg, P.; Rice, S.A.; Kjelleberg, S. Biofilms: An emergent form of bacterial life. *Nat. Rev. Microbiol*. **2016**, 14, 563–575. [CrossRef] [PubMed]

30. Turchi, B.; Bertelloni, F.; Marzoli, F.; Cerri, D.; Tola, S.; Azara, E.; Longheu, C.M.; Tassi, R.; Schiavo, M.; Cilia, G.; et al. Coagulase negative staphylococci from ovine milk: Genotypic and phenotypic characterization of susceptibility to antibiotics, disinfectants and biofilm production. *Small Rumin. Res.* **2020**, 183, 106030. [CrossRef]

31. Buttner, H.; Dietrich, M.; Rohde, H. Structural basis of *Staphylococcus epidermidis* biofilm formation: Mechanisms and molecular interactions. *Front. Cell. Infect. Microbiol*. **2015**, 5, 14. [CrossRef]

32. Arciola, C.R.; Campoccia, D.; Ravaioli, S.; Montanaro, L. Polysaccharide intercellular adhesin in biofilm: Structural and regulatory aspects. *Front. Cell. Infect. Microbiol*. **2015**, 5, 7. [CrossRef]

33. Heilmann, C. Adhesion Mechanisms of Staphylococci. Adv. Exp. Med. Biol. **2011**, 715, 105–123. [CrossRef]

34. Otto, M. Staphylococcal Biofilms. *Microbiol. Spectr*. **2018**, 6. [CrossRef]

35. Cucarella, C.; Solano, C.; Valle, J.; Amorena, B.; Lasa, I.N.I.; Penadés, J.R. Bap, a *Staphylococcus aureus* Surface Protein Involved in Biofilm Formation. *J. Bacteriol*. **2001**, 183, 2888–2896. [CrossRef] [PubMed]

36. Ceniti, C.; Britti, D.; Santoro, A.M.L.; Musarella, R.; Ciambrone, L.; Casalinuovo, F.; Costanzo, N. Phenotypic antimicrobial resistance profile of isolates causing clinical mastitis in dairy animals. *Ital. J. Food Saf*. **2017**, 6. [CrossRef]

37. Peixoto, R.D.M.; França, C.A.D.; Souza Júnior, A.F.D.; Veschi, J.L.A.; Costa, M.M.D. Etiologia e perfil de sensibilidade antimicrobiana dos isolados bacterianos da mastite em pequenos ruminantes e concordância de técnicas empregadas no diagnóstico. *Pesqui. Veterinária Bras.* **2010**, 30, 735–740. [CrossRef]

38. Andrade, N.P.C.; Peixoto, R.M.; Nogueira, D.M.; Krewer, C.C.; Costa, M.M. Perfil de sensibilidade aos antimicrobianos de *Staphylococcus* spp. coagulase negativa de um rebanho leiteiro caprino em Santa Maria da Boa Vista-PE. *Med. Veterinária* **2012**, 6, 1–6.

39. Al-Ashmawy, M.A.; Sallam, K.I.; Abd-Elghany, S.M.; Elhadidy, M.; Tamura, T. Prevalence, Molecular Characterization, and Antimicrobial Susceptibility of Methicillin-Resistant *Staphylococcus aureus* isolated from Milk and Dairy Products. *Foodborne Pathog. Dis*. **2016**, 13, 156–162. [CrossRef] [PubMed]

40. Merz, A.; Stephan, R.; Johler, S. *Staphylococcus aureus* Isolates from Goat and Sheep Milk Seem to Be Closely Related and Differ from Isolates Detected from Bovine Milk. *Front. Microbiol*. **2016**, 7, 319. [CrossRef] [PubMed]

41. Akkou, M.; Bentayeb, L.; Ferdji, K.; Medrouh, B.; Bachtarzi, M.-A.; Ziane, H.; Kaidi, R.; Tazir, M. Phenotypic characterization of Staphylococci causing mastitis in goats and microarray-based genotyping of *Staphylococcus aureus* isolates. *Small Rumin. Res*. **2018**, 169, 29–33. [CrossRef]

42. Lee, J.H. Methicillin (Oxacillin)—Resistant *Staphylococcus aureus* Strains Isolated from Major Food Animals and Their Potential Transmission to Humans. *Appl. Environ. Microbiol*. **2003**, 69, 6489–6494. [CrossRef]

43. Persson, Y.; Nyman, A.-K.; Söderquist, L.; Tomic, N.; Waller, K.P. Intramammary infections and somatic cell counts in meat and pelt producing ewes with clinically healthy udders. *Small Rumin. Res*. **2017**, 156, 66–72. [CrossRef]

44. Queiroga, M.C.; Laranjo, M.; Duarte, E.L. Mastitis in Sheep: A disease to be Taken Seriously. In *Sheep Diseases*; *Papst*, M., Ed.; Nova Science Publisher, Inc: New York, NY, USA, 2019; pp. 1–91.

45. Barrero-Domínguez, B.; Luque, I.; Galán-Relaño, Á.; Vega-Pla, J.L.; Huerta, B.; Román, F.; Astorga, R.J. Antimicrobial Resistance and Distribution of *Staphylococcus* spp. Pulsotypes Isolated from Goat and Sheep Bulk Tank Milk in Southern Spain. *Foodborne Pathog. Dis*. **2019**, 16, 723–730. [CrossRef]

46. Gosselin, V.B.; Lovstad, J.; Dufour, S.; Adkins, P.R.F.; Middleton, J.R. Use of MALDI-TOF to characterize staphylococcal intramammary infections in dairy goats. J. Dairy Sci. **2018**, 101, 6262–6270. [CrossRef] [PubMed]

47. Bergonier, D.; Berthelot, X. New advances in epizootiology and control of ewe mastitis. *Livest. Prod. Sci.* **2003**, 79, 1–16. [CrossRef]

48. Martins, K.B.; Faccioli, P.Y.; Bonesso, M.F.; Fernandes, S.; Oliveira, A.A.; Dantas, A.; Zafalon, L.F.; Cunha, M.D.L.R.S. Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. *J. Dairy Sci.* **2017**, 100, 2184–2195. [CrossRef] [PubMed]

49. Park, J.Y.; Fox, L.K.; Seo, K.S.; McGuire, M.A.; Park, Y.H.; Rurangirwa, F.R.; Sischo,W.M.; Bohach, G.A. Comparison of phenotypic and genotypic methods for the species identification of coagulase-negative staphylococcal isolates from bovine intramammary infections. *Vet. Microbiol*. **2011**, 147, 142–148. [CrossRef]

50. Becker, K.; Heilmann, C.; Peters, G. Coagulase-negative staphylococci. *Clin. Microbiol. Rev*. **2014**, 27, 870–926. [CrossRef] [PubMed]

51. Queiroga, M.C.; Duarte, E.L.; Laranjo, M. Sheep mastitis *Staphylococcus epidermidis* biofilm effects on cell adhesion and inflammatory changes. *Small Rumin. Res*. **2018**, 168, 6–11. [CrossRef]

52. Aher, T.; Roy, A.; Kumar, P. Molecular detection of virulence genes associated with pathogenicity of Gram positive isolates obtained from respiratory tract of apparently healthy as well as sick goats. *Vet. World* **2012**, 5, 676. [CrossRef]

53. Saei, H.D. Coa types and antimicrobial resistance profile of *Staphylococcus aureus* isolates from cases of bovine mastitis. *Comp. Clin. Pathol*. **2012**, 21, 301–307. [CrossRef]

54. Aarestrup, F.M.; Dangler, C.A.; Sordillo, L.M. Prevalence of coagulase gene polymorphism in *Staphylococcus aureus* isolates causing bovine mastitis. *Can. J. Vet. Res*. **1995**, 59, 124–128.

55. Hookey, J.V.; Richardson, J.F.; Cookson, B.D. Molecular typing of *Staphylococcus aureus* based on PCR restriction fragment length polymorphism and DNA sequence analysis of the coagulase gene. *J. Clin. Microbiol*. **1998**, 36, 1083–1089. [CrossRef] [PubMed]

56. Soltan Dallal, M.M.; Khoramizadeh, M.R.; Amiri, S.A.; Saboor Yaraghi, A.A.; Mazaheri Nezhad Fard, R. Coagulase gene polymorphism of *Staphylococcus aureus* isolates: A study on dairy food products and other foods in Tehran, Iran. *Food Sci. Hum. Wellness* **2016**, 5, 186–190. [CrossRef]

57. Shopsin, B.; Gomez, M.; Waddington, M.; Riehman, M.; Kreiswirth, B.N. Use of Coagulase Gene (*coa*) Repeat Region Nucleotide Sequences for Typing of Methicillin-Resistant *Staphylococcus aureus* Strains. *J. Clin. Microbiol*. **2000**, 38, 3453–3456. [CrossRef] [PubMed]

58. Tiwari, H.K.; Sapkota, D.; Gaur, A.; Mathuria, J.P.; Singh, A.; Sen, M.R. Molecular typing of clinical *Staphylococcus aureus* isolates from northern India using coagulase gene PCR-RFLP. *Southeast Asian J. Trop. Med. Public Health* **2008**, 39, 467–473.

59. Xu, J.; Tan, X.; Zhang, X.; Xia, X.; Sun, H. The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. *Microb. Pathog*. **2015**, 88, 29–38. [CrossRef] [PubMed]

60. Van Leeuwen,W.; Roorda, L.; Hendriks,W.; Francois, P.; Schrenzel, J. A nuc-deficient methicillin-resistant *Staphylococcus aureus* strain. *FEMS Immunol. Med. Microbiol*. **2008**, 54, 157. [CrossRef] [PubMed]

61. Hirotaki, S.; Sasaki, T.; Kuwahara-Arai, K.; Hiramatsu, K. Rapid and Accurate Identification of Human-Associated Staphylococci by Use of Multiplex PCR. *J. Clin. Microbiol*. **2011**, 49, 3627–3631. [CrossRef]

62. Silva,W.P.D.; Silva, J.A.; Macedo, M.R.P.D.; Araújo, M.R.D.; Mata, M.M.; Gandra, E.A. Identification of *Staphylococcus aureus*, *S. intermedius* and *S. hyicus* by PCR amplification of *coa* and *nuc* genes. *Braz. J. Microbiol*. **2003**, 34, 125–127. [CrossRef]

63. Cucarella, C.; Tormo, M.A.; Úbeda, C.; Trotonda, M.P.; Monzón, M.; Peris, C.; Amorena, B.; Lasa, I.; Penadés, J.R. Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*. *Infect. Immun*. **2004**, 72, 2177–2185. [CrossRef]

64. Vautor, E.; Magnone, V.; Rios, G.; Le Brigand, K.; Bergonier, D.; Lina, G.; Meugnier, H.; Barbry, P.; Thiéry, R.; Pépin, M. Genetic differences among *Staphylococcus aureus* isolates from dairy ruminant species: A single-dye DNA microarray approach. *Vet. Microbiol*. **2009**, 133, 105–114. [CrossRef]

65. Martins, K.B.; Faccioli-Martins, P.Y.; Riboli, D.F.; Pereira, V.C.; Fernandes, S.; Oliveira, A.A.; Dantas, A.; Zafalon, L.F.; da Cunha, M.E.L. Clonal profile, virulence and resistance of *Staphylococcus aureus* isolated from sheep milk. *Braz. J. Microbiol*. **2015**, 46, 535–543. [CrossRef]

66. Tormo, M.Á.; Knecht, E.; Götz, F.; Lasa, I.; Penadés, J.R. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: Evidence of horizontal gene transfer? *Microbiology* **2005**, 151, 2465–2475. [CrossRef] [PubMed]

67. Szweda, P.; Schielmann, M.; Milewski, S.; Frankowska, A.; Jakubczak, A. Biofilm production and presence of *ica* and *bap* genes in *Staphylococcus aureus* strains isolated from cows with mastitis in the eastern Poland. *Pol. J. Microbiol*. **2012**, 61, 65–69. [CrossRef] [PubMed]

68. Marques, V.F.; Motta, C.C.D.; Soares, B.D.S.; Melo, D.A.D.; Coelho, S.D.M.D.O.; Coelho, I.D.S.; Barbosa, H.S.; Souza, M.M.S.D. Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. Braz. *J. Microbiol*. **2017**, 48, 118–124. [CrossRef] [PubMed]

69. Khoramian, B.; Jabalameli, F.; Niasari-Naslaji, A.; Taherikalani, M.; Emaneini, M. Comparison of virulence factors and biofilm formation among *Staphylococcus aureus* strains isolated from human and bovine infections. *Microb. Pathog*. **2015**, 88, 73–77. [CrossRef]

70. Vitale, M.; Galluzzo, P.; Buffa, P.G.; Carlino, E.; Spezia, O.; Alduina, R. Comparison of Antibiotic Resistance Profile and Biofilm Production of *Staphylococcus aureus* Isolates Derived from Human Specimens and Animal-Derived Samples. *Antibiotics* **2019**, 8, 97. [CrossRef]

71. Zuniga, E.; Melville, P.A.; Saidenberg, A.B.S.; Laes, M.A.; Gonsales, F.F.; Salaberry, S.R.S.; Gregori, F.; Brandão, P.E.; dos Santos, F.G.B.; Lincopan, N.E.; et al. Occurrence of genes coding for MSCRAMM and biofilm-associated protein Bap in *Staphylococcus* spp. isolated from bovine subclinical mastitis and relationship with somatic cell counts. *Microb. Pathog.* **2015**, 89, 1–6. [CrossRef]

72. Notcovich, S.; DeNicolo, G.; Flint, S.; Williamson, N.; Gedye, K.; Grinberg, A.; Lopez-Villalobos, N. Biofilm-Forming Potential of *Staphylococcus aureus* Isolated from Bovine Mastitis in New Zealand. *Vet. Sci.* **2018**, 5, 8. [CrossRef]

73. Magiorakos, A.P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Harbarth, S.; Hindler, J.F.; Kahlmeter, G.; Olsson-Liljequist, B.; et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol*. Infect. **2012**, 18, 268–281. [CrossRef]

74. França, C.A.; Peixoto, R.M.; Cavalcante, M.B.; Melo, N.F.; Oliveira, C.J.B.; Veschi, J.A.; Mota, R.A.; Costa, M.M. Antimicrobial resistance of *Staphylococcus* spp. from small ruminant mastitis in Brazil. *Pesqui. Veterinária Bras*. **2012**, 32, 747–753. [CrossRef]

75. Taponen, S.; Pyorala, S. Coagulase-negative staphylococci as cause of bovine mastitis—Not so different from *Staphylococcus aureus*? *Vet. Microbiol.* **2009**, 134, 29–36. [CrossRef] [PubMed]

76. Vasileiou, N.G.C.; Sarrou, S.; Papagiannitsis, C.; Chatzopoulos, D.C.; Malli, E.; Mavrogianni, V.S.; Petinaki, E.; Fthenakis, G.C. Antimicrobial Agent Susceptibility and Typing of Staphylococcal Isolates from Subclinical Mastitis in Ewes. *Microb. Drug Resist*. **2019**, 25, 1099–1110. [CrossRef] [PubMed]

77. Queiroga, M.C.; Andrade, N.; Laranjo, M. Antimicrobial Action of Propolis Extracts Against Staphylococci. In *Understanding Microbial Pathogens: Current Knowledge and Educational Ideas on Antimicrobial Research*; Torres-Hergueta, E., Méndez-Vilas, A., Eds.; Formatex Research Center: Badajoz, Spain, 2018; pp. 28–35.

78. CLSI. M100S-*Performance Standards for Antimicrobial Susceptibility Testing-Approved Standard*, 26th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2016.

79. Reading, C.; Cole, M. Clavulanic acid: A beta-lactamase-inhiting beta-lactam from *Streptomyces clavuligerus*. Antimicrob. *Agents Chemother*. **1977**, 11, 852–857. [CrossRef] [PubMed]

80. Barbier, F.; Ruppé, E.; Hernandez, D.; Lebeaux, D.; Francois, P.; Felix, B.; Desprez, A.; Maiga, A.; Woerther, P.L.; Gaillard, K.; et al. Methicillin-Resistant Coagulase-Negative Staphylococci in the Community: High Homology of SCCmec IVa between *Staphylococcus epidermidis* and Major Clones of Methicillin-Resistant *Staphylococcus aureus*. *J. Infect. Dis.* **2010**, 202, 270–281. [CrossRef] [PubMed]

81. Yamada, K.; Namikawa, H.; Fujimoto, H.; Nakaie, K.; Takizawa, E.; Okada, Y.; Fujita, A.; Kawaguchi, H.; Nakamura, Y.; Abe, J.; et al. Clinical Characteristics of Methicillin-resistant Coagulase-negative Staphylococcal Bacteremia in a Tertiary Hospital. *Intern. Med*. **2017**, 56, 781–785. [CrossRef] [PubMed]

82. El-Razik, K.A.A.; Arafa, A.A.; Hedia, R.H.; Ibrahim, E.S. Tetracycline resistance phenotypes and genotypes of coagulase-negative staphylococcal isolates from bubaline mastitis in Egypt. *Vet. World* **2017**, 10, 702–710. [CrossRef]

83. El Feghaly, R.E.; Stamm, J.E.; Fritz, S.A.; Burnham, C.-A.D. Presence of the *blaZ* beta-lactamase gene in isolates of *Staphylococcus aureus* that appear penicillin susceptible by conventional phenotypic methods. *Diagn. Microbiol. Infect. Dis.* **2012**, 74, 388–393. [CrossRef]

84. Paterson, G.K.; Harrison, E.M.; Holmes, M.A. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol*. **2014**, 22, 42–47. [CrossRef]

85. Xu, Z.; Shi, L.; Alam, M.J.; Li, L.; Yamasaki, S. Integron-bearing methicillin-resistant coagulase-negative staphylococci in South China, 2001–2004. *FEMS Microbiol. Lett*. **2008**, 278, 223–230. [CrossRef]

86. Becker, K.; van Alen, S.; Idelevich, E.A.; Schleimer, N.; Seggewiß, J.; Mellmann, A.; Kaspar, U.; Peters, G. Plasmid-Encoded Transferable mecB-Mediated Methicillin Resistance in *Staphylococcus aureus*. *Emerg. Infect. Dis*. **2018**, 24, 242–248. [CrossRef]

87. Akpaka, P.E.; Roberts, R.; Monecke, S. Molecular characterization of antimicrobial resistance genes against *Staphylococcus aureus* isolates from Trinidad and Tobago. *J. Infect. Public Health* **2017**, 10, 316–323. [CrossRef]

88. Yılmaz, E.¸S.; Aslanta¸s, Ö. Antimicrobial resistance and underlying mechanisms in *Staphylococcus aureus* isolates. Asian Pac. *J. Trop. Med*. **2017**, 10, 1059–1064. [CrossRef] [PubMed]

89. Markey, B.; Leonard, F.; Archambault, M.; Cullinane, A.; Maguire, D. *Clinical Veterinary Microbiology*, 2nd ed.; Mosby Ltd.: London, UK, 2013.

90. Laranjo, M.; Machado, J.; Young, J.P.W.; Oliveira, S. High diversity of chickpea Mesorhizobium species isolated in a Portuguese agricultural region. *FEMS Microbiol. Ecol*. **2004**, 48, 101–107. [CrossRef] [PubMed]

91. Merino, N.; Toledo-Arana, A.; Vergara-Irigaray, M.; Valle, J.; Solano, C.; Calvo, E.; Lopez, J.A.; Foster, T.J.; Penadés, J.R.; Lasa, I. Protein A-mediated multicellular behavior in *Staphylococcus aureus*. *J. Bacteriol*. **2009**, 191, 832–843. [CrossRef] [PubMed]

92. CLSI. M02-A11-*Performance Standards for Antimicrobial Disk Susceptibility Tests-Approved Standard-Eleventh Edition*; Clinical and Laboratory Standards Institute (CLSI):Wayne, PA, USA, 2012.

93. EUCAST. *European Committee on Antimicrobial Susceptibility Testing-Breakpoint Tables for Interpretation of MICs and Zone Diameters*; Version 10.0; UCAST: Växjö, Sweden, 2020.

94. Rivas, R.; Vizcaino, N.; Buey, R.M.; Mateos, P.F.; Martinez-Molina, E.; Velazquez, E. An effective, rapid and simple method for total RNA extraction from bacteria and yeast. *J. Microbiol. Methods* **2001**, 47, 59–63. [CrossRef]

95. Brakstad, O.D.D.G.; Aasbakk, K.; Maeland, J.A. Detection of *Staphylococcus aureus* by Polymerase Chain Reaction Amplification of the *nuc* Gene. *J. Clin. Microbiol*. **1992**, 30, 1654–1660. [CrossRef] [PubMed]

96. Vasudevan, P.; Nair, M.K.; Annamalai, T.; Venkitanarayanan, K.S. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. *Vet. Microbiol*. **2003**, 92, 179–185. [CrossRef]

97. Sawant, A.; Gillespie, B.; Oliver, S. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Vet. Microbiol*. **2009**, 134, 73–81. [CrossRef]

98. Strommenger, B.; Kettlitz, C.;Werner, G. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. *J. Clin. Microbiol*. **2003**, 41, 4089–4094. [CrossRef]

99. Stegger, M.; Andersen, P.S.; Kearns, A.; Pichon, B.; Holmes, M.A.; Edwards, G.; Laurent, F.; Teale, C.; Skov, R.; Larsen, A.R. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue mecALGA251. *Clin. Microbiol. Infect*. **2012**, 18, 395–400. [CrossRef]

100. Ferreira, A.M.; Martins, K.B.; Silva, V.R.D.; Mondelli, A.L.; Cunha, M.D.L.R.D.S.D. Correlation of phenotypic tests with the presence of the *blaZ* gene for detection of beta-lactamase. *Braz. J. Microbiol.* **2017**, 48, 159–166. [CrossRef]

101. Horstkotte, M.A.; Knobloch, J.K.M.; Rohde, H.; Mack, D. Rapid Detection of Methicillin Resistance in Coagulase-Negative Staphylococci by a Penicillin-Binding Protein 2a-Specific Latex Agglutination Test. *J. Clin. Microbiol*. **2001**, 39, 3700–3702. [CrossRef] [PubMed]

102. Coelho, S.D.M.D.O.; Moraes, R.A.M.; Soares, L.D.C.; Pereira, I.A.; Gomes, L.P.; Souza, M.M.S.D. Mapeamento do perfil de resistência e detecção do gene *mecA* em *Staphylococcus aureus* e *Staphylococcus intermedius* oxacilina-resistentes isolados de espécies humanas e animais. *Ciência Rural* **2007**, 37, 195–200. [CrossRef]

Chapter 4

4. The use of propolis for mastitis control

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The use of propolis for mastitis control

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Abstract

Propolis is a resinous mass produced by honeybees for the protection of the honeycomb. Propolis has become very popular in Europe due to its antibacterial activity. Recently propolis activity against bacterial biofilms was reported. Mastitis control greatly depends on the use of antimicrobials. Antimicrobial resistance and bacterial ability to produce biofilm increase microbial survival and contribute to pathogens' persistence in the farm. Furthermore, the transfer of resistance genes from mastitis causing pathogens to bacteria belonging to the human natural microbiota fully justifies the search for alternative products with antimicrobial activity. The aim of this study was to assess propolis components accountable for bactericidal and antibiofilm activities against *Staphylococcus* spp. isolated from the milk of sheep and goats with mastitis. Ten propolis batches (seven from Brazil and three from Portugal) were collected in different regions and were used to produce 30% propolis ethanol extracts (PEE). Total phenolics, flavonoids, anthocyanins and condensed tannins contents were assessed. Thirty-five *Staphylococcus aureus* and 104 coagulase-negative staphylococci (CNS) were analysed for *in vitro* susceptibility to PEE and minimum bactericidal concentration (MBC) was performed with a 96-pins microplate replicator. These PEE were also assessed for biofilm formation inhibition and biofilm disruption on 44 biofilm producing *Staphylococcus* isolates. To identify the propolis components, which are probably associated with antimicrobial and antibiofilm activities,

statistical analyses were performed. Total phenolics content varied from 67.6 to 365.4 mg GAE/g extract, flavonoids content from 54.8 to 141.2 mg QE/g extract, anthocyanins content between not detected and 47.6 mg/g extract, and condensed tannins content between 36.9 and 395.6 mg ECC/g extract. All 139 staphylococci analysed (100%) showed to be susceptible to all PEEs, except Brown4, with concentrations varying between 0.026 and 13.37 mg/mL. Concerning antibiofilm activity, nine PEE inhibited biofilm formation in most of the 44 biofilm-forming isolates. The mean inhibition percentage for each PEE, varied between 33.5 and 80.2%. Regarding biofilm disruption, all ten PEEs partially or totally destroyed the biofilm produced by all 44 staphylococci isolates. The mean PEE activity on biofilm disruption varied between 28.4 and 79.5%. According to these results, propolis deserves to be considered for the control of mastitis.

Keywords: antibiofilm, antimicrobial, mastitis, propolis

1. Introduction

Propolis is a resinous mass produced by honeybees *Apis mellifera* for the protection of the honeycomb. The word *propolis* derives from the Greek *pro* (at the entrance to) and *polis* (community) and refers to a substance for the defense of the hive. Propolis is made of materials collected by worker bees from the leaf buds of numerous plant species and substances actively secreted by plants, or exuded from plant wounds, enriched with bee salivary and enzymatic secretions (Castaldo and Capasso 2002).

Plant secondary metabolites can be divided into three groups: (i) flavonoids and other phenolic and polyphenolic compounds, (ii) terpenoids and (iii) nitrogen-containing alkaloids and sulphur-containing compounds (Crozier et al., 2007). They seem to have a key role in protecting plants from herbivores and microbial infection, among other functions. Secondary metabolites are use for dyes, fibres, glues, oils, waxes, flavouring agents, drugs and perfumes, and they are viewed as potential sources of new natural drugs, antibiotics, insecticides and herbicides (Croteau et al., 2000).

Propolis is rich in flavonoids and other phenolic compounds and may also contain terpenoids (Huang et al., 2014). It has been used around the world in the preparation of medicines to treat different pathologies and more recently became very popular in Europe due to its antibacterial activity (Wojtyczka et al., 2013; Santana et al., 2012; Lu et al., 2005). Recently propolis activity against bacterial biofilms was reported (De Marco et al., 2017; Veloz et al., 2015; Wojtyczka et al., 2013; Doganli, 2016).

Mastitis in small ruminants is responsible for decreased milk yield (Gelasakis et al., 2016; Oravcová et al., 2018), and changed milk composition, which affects cheese-making aptitude and quality of the products (Rovai et al., 2015a; Rovai et al. 2015b ; Raynal-Ljutovac et al., 2007; Paschino et al., 2018). Mastitis control is based on hygienic management of animals, but also greatly depends on the use of antimicrobials. However, some researchers report high rates of antimicrobial resistance (AMR) in pathogens isolated from ewes and goats with subclinical mastitis (Onni et al., 2011; Šiugždaitė et al., 2016; Virdis et al. 2010). Furthermore, the transfer of resistance genes from mastitis causing pathogens to bacteria belonging to the human natural microbiota (Woolhouse et al. 2015) fully justifies the search for alternative products with antimicrobial activity.

Brackman and Coenye (2015) estimated that biofilms are associated with 80% of microbial infections. Biofilm major components are an exopolysaccharide matrix (slime), proteins and environmental DNA (eDNA) along with the bacterial cells (Buttner, Dietrich, and Rohde 2015). Regarding mastitis pathology, bacterial ability to produce biofilm increases microbial survival and contribute to pathogens' persistence in the farm (Gomes et al., 2016). Although some studies showed that S. *aureus* isolated from cows with persistent and non-persistent intramammary infection did not differ in biofilm-forming ability in vitro (Pereyra et al., 2016), other reports state that S. *aureus* strains persisting in the bovine mammary gland through the dry period produced significantly more biofilm *in vitro* than strains that do not persist after calving (Veh et al., 2015). Moreover a strong biofilm forming *S. aureus* isolated from bovine mastitis produced significantly more acute mastitic lesions in mammary glands of mice inoculated than a weak biofilm-forming *S.*

aureus strain (Gogoi-Tiwari et al., 2017). In sheep, Vasileiou et al., (2018) reported 80.4% of all cases of staphylococcal subclinical mastitis due to slime producing strains.

The need to control biofilm associated with mammary infection seems to be of major importance. As revised by Melchior and co-workers (2006) several researchers have shown that bacteria growing in a biofilm can become 10–1 000 times more resistant to antimicrobials than the same strain planktonic growing bacteria. Likewise, Costerton (1999) refers to biofilm-forming bacteria as resisting to host defence mechanisms and surviving in the presence of disinfectants and/or antibiotics at concentrations 1000 to 1500 times higher than concentrations that kill planktonic cells of the same species.

In previous studies we showed that propolis ethanol extracts may have a strong bactericidal activity against *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) isolated from sheep and goats with mastitis (Queiroga et al., 2018). We also showed propolis ethanol extracts (PEE) inhibitory action on biofilm formation and PEE ability to eliminate established biofilms (Laranjo et al., 2018). A key aspect to keep in mind, considering mastitis treatment, is that the disruption of biofilm must be associated with bacterial inhibition, since the disassemble of biofilm would be responsible for the release of living bacteria that would colonize additional body sites (Buttner et al., 2015).

The aim of this study was to assess propolis components accountable for bactericidal and antibiofilm activities against *Staphylococcus* spp. isolated from the milk of sheep and goats with mastitis.

2. Methods

2.1. Propolis Collection and Origin

Seven batches of raw propolis (one Green, two Red and four Brown) were collected in apiaries of four Brazilian (BR) States with different climates and vegetation (Table 4.1, Figure 4.1A).

Table 4.1. Propolis collection and origin information

Propolis	Country	Location	Latitude	Longitude	Production	Vegetation
Green	BR	Minas Gerais SE	-20,2752	-45,2535	propolis honey	*Baccharis dracunculifolia* *Eucalyptus* sp.
Red1	BR	Alagoas NE	-9,4237	-35,5342	propolis	*Schinus terebinthifolius* *Rhizophora mangle* *Dalbergia ecastophyllum*
Red2	BR	Alagoas NE	-9,4237	-35,5342	propolis	*Schinus terebinthifolius* *Rhizophora mangle* *Dalbergia ecastophyllum*
Brown1	BR	Ceará NE	-5.74312	-39,6285	honey	*Schinus terebinthifolius* *Ziziphus joazeiro* *Mimosa hostilis* *Macrosiphonia velame* *Scoparia dulcis*
Brown2	BR	Ceará NE	-5.74312	-39,6285	honey	*Schinus terebinthifolius* *Ziziphus joazeiro* *Mimosa hostilis* *Macrosiphonia velame* *Scoparia dulcis*
Brown3	BR	Minas Gerais SE	-21,0137	-46,7439	propolis honey	*Eucalyptus* sp. *Copaifera langsdorffii* *Pinus pinea* *Vernonia polysphaera* *Baccharis dracunculifolia*
Brown4	BR	Pernambuco NE	-9,2189	-40,4530	honey	*Schinus terebinthifolius* *Schinopsis brasiliensis* *Anadenanthera macrocarpa* *Spondias tuberosa* *Prosopis juliflora* *Tamarindus indica*
Brown5	PT	Alentejo S	38,5774	-7,8614	honey	*Quercus suber* *Baccharis dracunculifolia*
Brown6	PT	Norte N	41,5153	-6,87799	honey	*Lavandula stoechas* among others
Brown7	PT	Algarve S	37,2859	-8,55594	honey	*Cistus ladanifer* *Arbutus unedo* *Lavandula stoechas* *Thymus serpyllum* *Eucalyptus* sp.

The Green propolis and Brown3 were collected in the cities of Formiga and Jacuí-MG in southeastern Brazil (SE), respectively. The Brown1 and Brown2 propolis were obtained in the city of Mombaça-CE in the northeast of Brazil (NE), in two apiaries 7 Km apart. The apiary where Brown2 propolis was collected was near a stream and the apiary where Brown1 propolis was collected had only a water box 600 m away. Brown4 propolis was collected in the city of Petrolina, PE backcountry (NE) and Red1 and Red2 propolis were obtained in the same apiary in Marechal Deodoro-AL (NE), with a two years gap between harvests (Red1 in 2014 and Red2 in 2012) and were kept at -20°C until extract preparation.

Three batches of raw propolis were collected in apiaries from diverse Portugal (PT) regions (NUTS 2), with different climates and vegetation (Table 4.1, Figure 4.1B). Brown5 was collected in Évora city in the Alentejo region (S), Brown6 was collected near the Macedo de Cavaleiros city in the North (N) and Brown7 near Monchique, district of Faro in the South (S).

Figure 4.1. Maps showing the collection sites of propolis samples: A-map of Portugal showing three propolis collection sites; B-map of Brazil showing the collection sites of seven propolis samples.

According to Abou-Shaara (2014), bees harvest resins in an area of about 2 km around the hive. Location of collection and vegetation around the apiary, as by beekeepers information, are shown in the Table 4.1.

2.2. Propolis Ethanol Extracts Preparation

Propolis ethanol extracts were prepared according to the official standards for extracts production in Normative Instruction No 3, 19/01/2001, published by the Department of Inspection of Animal Products of the Ministry of Agriculture, Livestock and Food Supply, Brazil (Brazil, 2001). Cold maceration of 300 g of raw propolis in 700 mL of 70° ethanol was performed, resulting in 30% PEE. The preparations were kept at room temperature, protected from light, for 45 days. After this period, extracts were filtered with a sterile funnel and filter paper and kept refrigerated at 4°C, in amber bottles, until use.

2.3. Propolis Ethanol Extracts Major Chemical Groups

Total phenolic, flavonoids, condensed tannins and anthocyanins contents of PEE were determined by spectrophotometric methods. For total phenolic assessment each PEE was diluted to 1mg/mL solution and the determination was performed by the spectrophotometric method of Folin-Ciocalteau (Slinkard and Singleton 1977) with modifications, using gallic acid as standard phenolic compound. Fifty µL of each ethanolic solution was added to 20 µL of de Folin-Ciocalteau and 870 µL distilled water and stirred for 1 min. Sixty µL of Na_2CO_3 (15%) were added and the mixture stirred for 30 seconds, resulting in a final concentration of 50 µg/mL for each sample.

After two hours rest in the absence of light, 300 µL of each solution were transferred to 96-well microtiter plates and readings were recorded at 760 nm. The analyses were performed in triplicate and total phenolic content was determined by interpolating the absorbance of the samples against a calibration curve constructed with gallic acid standards (5.0 to 30.0 µg/mL) and expressed as milligrams of equivalent acid gallic for gram of extract (mg GAE/g), considering the mean standard error. The equation of gallic acid calibration

curve was y = 0.054x + 0.192, with the correlation coefficient r^2= 0.995, where *x* is the concentration of gallic acid and *y* is the absorbance at 760 nm.

Total flavonoid content was determined using the method of Vermerris and Nicholson (2006) with modifications. Briefly, 500 µL of 5% methanol aluminium chloride ($AlCl_3$) were mixed with suitable volumes of samples and the volume was made up to 1,000 µL with distilled water. After 10 minutes, absorbance was measured in UV-Vis ELISA spectrophotometer at 425 nm in 96-well microtiter plates. The analyses were performed in triplicate and total flavonoid content was determined by interpolating the absorbance of the samples against a calibration curve constructed with standard solutions of quercetin (from 2.5 to 30.0 µg/mL) and expressed as equivalent milligrams of quercetin per gram of PEE (mg QE/g), considering mean standard error. The equation of quercetin calibration curve was y = 0.0625x – 0.0023, with the correlation coefficient r^2= 0.997, where *x* is the concentration of quercetin and *y* is the absorbance at 425nm.

Condensed tannins content determination was performed using colorimetric method based on the reaction of vanillin with tannins in acidic medium (Vermerris and Nicholson, 2006). Briefly 300 µL of a vanillin solution in 70% sulfuric acid (1% w/v, freshly prepared) was added to the samples, diluted in ethanol and incubated at 20°C in a water bath for 15 minutes. The absorbance was measured in 96-well plates on an Elisa UV-Vis spectrophotometer at 500 nm. Analyses were performed in triplicate and the content of condensed tannins (proanthocyanidins) was determined by interpolating the absorbance of the samples against a calibration curve constructed with solutions of the catechin standard at various concentrations (5.0 to 40.0 µg/mL) and expressed as milligram epicatechin content per gram of extract (mg ECC/g), considering the standard error of the mean (SEM). The equation of the catechin calibration curve was: y = 0.0133x + 0.2241, with the correlation coefficient r^2 = 0.989, where *x* is the catechin concentration and *y* is the absorbance at 500 nm.

Determination of the anthocyanin content was performed by the differential pH method (Giusti and Wrolstad, 2001). Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra.

The pH-differential method is based on this reaction and permits accurate and rapid measurement of the total anthocyanins by differences in spectrophotometer absorbance values.

Dilutions of the samples were prepared in triplicate, one with 0.025M potassium chloride buffer pH 1, and the other with 0.4M sodium acetate buffer pH 4.5. The pH was adjusted with concentrated hydrochloric acid using a pH meter. Dilutions were allowed to equilibrate for 15 minutes and samples were read in a 510 nm and 700 nm in glass cuvettes in a UV-visible spectrophotometer (LAMBDA 45, Perkin Elmer). The absorbances of the dilutions were calculated using the following equation:

$A = (A_{510nm} – A_{700nm})\ pH1.0 - (A_{510nm} - A_{700nm})$

Monomeric anthocyanins concentration of the original sample was calculated by the following formula:

Monomeric anthocyanins (mg/L) = (A x WM x FD x 1000) / (ε x 1)

Where WM is the molecular weight, FD is the dilution factor and ε is the molar absorptivity of cyanidin-3-glycoside. (MW = 449g, ε = 26,900).

2.4. Propolis Ethanol Extracts Chemical Profile

The ultra-high-pressure liquid chromatography along with quadrupole time of flight mass spectrometry (UPLC-QTOF MS^E) experiments were performed to identify some individual compounds from PEE. The UPLC-Photo Diode Array (PDA) phenolic profile of PEE was recorded at 290 nm and the compounds were tentatively identified by UPLC-QTOF-MS/MS as flavonoids (flavonol/flavone, isoflavone, flavanone and chalcones), non-flavonoids phenolics and triterpenes, based on their characteristic UV-vis spectra peaks and mass detection as well as the accurate mass measurement of the precursor and product ions.

Chromatographic analyses were carried out as follows. One hundred mg of each propolis sample were solubilized in 0,5 mL of HPLC grade methanol and 0.5 mL of MilliQ

water pH 2.0 (adjusted with HCl). This solution was eluted in a Strata SPE-C18 cartridge (San Diego, CA, USA) previously activated. After washing with MilliQ water, 2.0 mL of methanol were used three times to elute the compounds of interest and evaporated. Five mg of each extract was solubilized in the mobile phase, filtered through a 0.45 µm Millipore membrane.

The XEVO-G2XSQTOF mass spectrometer (Waters, USA) was connected to the Acquity UPLC system (Waters, USA) via an electrospray ionization interface (ESI). The chromatographic separation of compounds was performed on the ACQUITY UPLC with a conditioned autosampler at 4 °C using a Waters Acquity UPLC BEH C18 (2.1 mm × 50 mm, 1.7 µm) (Waters, USA).

The mobile phase consisting of water with 0.1% formic acid (Sigma, USA) in water (solvent A) and acetonitrile (Sigma, USA) 0.1% formic acid (solvent B) was pumped at a flow rate of 0.4 mL min^{-1}.

The gradient elution program was as follows: 0-5 min, 5-10% B; 5-9 min, 10-95% B. The injection volume was 5-10 µL. MS analysis was performed on the Xevo G2 QTOF, a quadrupole time-of-flight tandem mass spectrometer coupled with an electrospray ionization source in negative ion mode. The scan range was from 50 to 1200 m/z for data acquisition.

In addition, MS^E experiments were carried which allows both precursor and product ion data to be acquired in one injection. Source conditions as follows: capillary voltage, 3.0 kV; sample cone, source temperature, 120 °C; desolvation temperature 250 °C; cone gas flow rate 50 Lh^{-1}; desolvation gas (N2) flow rate 800 Lh^{-1}. All analyses were performed using the lockspray, which ensured accuracy and reproducibility.

Leucine-enkephalin (5 ng·mL^{-1}) was used as a standard or reference compound to calibrate mass spectrometers during analysis. All the acquisition and analysis of data were controlled using Waters MassLynx v 4.1 software.

2.5. Bacterial Isolates

Milk samples were aseptically collected from different mammary glands of sheep and goats, belonging to different flocks, with clinical and subclinical mastitis. Clinical mastitis was recognized based on udder and/or milk changes and subclinical mastitis was assessed according to milk somatic cell discharge using California mastitis test (CMT), which results were recorded as negative, "traces" (T), 1+, 2+ and 3 +. Clinical mastitis (CM) was considered the highest grade of mammary inflammation.

Milk samples were immediately refrigerated until processed, within no more than 12 hours. Bacteriological analyses were undertaken according to the National Mastitis Council methodology (2004) and isolates were identified to the species level using API-Staph (Biomérieux) or Vitek 2 Compac (Biomérieux).

A total of 139 staphylococci field isolates, 35 *Staphylococcus aureus*, 104 coagulase-negative staphylococci (CNS) and seven reference strains: five *S. aureus* (ATCC 25923, ATCC 29213, COL, FRI 472, and FRI 913) and two *S. epidermidis* (ATCC 12228, and ATCC 35984) were used to assess antimicrobial activity of PEE.

For PEE antibiofilm activity assessment, 44 biofilm producing *Staphylococcus* field isolates (26 *S. aureus*, seven *S. chromogenes*, four *S. warneri*, three *S. auricularis*, two *S. simulans*, one *S. caprae*, one *S. capitis*), and one biofilm-producing reference *S. epidermidis* strain (ATCC 35984) were included in this study. Biofilm production was evaluated according to Merino et al. (2009) with some modifications, as previously described (Laranjo et al., 2018).

2.6. Antimicrobial and Antibiofilm Assessment

In vitro antimicrobial activity of propolis ethanol extracts was performed according to the CLSI protocol M07-A9 (2012), as described by Queiroga and co-workers (2018). Minimum bactericidal concentration (MBC) was determined with a 96-pin microplate replicator (Boekel Scientific) which inoculates approximately 10 µl of each dilution onto a 150 mm diameter Petri dish with Mueller-Hinton Agar (Queiroga et al., 2018).

To investigate the *in vitro* activity of PEE against biofilms produced by staphylococci isolates, the inhibitory action on biofilm formation and the PEE ability to eliminate established biofilms were evaluated in terms of % of affected isolates and mean % of inhibition and disruption, respectively. The inhibitory effect on biofilm formation was assessed by growing isolates' suspensions with half minimum bactericidal concentration (1/2 MBC) from each PEE, as previously described (Laranjo et al., 2018). To evaluate the PEE ability to eliminate established biofilms, the same isolates were grown to produce biofilms, which were, then, exposed to the action of the corresponding 1/2 MBC of PEE (Laranjo et al., 2018).

2.7. Data Analysis

These analyses were performed in order to tentatively answer the following questions: (1) Does the ability of a *Staphylococcus* isolate to produce biofilm affect mammary inflammation? (2) Is the ability of a *Staphylococcus* isolate to produce biofilm important for its resistance to PEE? (3) Which PEE has shown the best bactericidal activity, biofilm inhibition and biofilm disruption performances? (4) Which PEE components are associated with their antimicrobial action? (5) Which PEE components are associated with their ability to inhibit or to disrupt biofilm?

Data for PEE major components were submitted to analysis of variance (ANOVA). Significant differences ($P<0.05$) were determined according to Tukey's Honest Significant Difference (HSD) test. Chi-square test of association was used to assess the association between bacterial species and biofilm production ability and the association between mammary gland inflammatory intensity and biofilm production. An ANOVA was performed to evaluate the effect of biofilm production on minimal bactericidal concentration.

In order to assess the best PEE activities, namely minimal bactericidal concentration, percentage of biofilm inhibition and percentage of biofilm disruption, an ANOVA was performed, with significant differences ($P<0.05$) determined according to Tukey's HSD test.

In addition, Box-whisker plots were performed to represent the distribution of the different PEE activities.

Data for antimicrobial and antibiofilm actions of PEE were analysed by Spearmann's correlations for a significance level of P=0.05. Data for antimicrobial and antibiofilm activities of each PEE were submitted to an ANOVA to assess PEE individual compounds accountable for those activities. Significant differences (P<0.05) were determined according to Tukey's HSD test.

The software StatisticaTM v.12.0 software (StatSoft Inc, Tulsa, OK, USA, 1984–2014) was used for all statistical analyses.

3. Results

3.1. Propolis Ethanol Extracts Major Chemical Groups

Table 4.2. Propolis ethanol extracts (PEE) major chemical groups concentration.

PEE	Total phenolics (mg GAE/g)	Flavonoids (mg QE/g)	Anthocyanins (mg/g)	Tannins (mg ECC/g)
Green	$283.6^{c} \pm 7.5$	$64.9^{a} \pm 1.6$	$7.1^{a} \pm 0.5$	$287.9^{e} \pm 5.7$
Red1	$232.1^{b} \pm 3.7$	$55.5^{a} \pm 1.4$	$22.6^{ab} \pm 13.4$	$256.2^{d} \pm 1.1$
Red2	$312.1^{c} \pm 95.3$	$96.1b^{c} \pm 0.9$	$47.6^{b} \pm 8.0$	$216.1^{c} \pm 1.0$
Brown1	$232.9^{b} \pm 6.7$	$109.1^{d} \pm 1.7$	$11.8^{a} \pm 2.1$	$188.6^{b} \pm 2.7$
Brown2	$216.2^{b} \pm 4.3$	$124.5^{e} \pm 1.2$	$11.8^{a} \pm 1.7$	$395.6^{f} \pm 7.7$
Brown3	$305.9^{c} \pm 9.2$	$141.2^{f} \pm 3.8$	$5.8^{a} \pm 0.7$	$247.2^{d} \pm 5.0$
Brown4	$246.6^{b} \pm 11.3$	$103.7^{cd} \pm 5.0$	$9.4^{a} \pm 0.3$	$199.2^{bc} \pm 2.2$
Brown5	$345.5^{d} \pm 1.2$	$86.3^{b} \pm 1.3$	ND	$48.5^{a} \pm 0.1$
Brown6	$365.4^{d} \pm 0.5$	$92.3^{bc} \pm 1.5$	ND	$50.9^{a} \pm 0.2$
Brown7	$67.6^{a} \pm 2.8$	$54.8^{a} \pm 1.7$	ND	$36.9^{a} \pm 0.4$

SEM - standard error of the mean; ND – not detected; Values are expressed as means ± SEM. Different letters in each column mean significant differences for P<0.05.

Chemical composition of the propolis ethanol extracts (PEE) was determined for the 10 PEE. Total phenolics content varied from 67.6 to 365.4 mg GAE/g, flavonoids content ranged from 54.8 to 141.2 mg QE/g, anthocyanins content varied between not detected, in

the Portuguese PEE (Brown5, Brown6, and Brown7), to 47.6 mg/g, and condensed tannins content ranged from 36.9 to 395.6 mg ECC/g (Table 4.2).

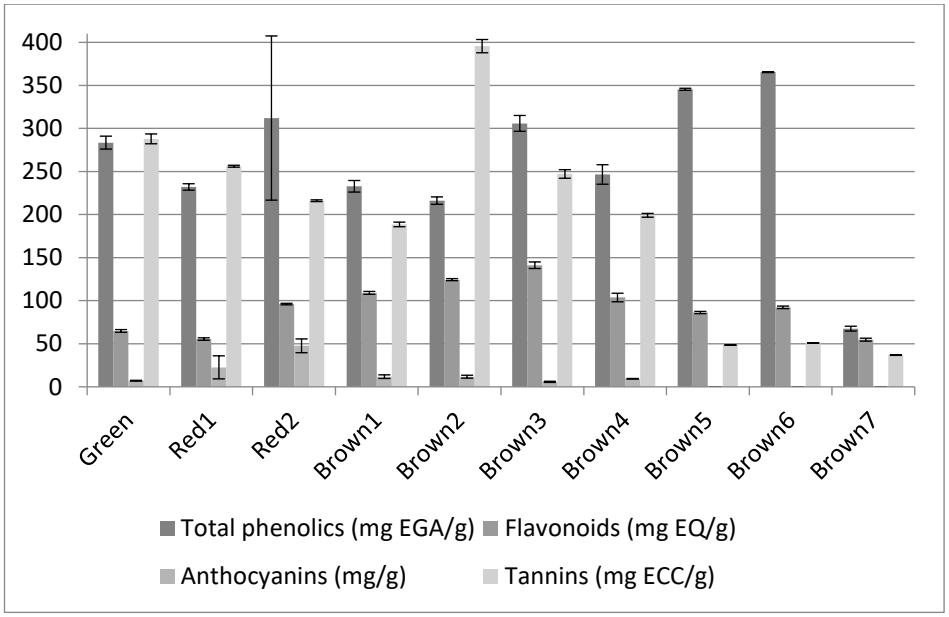

Figure 4.2. Propolis ethanol extracts (PEE) major components.

There are significant differences between the total phenolics, flavonoids, anthocyanins, and condensed tannins contents of the studied PEE ($P<0.001$). A positive correlation was found between anthocyanins and tannins ($P<0.05$). Moreover, significant differences in the content of flavonoids and anthocyanins were found for distinct propolis colour. Red propolis PEE have a significantly higher content in anthocyanins compared to green and brown propolis PEE ($P<0.05$) (Figure 4.2).

3.2. Propolis Ethanol Extracts Chemical Profile

The chemical profile of the 10 PEE was assessed by UPLC-QTOF-MS/MS as exemplified in Figure 4.3 for Green PEE. The peaks not cited are from unidentified compounds. The identification of individual compounds in the different PEE allowed their grouping into six (I-VI) groups as shown in Table 4.3: I-Green; II-Red1 and Red2; III-Brown1 and Brown2; IV-Brown3 and Brown 4; V-Brown5 and Brown 6; and VI-Brown7.

All individual compounds that were identified are flavonoids (chalcones, flavanones, flavanonols, flavones, flavonols, isodihydroflavones, isoflavans), non-flavonoid phenolics and triterpenes.

Green propolis PEE (group I) has two flavonoids and eight non-flavonoid phenolics (among which seven phenolic acids). Red propolis PEE (group II) has nine flavonoids. Brown propolis PEE are divided into four groups: group III (Brown1 and Brown2) PEE has 25 flavonoids; group IV (Brown3 and Brown4) PEE has one flavonoid and four triterpenes; group V (Brown5 and Brown6) PEE has 12 flavonoids and four non-flavonoids phenolics (among which one phenolic acid); and group VI (Brown7) has six flavonoids.

The most widespread individual compounds across the PEE are naringenin (Red1, Red2, Brown1 and Brown2), kaempferol methyl ether and quercetin dimethyl ether (Brown1, Brown2, Brown5 and Brown6), all flavonoids, which were detected in four out of 10 PEE, followed by pinobanksin, chrysin and acacetin, found only in the three Portuguese brown propolis. All other individual compounds are found in two PEE, with the exception all Green PEE compounds, which are exclusive.

3.3. *Staphylococcus* Species and Biofilm Production

Regarding biofilm production ability, according to the Chi-square test of association, an association was found between bacterial species and biofilm production ability ($\chi 2 = 60.671$, $df = 13$, $P<0.001$) (Table 4.4).

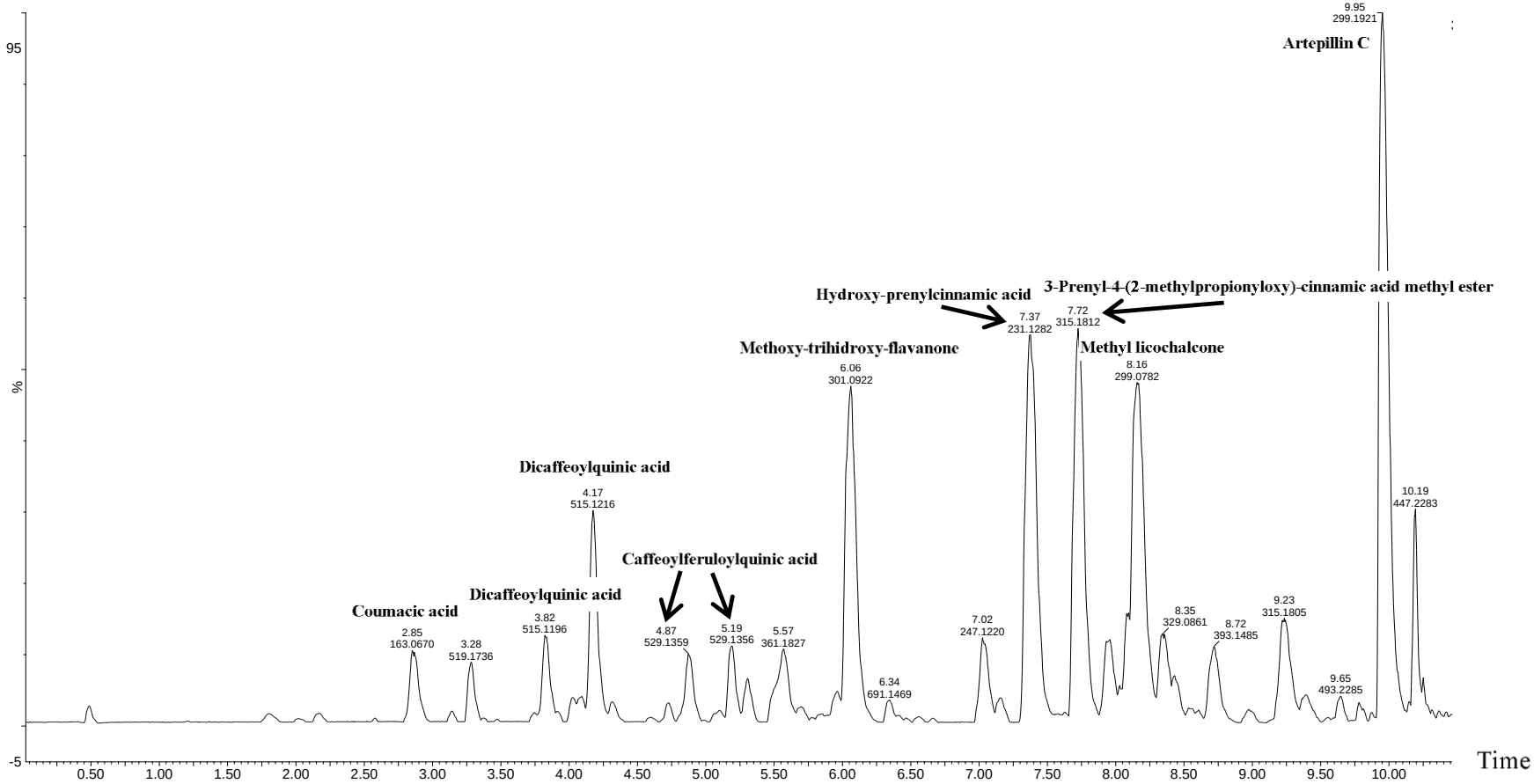

Figure 4.3. UPLC-QTOF-MS/MS profile of Green PEE.

Table 4.3. Identification of some propolis individual compounds

λmax (nm)	[M-H]- (*m/z*)	[M-H]- (*m/z*) calculated	Compound	Green	Red1	Red2	Brown1	Brown2	Brown3	Brown4	Brown5	Brown6	Brown7
308	163.0419	163.0400	Coumaric acid	x									
326	515.1196	515.1195	Dicaffeoylquinic acid (1)	x									
325	515.1227	515.1195	Dicaffeoylquinic acid (2)	x									
325	529.1359	529.1352	Caffeoylferuloylquinic acid (1)	x									
328	529.1356	529.1352	Caffeoylferuloylquinic acid (2)	x									
290	301.0771	301.0717	Methoxy-trihidroxy-flavanone	x									
314	231.1026	231.1026	Hydroxy-prenylcinnamic acid	x									
312	315.1601	315.1601	3-Prenyl-4-(2-methylpropionyloxy)-cinnamic acid methyl ester	x									
364	299.0782	299.0924	Methyl licochalcone	x									
313	299.1658	299.1652	Hydroxy-diprenylcinnamic acid (artepillin C)	x									
274, 314	255.0662	255.0661	Liquiritigenin		x	x							
283	283.0692	283.0611	2´-Hydroxyformononetin		x	x							
284	285.0863	285.0768	Pinobanksin methyl ether		x	x							
372	255.0627	255.0640	Isoliquiritigenin		x	x							
297	267.0792	267.0662	Formononetin/Isoformononetin		x	x							
280	271.0950	271.0611	Vestitol		x	x							
277, 339	283.0793	283.0611	Biochanin A		x	x							
280	271.0943	271.0611	Naringenin *		x	x	x	x					
279	285.1091	285.0768	Vestitone		x	x							
283	285.0757	285.0768	Trimethoxy flavanone				x	x					
373	301.0356	301.0354	Quercetin *				x	x					

λmax (nm)	[M-H]- (*m/z*)	[M-H]- (*m/z*) calculated	Compound	Green	Red1	Red2	Brown1	Brown2	Brown3	Brown4	Brown5	Brown6	Brown7
246	315.0500	315.0510	Quercetin methyl ether				x	x					
287	301.0342	301.0717	Trihydroxy-methoxy-flavanonol				x	x					
356	315.0500	315.0510	3-O-Methyl-quercetin				x	x					
356	345.0602	345.0616	Myricetin dimethyl-ether				x	x					
364	285.0405	285.0404	Herbacetin				x	x					
343	299.0541	299.0561	Kaempferol methyl ether (1)				x	x					
361	315.0504	315.0510	Isorhamnetin *				x	x					
346	299.0545	299.0561	Kaempferol methyl ether (2)				x	x			x	x	
287	315.0500	315.0874	Aromadendrin dimethyl ether				x	x					
345	329.0654	329.0667	Quercetin dimethyl ether (1)				x	x					
361	329.0657	329.0667	Quercetin dimethyl ether (2)				x	x					
369	285.0742	285.0404	Hydroxy-methoxy-chalcone				x	x					
296	301.0700	301.0717	Trihydroxy-methoxy-flavanone				x	x					
275	269.0800	269.0819	Hydroxy-methoxy-flavanone				x	x					
333	313.0703	313.0718	Kaempferol dimethyl ether (1)				x	x					
358	329.0642	329.0667	Quercetin dimethyl ether				x	x			x	x	
341	343.0805	343.0823	Quercetin trimethyl ether				x	x					
287	285.0741	285.0768	7-*O*-methyl naringenin (sakuranetin) *				x	x					
339	283.0605	283.0612	Dihydroxy methoxy flavone				x	x					
346	313.0703	313.0718	Kaempferol dimethyl ether (2)				x	x					
364	269.0814	269.0819	Dihydroxy methoxy chalcone				x	x					
286	299.0914	299.0925	Naringenin dimethyl ether				x	x					
ND	339.1237	339.1238	Trihydroxy-phenyl-flavonone						x	x			

λmax (nm)	[M-H]- (*m/z*)	[M-H]- (*m/z*) calculated	Compound	Green	Red1	Red2	Brown1	Brown2	Brown3	Brown4	Brown5	Brown6	Brown7
ND	767.4587	767.4587	Triterpene hexoside derivative						x	x			
ND	373.2704	373.2748	Steroid derivative						x	x			
ND	471.3481	471.3480	Triterpene acid derivative (1)						x	x			
ND	469.3313	469.3329	Triterpene acid derivative (2)						x	x			
322	179.0623	179.0349	Caffeic acid								x	x	
289	285.0654	285.0768	Pinocembrin-methyl ether								x	x	
357	315.0721	315.0510	Quercetin-methyl ether								x	x	
290	271.0662	271.0611	Pinobanksin								x	x	x
294, 325	247.0379	247.0975	Caffeic acid isoprenyl ester								x	x	
266, 313	253.0756	253.0506	Chrysin								x	x	x
325	283.0845	283.0975	Caffeic acid phenylethyl ester								x	x	
354	269.0668	269.0455	Galangin								x	x	
292	313.0817	313.0717	Pinobanksin-*O*-acetate								x	x	
326	283.0817	283.0617	Acacetin								x	x	x
325	295.0832	295.0975	Caffeic acid cinnamyl ester								x	x	
292	341.1254	341.1394	Pinobanksin-*O*-butyrate or isobutyrate								x	x	
290	355.1385	355.1550	Pinobanksin-3-*O*-pentanoate or-2-methylbutyrate								x	x	
290	403.1329	403.1550	Pinobanksin-*O*-phenylpropionate								x	x	
339	269.0714	269.0455	Apigenin										x
289	255.0926	255.0662	Pinocembrin										x
345	313.0936	313.0717	Kaempferol-dimethyl-ether (3)										x

Compared with references (Almeida et al., 2017; Carvalho et al., 2011; Daugsch et al., 2008; Falcão, Tomás, et al., 2013; Falcão, Vale, et al., 2013; Ferreira et al., 2017).

* Compared with standard samples.

Table 4.4. *Staphylococcus* species and biofilm production ability.

***Staphylococcus* species**	**Biofilm production**		**Total**
	positive	**negative**	
S. aureus	26	9	35
S. warneri	4	3	7
S. caprae	1	23	24
S. simulans	2	10	12
S. auricularis	3	1	4
S. epidermidis	0	14	14
S. capitis	1	3	4
Staphylococcus sp.	0	2	2
S. lentus	0	5	5
S. chromogenes	7	12	19
S. hominis	0	4	4
S. hyicus	0	3	3
S. equorum	0	1	1
S. haemolyticus	0	5	5
Total	**44**	**95**	**139**

Most (74%) *S. aureus* isolates (26/35) have the ability to produce biofilm, while all *S. epidermidis* (14/14), *S. simulans* (12/12), *S. lentus* (5/5), *S. haemolyticus* (5/5), *S. hominis* (4/4), *S. hyicus* (3/3) and *S. equorum* (1/1) are non-producers. *S. caprae* isolates are mostly non-producers (23/24) and 37% *S. chromogenes* isolates (7/19) produce biofilm.

3.4. Biofilm Effect on Mammary Inflammation and on In vitro Susceptibility to PEE

Regarding biofilm production effect on mammary inflammation, for a group of 139 staphylococci field isolates (Table 4.5), there is an association between mammary gland inflammatory intensity, according to CM/CMT, and biofilm production ($\chi^2 = 24.042$, df = 5, $P<0.001$). The most exuberant inflammatory reactions (CM and 3+) as assessed by CMT seem to have been caused by biofilm-producing pathogens. The ability of a *Staphylococcus* isolate to produce biofilm does not seem to influence its susceptibility to PEE ($P>0.05$).

Table 4.5. Biofilm production effect on mammary inflammation

Biofilm production	Mammary inflammation intensity						Total
	CM	3+	2+	1+	T	neg	
positive	8	13	10	7	3	3	44
negative	4	8	20	39	17	7	95
Total	12	21	30	46	20	10	139

3.5. PEE/PEE Components and Bactericidal Activity

The PEE with the worst bactericidal activity is Brown4, which significantly different from all other PEE (P<0.05). Although not significantly different from all PEE, except Brown4, Brown7 is the PEE with the best bactericidal action. This PEE has six flavonoids: apigenin, pinobanksin, chrysin, pinocembrin, acacetin, kaempferol-dimethyl-ether (Table 4.3). The bactericidal activity of the different PEE is shown on Figure 4.4 with a box-whisker distribution plot.

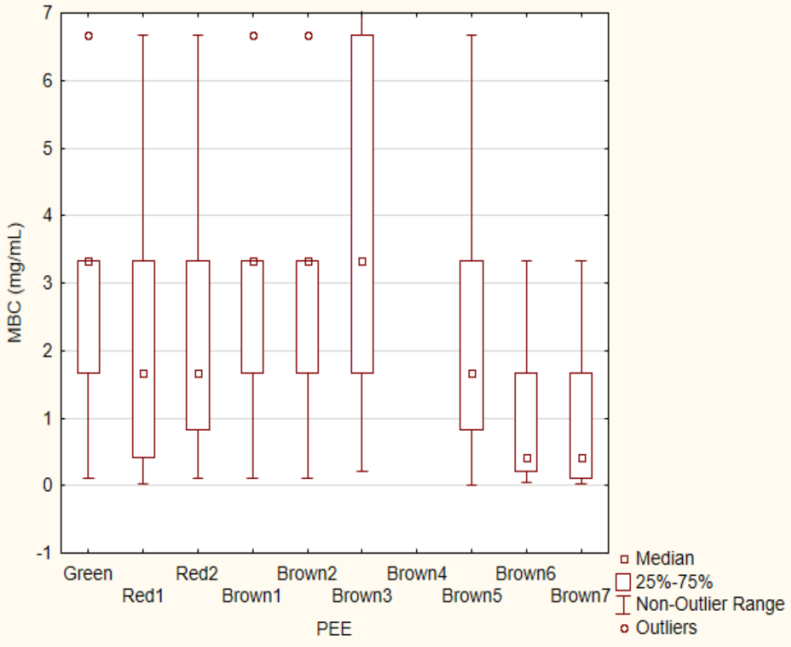

Figure 4.4. Box-whisker plot of PEE antimicrobial activity.

For the 139 staphylococci field isolates studied there is a positive correlation between the content in flavonoids, anthocyanins and condensed tannins and the minimal bactericidal concentration (MBC) (P<0.05). The concentration of these major chemical

groups is directly proportional to the MBC, meaning that the less flavonoids, anthocyanins and condensed tannins, lower the MBC and the more effective the PEE.

Nevertheless, when considering the PEE chemical profiles, the presence of individual compounds showed to positively influence the antibacterial activity, for all identified phenolic compounds (n=61), from which 49 are flavonoids. On the other hand, the presence of triterpenes negatively influences the antimicrobial activity ($P>0.05$).

3.6. PEE/PEE Components and Antibiofilm Activity

3.6.1. Biofilm inhibition

Table4. 6. Mean biofilm inhibition percentage of different PEE

PEE	Mean biofilm inhibition percentage
Brown2	80.2^{a}
Brown3	77.4^{a}
Brown1	72.9^{ab}
Red2	72.2^{ab}
Red1	67.8^{ab}
Green	65.2^{ab}
Brown5	58.6^{ab}
Brown7	53.4^{bc}
Brown6	33.5^{c}
Brown4	0.0^{d}

Different letters denote significantly different means ($P<0.05$).

Regarding PEE inhibition activity, Brown2 PEE showed to be the best PEE, although not significantly different from Brown3, Brown1, Red2, Red1, Green and Brown5. Brown4 showed to be the worst PEE significantly different from all other PEE (Table 4.6).

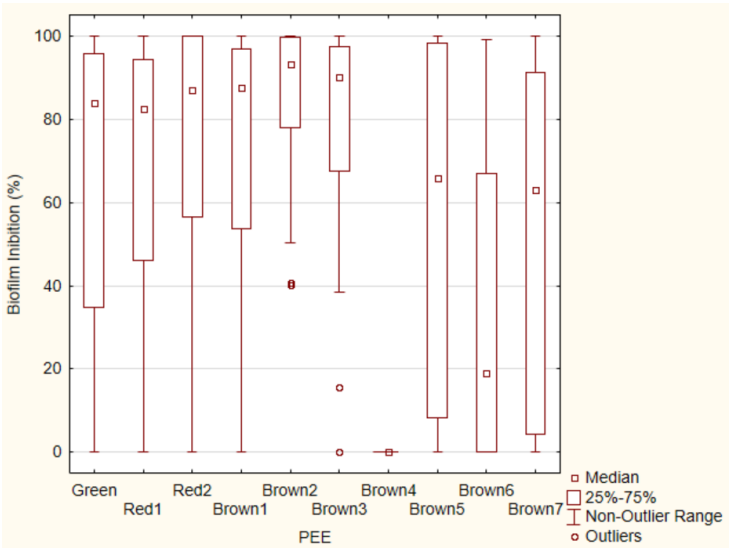

Figure 4.5. Box-whisker plot of PEE biofilm inhibition.

PEE biofilm inhibition percentage is shown on Figure 4.5 with a box-whisker distribution plot. For the 44 biofilm producing *Staphylococcus* field isolates, biofilm mean % of inhibition is positively correlated with the content in major chemical groups flavonoids, anthocyanins and condensed tannins ($P<0.05$). Regarding the PEE chemical profiles, the presence of different individual compounds showed different influence on biofilm inhibition (Table 4.7).

Considering the 65 individual compounds identified on the studied PEE, 31, all flavonoids, showed to be associated with inhibitory effect on biofilm formation. All triterpenes identified, four non-flavonoid phenolics and 11 flavonoids are associated with a decrease in biofilm inhibition, when present on PEE. No influence on this ability was shown by eight non-flavonoid phenolics and seven flavonoids.

Table 4.7. Effect of the presence of individual compounds on the inhibition of biofilm formation

Increase in inhibition %	Decrease in inhibition %	No influence
Liquiritigenin	Trihydroxy-phenyl-flavanone	Coumaric acid
2´-Hydroxyformononetin	Triterpene hexoside derivative	Dicaffeoylquinic acid (1)
Pinobanksin methyl ether	Steroid derivative	Dicaffeoylquinic acid (2)
Isoliquiritigenin	Triterpene acid derivative (1)	Caffeoylferuloylquinic acid (1)
Formononetin/Isoformononetin	Triterpene acid derivative (2)	Caffeoylferuloylquinic acid (2)
Vestitol	Caffeic acid	Methoxy-trihidroxy-flavanone
Biochanin A	Pinocembrin-methyl ether	Hydroxy-prenylcinnamic acid

Naringenin	Quercetin-methyl ether	3-Prenyl-4-(2-methylpropionyloxy)-cinnamic acid methyl ester
Vestitone	Pinobanksin	Methyl licochalcone
Trimethoxy flavanone	Caffeic acid isoprenyl ester	Hydroxy-diprenylcinnamic acid (artepillin C)
Quercetin	Chrysin	Kaempferol methyl ether (2)
Quercetin methyl ether	Caffeic acid phenylethyl ester	Quercetin dimethyl ether
Trihydroxy-methoxy-flavanonol	Galangin	Apigenin
3-O-Methyl-quercetin	Pinobanksin-O-acetate	Pinocembrin
Myricetin dimethyl-ether	Acacetin	Kaempferol-dimethyl-ether (3)
Herbacetin	Caffeic acid cinnamyl ester	
Kaempferol methyl ether (1)	Pinobanksin-O-butyrate or isobutyrate	
Isorhamnetin	Pinobanksin-3-O-pentanoate or-2-methylbutyrate	
Aromadendrin dimethyl ether	Pinobanksin-O-phenylpropionate	
Quercetin dimethyl ether (1)		
Quercetin dimethyl ether (2)		
Hydroxy-methoxy-chalcone		
Trihydroxy-methoxy-flavanone		
Hydroxy-methoxy-flavanone		
Kaempferol dimethyl ether (1)		
Quercetin trimethyl ether		
7-O-methyl naringenin (sakuranetin)		
Dihydroxy methoxy flavone		
Kaempferol dimethyl ether (2)		
Dihydroxy methoxy chalcone		
Naringenin dimethyl ether		

3.6.2. Biofilm Disruption

Concerning PEE disruption activity, Brown2 PEE showed to be the best PEE, although not significantly different from Brown7, Green, Red2, Red1 and Brown6. Brown1 showed to be the worst PEE also not significantly different from Brown3, Brown5, Brown4, Brown6 and Red1 (Table 4.8). PEE biofilm disruption percentage is shown on Figure 4.6 with a box-whisker distribution plot. Biofilm mean % of disruption is positively correlated with the content in condensed tannins and negatively correlated with total phenolics and flavonoids ($P<0.05$). The presence of different individual compounds showed different influence on biofilm disruption (Table 4.9).

Table 4.8. Mean biofilm disruption percentage of different PEE

PEE	Mean biofilm disruption percentage
Brown2	79.5[a]
Brown7	72.4[ab]
Green	67.8[abc]
Red2	56.0[abcd]
Red1	55.5[abcde]
Brown6	54.4[abcde]
Brown4	45.4[bcde]
Brown5	44.4cde
Brown3	37.6[de]
Brown1	28.4[e]

Different letters denote significantly different means (P<0.05).

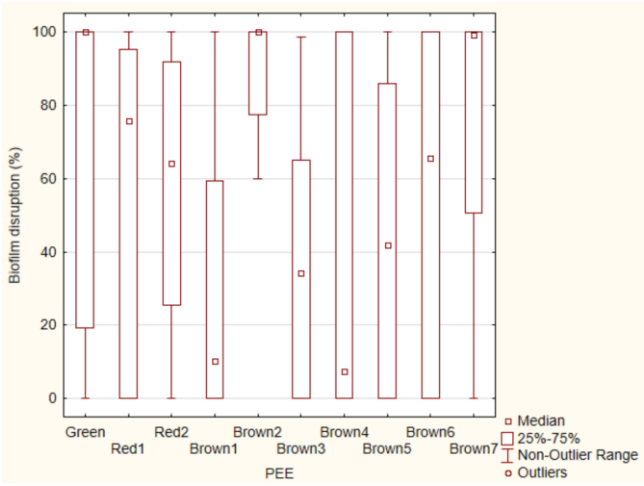

Figure 4.6. Box-whisker plot of PEE biofilm disruption.

Table 4.9. Effect of the presence of individual compounds on the biofilm disruption

Increase in disruption %	Decrease in disruption %	No influence
Coumaric acid	Trihydroxy-phenyl-flavanone	Liquiritigenin
Dicaffeoylquinic acid (1)	Triterpene hexoside derivative	2´-Hydroxyformononetin
Dicaffeoylquinic acid (2)	Steroid derivative	Pinobanksin methyl ether
Caffeoylferuloylquinic acid (1)	Triterpene acid derivative (1)	Isoliquiritigenin
Caffeoylferuloylquinic acid (2)	Triterpene acid derivative (2)	Formononetin/Isoformononetin
Methoxy-trihidroxy-flavanone		Vestitol
Hydroxy-prenylcinnamic acid		Biochanin A
3-Prenyl-4-(2-methylpropionyloxy)-cinnamic acid methyl ester		Naringenin
Methyl licochalcone		Vestitone
Hydroxy-diprenylcinnamic acid (artepillin C)		Trimethoxy flavanone
Apigenin		Quercetin
Pinocembrin		Quercetin methyl ether
Kaempferol-dimethyl-ether (3)		Trihydroxy-methoxy-flavanonol
		3-O-Methyl-quercetin

		Myricetin dimethyl-ether
		Herbacetin
		Kaempferol methyl ether (1)
		Isorhamnetin
		Kaempferol methyl ether (2)
		Aromadendrin dimethyl ether
		Quercetin dimethyl ether (1)
		Quercetin dimethyl ether (2)
		Hydroxy-methoxy-chalcone
		Trihydroxy-methoxy-flavanone
		Hydroxy-methoxy-flavanone
		Kaempferol dimethyl ether (1)
		Quercetin dimethyl ether
		Quercetin trimethyl ether
		7-O-methyl naringenin (sakuranetin)
		Dihydroxy methoxy flavone
		Kaempferol dimethyl ether (2)
		Dihydroxy methoxy chalcone
		Naringenin dimethyl ether
		Caffeic acid
		Pinocembrin-methyl ether
		Quercetin-methyl ether
		Pinobanksin
		Caffeic acid isoprenyl ester
		Chrysin
		Caffeic acid phenylethyl ester
		Galangin
		Pinobanksin-O-acetate
		Acacetin
		Caffeic acid cinnamyl ester
		Pinobanksin-O-butyrate or isobutyrate
		Pinobanksin-3-O-pentanoate or-2-methylbutyrate
		Pinobanksin-O-phenylpropionate

From all individual compounds identified, eight non-flavonoid phenolics and five flavonoids showed to be associated with biofilm disruption effect. All triterpenes identified and one flavonoid are associated with a decrease in biofilm disruption activity. No influence on this ability was shown by 43 flavonoids and four non-flavonoid phenolics.

4. Discussion

The present study evaluated 10 propolis ethanol extracts (PEE) with the purpose of assessing the influence of their composition on bactericidal, biofilm inhibition and biofilm

disruption activities against *Staphylococcus* species isolated from the milk of sheep and goats with mastitis.

Regarding PEE chemical composition, a positive correlation was found between anthocyanins and condensed tannins ($P<0.05$). A strong relationship between tannin and anthocyanin was also demonstrated in wine (Kilmister et al., 2014). According to the same authors, the fruit anthocyanin concentration correlates with wine tannin concentration and wine colour (Kilmister et al., 2014). According to the same authors, the fruit anthocyanin concentration correlates with wine tannin concentration and wine colour. Similarly, a correlation was found in the present study between anthocyanin content and propolis colour with significantly higher values for red propolis samples.

Total phenolics, flavonoids and condensed tannins contents for Red1 and Red2 PEE were significantly different ($P<0.05$). These two propolis batches were collected in the same apiary with a two years gap between harvests. Other authors report total polyphenols and flavonoids variations depending on the month of collection (Isla et al., 2009; Montenegro et al., 2001) and quantitative and qualitative variations in the composition of the polyphenol-rich extracts from propolis collected in the same month along three different years (Veloz et al., 2015). As mentioned by Costa et al., (2013), propolis composition and quality depends on the region vegetation and on other environmental factors.

Individual compounds identified in the studied PEE are mostly flavonoids and other phenolics compounds. Naringenin, kaempferol methyl ether and quercetin dimethyl ether were detected in four out of 10 PEE, pinobanksin, chrysin and acacetin were found in the three Portuguese brown propolis. These three compounds were found only in the Portuguese propolis. Apigenin, acacetin, and kaempferol derivatives were found before in Portuguese propolis sample (Falcão, Vale, et al., 2013).

Several authors have reported that Asian, African, and European PEE predominantly contain phenolics and flavonoids, such as naringenin, galangin, pinocembrin, apigenin, quercetin, cinnamic acid and its esters, kaempferol, chrysin, cinnamyl caffeate, cinnamylidene acetic acid, caffeic acid, p-coumaric acid, aromatic acids and their esters, among others (Huang et al., 2014; AL-Ani et al., 2018).

In this work, Green PEE Individual compounds are mainly non-flavonoid phenolics. The composition of Brazilian Green propolis includes flavonoids and cinnamic acid derivatives (Park, Alencar, and Aguiar 2002). In fact, in green propolis, p-coumaric acid and artepillin C are reported to be typical components of green Brazilian propolis (Machado et al., 2016). Green propolis are very typical and significantly differ from other types of propolis, as also confirmed by the results obtained for our Green PEE.

The typical Brazilian Red propolis composition (Daugsch et al., 2008) includes liquiritigenin, daidzein, dalbergin, isoliquiritigenin, formononetin and biochanin A. This composition is similar to that of our Red1 and Red2 PEE.

According to Zaccaria et al., (2017), flavonoids are the major components of European brown propolis, with flavones (chrysin and apigenin), flavanones (pinocembrin) and flavonols (galangin) being the most common individual compounds (Volpi and Bergonzini 2006), which is in agreement with the composition of our Portuguese brown PEE.

Concerning bacterial species and biofilm production ability, most *S. aureus* isolates produce biofilm, while most coagulase-negative staphylococci are non-biofilm producers. Similarly, only 8% *S. epidermidis* isolates expressed the capacity to produce biofilm, using tissue culture plate (TCP) method, in a study comprising 109 *S. epidermidis* isolates from milk samples collected from 90 sheep belonging to 14 different flocks (Queiroga, Duarte, and Laranjo 2018). Nevertheless, studies on bovine mastitis isolates, using the same methodology for biofilm assessment, report different results, with higher percentage of biofilm producer CNS, namely 37.5% biofilm producing *S. epidermidis* to 18.8% biofilm producing *S. aureus* (Oliveira et al., 2006), 31.3% biofilm producing CNS (Simojoki et al., 2012), and 85.1% biofilm producing CNS (Tremblay et al., 2013).

Considering the effect of biofilm on mammary inflammation, the results showed a significant association between mammary gland inflammatory intensity, according to CM/CMT, and biofilm production. These results are in agreement with other studies in bovine mastitis caused by *S. aureus* (Cucarella et al., 2004; Zuniga et al., 2015) and in the mouse model mammary gland inoculated with *S. aureus* (Gogoi-Tiwari et al., 2017). However, also in bovine mastitis causing CNS, biofilm production was not associated with the intensity of inflammation (Simojoki et al., 2012) and in a study on sheep mastitis *S.*

epidermidis biofilm effects on inflammatory changes, this association was not detected (Queiroga et al., 2018).

According to our results, Brown7 PEE shows the lowest content in flavonoids, anthocyanins and condensed tannins. This is the PEE with best antimicrobial activity, while the one with less antimicrobial action is Brown4 (Queiroga, Andrade, and Laranjo 2018), which exhibited high values for those components in this work. Moura (2000) found greater antimicrobial activity in extracts of propolis collected in NE Brazil, with lower flavonoid contents. Similar results were mentioned for propolis, also from NE Brazil, with low levels of flavonoids and phenolic compounds, which showed highly efficient bacteriostatic and bactericidal activity in comparison to propolis found in the Southeast (SE) of Brazil (Cabral et al., 2012). Nonetheless, Bonvehí and Gutiérrez (2012) reported a strong correlation between the total phenolic and flavonoids content and antimicrobial activity in propolis from Spain. Also Uzel et al., (2005) refer high flavonoids content propolis and significant antimicrobial action on Gram-positive bacteria. Silva et al., (2006) found a strong linear relationship between total phenol contents and the growth inhibition of *S. aureus* in one group of extracts, but in the other group this relationship was weaker. Additionally, no direct correlation between the content in total phenolics and flavonoids and MIC was reported in propolis from Brazil (Silva et al., 2006) and Greece and Cyprus (Kalogeropoulos et al., 2009).

The propolis flavonoid contents has been related with its antimicrobial activity (Cushnie and Lamb 2005; Marcucci, Ferreres, and Garcı 2001; Yong Kun Park and Ikegaki 1998), namely due to inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and inhibition of energy metabolism (Cushnie and Lamb 2005). Nevertheless, other propolis components may as well cause bacterial inhibition, some are also phenolic compounds (Bankova 2009; Bogdanov 2017).

Our results have shown that higher contents in total phenolics and flavonoids (including anthocyanins and condensed tannins) are related to a lower bactericidal activity. Nevertheless, all flavonoids and other phenolics identified in this work are associated with a higher bactericidal activity. In fact, the concentration in total phenolics and flavonoids does not directly reflect all biological activities, antimicrobial activity included, of PEE (Cabral et al., 2012). The methods used for the determination of total phenolics and

flavonoids are not accurate and may under- or overestimate their content depending on the PEE composition in individual compounds (Cabral et al., 2012). This may justify the fact that in the present study, although total flavonoid content is associated with a lower bactericidal activity, the presence of each individual flavonoid (n=49) positively influences antimicrobial activity. Manner et al., (2013) studied the anti-*S. aureus* biofilm activity of 500 natural and synthetic flavonoids and stated that they had different degrees of activity, some highly active although more than 80% (443) were declared inactive. Furthermore, the synergism of propolis components regarding antimicrobial activity, compared to isolated compounds, has been reported before (Król et al., 1993; Vassya Bankova 2009), and could also be partly responsible for this apparent discrepancy.

The antimicrobial activity of propolis is generally attributed to to flavonoids and phenolic acids and their esters. In particular, the antimicrobial activity of Iranian propolis may be due to the presence of the most effective flavonoid agents, including pinocembrin (Afrouzan et al., 2018). In Croatian PEE, apigenin, chrysin, pinocembrin, and kaempferol were found to be significantly correlated with antimicrobial activity. Additionally, it has been shown that flavonoids, and particularly kaempferide, and pinocembrin, interfere with bacterial RNA polymerase and cause its inhibition (Gajger et al., 2017). Similarly, Uzel et al., (2005) refer that pinocembrin is among the most potent microbicidal compounds in propolis. In the present work, Brown7 PEE, which showed the best bactericidal activity, is the only one where pinocembrin was detected.

Bankova et al., (1995) suggested that the biological activities of Brazilian propolis could be due to the high levels of phenolic acids, whereas flavonoids might be responsible for the activity of European propolis extracts (Hegazi et al., 2000).

Regarding the antibiofilm role of PEE, the present results have shown a remarkable inhibitory effect on staphylococcal biofilm formation. Other authors have reported an effect on biofilm inhibition from Poland PEE, which were efficient against all 10 *Staphylococcus epidermidis* isolates Wojtyczka et al., (2013). Brown2 is the best PEE for its ability to inhibit the formation of biofilm, while Brown4 is the worst, as reported in a previous study (Laranjo et al, 2018).

On the other hand, regarding the ability of PEE to disrupt the newly formed biofilm, a positive association was found between condensed tannins and biofilm mean percentage

of disruption, and a negative association between total phenolics and flavonoids and biofilm mean % of disruption. Brown2 PEE exhibited the higher content on condensed tannins and in a previous study had shown the best performance in terms of mean % of disruption (Laranjo et al., 2018).

PEE Brown4 showed the lowest performance of all studied PEE: antimicrobial and in biofilm production (Laranjo, Andrade, and Queiroga 2018; M.C. Queiroga, Andrade, and Laranjo 2018). This might be related to the different chemical composition of the Brown4 PEE, composed mainly of triterpenes. According to the ANOVA performed, triterpenes negatively influence bactericidal activity, biofilm inhibition and biofilm disruption for *Staphylococcus* spp. Nevertheless, tannins (condensed and hydrolysable) are capable of inhibiting the formation of biofilms in Gram-negative bacteria, rupturing the bacterial membrane and making it difficult to produce exopolysaccharide matrix (Trentin et al., 2013).

In this study, biofilm mean % of inhibition is positively correlated with the content in flavonoids, anthocyanins and condensed tannins. On the other hand, biofilm mean % of disruption is positively correlated with the content in condensed tannins and negatively correlated with total phenolics and flavonoids. Moreover, all individual compounds which presence is related to biofilm formation inhibition do not influence biofilm disruption and all individual compounds which presence is related to biofilm disruption do not influence biofilm formation.

Inhibition of biofilm formation by propolis extracts has been attributed to flavonoids and total phenolic contents, regarding *S. aureus* biofilm (Doganli 2016) and *Streptococcus mutans* biofilm (Veloz et al., 2015). Doganli (2016) studied the antibiofilm activity of three different propolis extract, both inhibitory and disruption activities and reported that extracts with the higher amount of phenolic contents were the ones that revealed the best biofilm reduction rates, stating that there is a notable consistency between the phenolic content and the biofilm inhibition rate/antibiofilm activity. However, an anti-*S. aureus* biofilm screening comprising 500 flavonoids, including both the inhibitory and disruption abilities, based on viability results, showed that in general, many flavonoids had strain-specific effects, and their biofilm disruption percentages were lower than inhibition percentages (Manner et al., 2013).

Our results strongly suggest that propolis is a promising product for mastitis control which also addresses One Health protection. The treatment of mastitis with antimicrobial alternatives is a priority to reduce the growing trend towards antimicrobial resistance (AMR) and its harmful repercussions on animal health and public health. The use of propolis for mastitis treatment and prevention has been addressed by other researchers. In 1980, Mirolyubov and Barskov described a propolis based intramammary preparation that improved the degree of restoration of milk yield and reduced bacterial counts in mammary secretion. More recently, one other intramammary propolis formulation has been developed, which reduced intramammary infection prevalence from 28.6% to 4.8% after two applications (Bačić et al., 2016a; Bačić et al., 2016b; Mačešić et al., 2016). Wang et al. (2016) studied the effect of Chinese propolis in a cell culture of bovine mammary epithelial cells and described that the treatment of the cells, prior to infection with mastitis pathogens, enhanced expressions of antioxidant genes and decreases in expressions of proinflammatory cytokines compared to non-treated challenged cells. Additionally, synergism between propolis extracts and antimicrobials, such as gentamicin, and those acting on cell wall synthesis, like vancomycin and oxacillin, has been reported (AL-Ani et al., 2018; Runyoro et a l., 2017).

5. Conclusions

The ability of a *Staphylococcus* isolate to produce biofilm seems to affect mammary inflammation, as evaluated by CMT/CM. However, the ability of a *Staphylococcus* isolate to produce biofilm does not seem to be important for its resistance to PEE.

Brown7 is the PEE with the best bactericidal activity, while Brown2 is the PEE that showed the best antibiofilm activity, both inhibiting the formation and disrupting the preformed biofilm.

The antibiofilm activity is probably important for a good efficiency in the treatment and prevention of intramammary infections, but it will be absolutely necessary that the PEE also has antimicrobial action; otherwise there is a risk of releasing viable bacteria that could spread the infection.

The presence of all PEE individual phenolics identified in the present study enhanced bactericidal activity, whereas triterpenes negatively influenced the antimicrobial activity.

As to antibiofilm activities, triterpenes also showed to be related with decreased activity, while diverse effects are associated with different flavonoids and other phenolic compounds.

Further studies to determine the concentration of each individual PEE compound are needed to better correlate these with the biological activities of propolis.

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References

1. Abou-Shaara, Hossam F. 2014. "The Foraging Behaviour of Honey Bees, Apis Mellifera: A Review." *Veterinarni Medicina* 59 (1): 1–10. doi:10.17221/7240-VETMED.
2. Afrouzan, Houshang, Azar Tahghighi, Sedigheh Zakeri, and Ali Es-Haghi. 2018. "Chemical Composition and Antimicrobial Activities of Iranian Propolis." *Iranian Biomedical Journal* 22 (1): 50–65. doi:10.22034/ibj.22.1.50.
3. AL-Ani, Issam, Stefan Zimmermann, Jürgen Reichling, and Michael Wink. 2018. "Antimicrobial Activities of European Propolis Collected from Various Geographic Origins Alone and in Combination with Antibiotics." *Medicines* 5 (1): 1–17. doi:10.3390/medicines5010002.
4. Almeida, Erika Tayse da Cruz, Maria Cristina Delgadodo Silva, José Marcos dos Santos Oliveira, Regianne Umeko Kamiya, Rodolfo Elleson dos Santos Arruda, Danilo Abreu Vieira, Valdemir da Costa Silva, Pierre Barnabé Escodro, Irinaldo Diniz Basílio-Júnior, and Ticiano Gomes Nascimento. 2017. "Chemical and Microbiological Characterization of Tinctures and Microcapsules Loaded with Brazilian Red Propolis Extract." *Journal of Pharmaceutical Analysis* 7 (5). Elsevier B.V.: 280–87. doi:10.1016/j.jpha.2017.03.004.
5. Bačić, 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): 159. doi:10.15406/jdvar.2016.03.00091.
6. Bačić, Goran, Nino Mačešić, Lada Radin, Jasna Aladrović, Krešimir Matanović, Tomislav Mašek, Diana Brozić, et al. 2016. "Intramammary Propolis Formulation for Prevention and Treatment of Mastitis in Dairy Ruminants (RC.2.2.08–0003)." *Journal of Animal Research* 6 (2): 47–49. doi:10.5958/2277-940x.2015.00165.5.
7. Bankova, V, R Christov, A Kujumgiev, Marcucci M C, and Popov S. 1995. "Chemical Composition and Antibacterial Activity of Brazilian Propolis." *Zeitschrift Für Naturforschung C*. doi:10.1515/znc-1995-3-402.
8. Bankova, Vassya. 2009. "Chemical Diversity of Propolis Makes It a Valuable Source of New Biologically Active Compounds." *Journal of ApiProduct and ApiMedical Science* 1 (2): 23–28. doi:10.3896/IBRA.4.01.2.01.
9. Bogdanov, Stefan. 2017. "Propolis : Composition , Health , Medicine : A Review." *Bee Product Science*, 1–44.
10. Bonvehí, Josep Serra, and Arrate Lacalle Gutiérrez. 2012. "The Antimicrobial Effects of Propolis Collected in Different Regions in the Basque Country (Northern Spain)." *World Journal of Microbiology and Biotechnology* 28 (4): 1351–58. doi:10.1007/s11274-011-0932-y.
11. Brackman, Gilles, and Tom Coenye. 2015. "Quorum Sensing Inhibitors as Anti-Biofilm Agents." *Current Pharmaceutical Design* 21 (1): 5–11. doi:10.2174/1381612820666140905114627.
12. Brasil. 2001. "Regulamentos Técnicos de Identidade E Qualidade de Apitoxina, Cera de Abelha, Geléia Real, Geléia Real Liofilizada, Pólen Apícola, Própolis E Extrato de Própolis." In *Instrução Normativa n.3*.
13. Buttner, Henning, Mack Dietrich, and Holger Rohde. 2015. "Structural Basis of *Staphylococcus Epidermidis* Biofilm Formation: Mechanisms and Molecular Interactions." *Frontiers in Cellular and Infection Microbiology* 5 (February): 1–15. doi:10.3389/fcimb.2015.00014.
14. Cabral, Ingridy Simone Ribeiro, Tatiane Luiza Cadorin Oldoni, Severino Matias de Alencar, Pedro Luiz Rosalen, and Masaharu Ikegaki. 2012. "The Correlation between the Phenolic Composition and

Biological Activities of Two Varieties of Brazilian Propolis (G6 and G12)." *Brazilian Journal of Pharmaceutical Sciences* 48 (3): 557–64. doi:10.1590/s1984-82502012000300023.

15. Carvalho, Adriana Andrade, Daiane Finger, Christiane Schinieder Machado, Eduardo Morgado Schmidt, Patrícia Marçal da Costa, Ana Paula Negreiros Nunes Alves, Thamires Maria Fontenele Morais, et al. 2011. "In Vivo Antitumoural Activity and Composition of an Oil Extract of Brazilian Propolis." *Food Chemistry* 126 (3). Elsevier Ltd: 1239–45. doi:10.1016/j.foodchem.2010.12.035.
16. Castaldo, Stefano, and Francesco Capasso. 2002. "Propolis, an Old Remedy Used in Modern Medicine." *Fitoterapia* 73 (SUPPL. 1): 1–6. doi:10.1016/S0367-326X(02)00185-5.
17. CLSI. 2012. "M07-A9—Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition." *Clinical and Laboratory Standards Institute (CLSI)–approved Standards*.
18. Costa, Aline Silva, Bruna Aparecida Souza Machado, Marcelo Andres Umsza-Guez, Marina Gonçalves Cirqueira, Silmar Baptista Nunes, and Francine Ferreira Padilha. 2013. "Levantamento Dos Estudos Realizados Com a Própolis Produzida No Estado Da Bahia." *SITIENTIBUS Série Ciências Biológicas* 13 (Bittencourt 2008): 1–7. doi:10.13102/scb324.
19. Costerton, J.W. 1999. "Introdution to Biofilm." *International Journal of Antimicrobial Agents* 11 (3–4): 217–21. doi:10.1016/S0924-8579(99)00018-7.
20. Croteau, R., T.M. Kutchan, and N.G. Lewis. 2000. "Natural Products (Secondary Metabolites)." In *Biochemistry and Molecular Biology of Plants*, edited by B. Buchanan, W Gruissem, and R Jones, 1250–1318. American Society of Plant Physiologists, Rockville, MD.
21. Crozier, Alan, Indu B. Jaganath, and Michael N. Clifford. 2007. "Phenols, Polyphenols and Tannins: An Overview." In *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*, 1–24. doi:10.1002/9780470988558.ch1.
22. Cucarella, Carme, M.A. Tormo, Carles Úbeda, M. Pilar Trotonda, Marta Monzón, Critòfol Peris, Beatriz Amorena, Iñigo Lasa, and José R. Penadés. 2004. "Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*." *Infection and Immunity* 72 (4): 2177–85. doi:10.1128/IAI.72.4.2177-2185.2004.
23. Cushnie, T. P.Tim, and Andrew J. Lamb. 2005. "Antimicrobial Activity of Flavonoids." *International Journal of Antimicrobial Agents* 26 (5): 343–56. doi:10.1016/j.ijantimicag.2005.09.002.
24. Daugsch, Andreas, Cleber S. Moraes, Patricia Fort, and Yong K. Park. 2008. "Brazilian Red Propolis - Chemical Composition and Botanical Origin." *Evidence-Based Complementary and Alternative Medicine* 5 (4): 435–41. doi:10.1093/ecam/nem057.
25. Doganli, Gulumser Acar. 2016. "Phenolic Content and Antibiofilm Activity of Propolis against Clinical MSSA Strains." *Records of Natural Products* 10 (5): 617–27.
26. Falcão, Soraia I., Andreia Tomás, Nuno Vale, Paula Gomes, Cristina Freire, and Miguel Vilas-Boas. 2013. "Phenolic Quantification and Botanical Origin of Portuguese Propolis." *Industrial Crops and Products* 49. Elsevier B.V.: 805–12. doi:10.1016/j.indcrop.2013.07.021.
27. Falcão, Soraia I., Nuno Vale, Paula Gomes, Maria R. M. Domingues, Cristina Freire, Susana M. Cardoso, and Miguel Vilas-Boas. 2013. "Phenolic Profiling of Portuguese Propolis by LC-MS Spectrometry: Uncommon Propolis Rich in Flavonoid Glycosides." *Phytochemical Analysis* 24 (4): 309–18. doi:10.1002/pca.2412.
28. Ferreira, Joselena M., Caroline C. Fernandes-Silva, Antonio Salatino, Giuseppina Negri, and Dejair Message. 2017. "New Propolis Type from North-East Brazil: Chemical Composition, Antioxidant Activity and Botanical Origin." *Journal of the Science of Food and Agriculture* 97 (11): 3552–58. doi:10.1002/jsfa.8210.
29. Gajger, Ivana Tlak, Iva Pavlović, Mirza Bojić, Ivan Kosalec, Siniša Srečec, Toni Vlainić, and Josipa Vlainić. 2017. "The Components Responsible for Antimicrobial Activity of Propolis from Continental and Mediterranean Regions in Croatian." *Czech Journal of Food Sciences* 35 (No. 5): 376–85. doi:10.17221/103/2017-cjfs.
30. Gelasakis, A.I., A.S. Angelidis, R. Giannakou, G. Filioussis, M.S. Kalamaki, and G. Arsenos. 2016. "Bacterial Subclinical Mastitis and Its Effect on Milk Yield in Low-Input Dairy Goat Herds." *Journal of Dairy Science* 99 (5). American Dairy Science Association: 3698–3708. doi:10.3168/jds.2015-10694.
31. Giusti, Mónica, and Ronald E. Wrolstad. 2001. "Anthocyanins by UV-Visible Spectroscopy." *Current Protocols in Food Analytical Chemistry*, F1.2.1-F1.2.13. doi:10.1002/0471142913.faf0102s00.
32. Gogoi-Tiwari, Jully, Vincent Williams, Charlene Babra Waryah, Paul Costantino, Hani Al-Salami, Sangeetha Mathavan, Kelsi Wells, et al. 2017. "Mammary Gland Pathology Subsequent to Acute Infection with Strong versus Weak Biofilm Forming *Staphylococcus aureus* Bovine Mastitis Isolates: A

Pilot Study Using Non-Invasive Mouse Mastitis Model." *Plos One* 12 (1): e0170668. doi:10.1371/journal.pone.0170668.

33. Gomes, Fernanda, Maria José Saavedra, and Mariana Henriques. 2016. "Bovine Mastitis Disease/pathogenicity: Evidence of the Potential Role of Microbial Biofilms." *Pathogens and Disease*, no. January: 1–19. doi:10.1093/femspd/ftw006.
34. Hegazi, Ahmed G, Abd El Hady, Faten K, and Fayrouz A M Abd Allah. 2000. "Chemical Composition and Antimicrobial Activity of European Propolis." *Zeitschrift Für Naturforschung*. doi:10.1515/znc-2000-1-214.
35. Huang, Shuai, Cui Ping Zhang, Kai Wang, George Q. Li, and Fu Liang Hu. 2014. "Recent Advances in the Chemical Composition of Propolis." *Molecules* 19 (12): 19610–32. doi:10.3390/molecules191219610.
36. Isla, MI, IC Zampini, RM Ordoñez, S Cuello, BC Juárez, JE Sayago, MIN Moreno, et al. 2009. "Effect of Seasonal Variations and Collection Form on Antioxidant Activity of Propolis from San Juan, Argentina." *Journal of Medical Food* 12 (6): 1334–1342. doi:10.1089/cmb.2014.0297.
37. Kalogeropoulos, Nick, Spyros J. Konteles, Elena Troullidou, Ioannis Mourtzinos, and Vaios T. Karathanos. 2009. "Chemical Composition, Antioxidant Activity and Antimicrobial Properties of Propolis Extracts from Greece and Cyprus." *Food Chemistry* 116 (2). Elsevier Ltd: 452–61. doi:10.1016/j.foodchem.2009.02.060.
38. Kilmister, Rachel L., Marica Mazza, Nardia K. Baker, Peta Faulkner, and Mark O. Downey. 2014. "A Role for Anthocyanin in Determining Wine Tannin Concentration in Shiraz." *Food Chemistry* 152: 475–82. doi:10.1016/j.foodchem.2013.12.007.
39. Król, Wojciech, S Scheller, J Shani, G Pietsz, and Zenon Czuba. 1993. *Synergistic Effect of Ethanol Extract of Propolis and Antibiotics on the Growth of Staphylococcus aureus*. *Arzneimittel-Forschung*. Vol. 43.
40. Laranjo, M., N. Andrade, and M.C. Queiroga. 2018. "Antibiofilm Activity of Propolis Extracts." In *Understanding Microbial Pathogens: Current Knowledge and Educational Ideas on Antimicrobial Research*, edited by A. Méndez-Vilas. Badajoz: Formatex Research Center.
41. Lu, Li-Chang, Yue-Wen Chen, and Cheng-Chun Chou. 2005. "Antibacterial Activity of Propolis against *Staphylococcus aureus*." *International Journal of Food Microbiology* 102 (2): 213–20. doi:10.1016/j.ijfoodmicro.2004.12.017.
42. Mačešić, Nino, Miroslav Benić, Diana Brozić, Jasna Aladrović, Jelena Šuran, Božo Radić, Tomislav Mašek, et al. 2016. "Intramammary Propolis Formulation for Prevention and Treatment of Mastitis in Dairy Ruminants (RC.2.2.08–0003)." *Journal of Animal Research* 6 (2): 227. doi:10.5958/2277-940x.2015.00165.5.
43. Machado, Bruna Aparecida Souza, Rejane Pina Dantas Silva, Gabriele De Abreu Barreto, Samantha Serra Costa, Danielle Figuerêdo Da Silva, Hugo Neves Brandão, José Luiz Carneiro Da Rocha, et al. 2016. "Chemical Composition and Biological Activity of Extracts Obtained by Supercritical Extraction and Ethanolic Extraction of Brown, Green and Red Propolis Derived from Different Geographic Regions in Brazil." *PLoS ONE* 11 (1): 1–26. doi:10.1371/journal.pone.0145954.
44. Manner, Suvi, Malena Skogman, Darla Goeres, Pia Vuorela, and Adyary Fallarero. 2013. "Systematic Exploration of Natural and Synthetic Flavonoids for the Inhibition of *Staphylococcus aureus* Biofilms." *International Journal of Molecular Sciences* 14 (10): 19434–51. doi:10.3390/ijms141019434.
45. Marco, Stefania De, Miranda Piccioni, Rita Pagiotti, and Donatella Pietrella. 2017. "Antibiofilm and Antioxidant Activity of Propolis and Bud Poplar Resins versus Pseudomonas Aeruginosa." *Evidence-Based Complementary and Alternative Medicine* 2017. doi:10.1155/2017/5163575.
46. Marcucci, M C, F Ferreres, and C Garcı. 2001. "Phenolic Compounds from Brazilian Propolis with Pharmacological Activities" 74: 105–12.
47. Melchior, M. B., H. Vaarkamp, and J. Fink-Gremmels. 2006. "Biofilms: A Role in Recurrent Mastitis Infections?" *Veterinary Journal* 171 (3): 398–407. doi:10.1016/j.tvjl.2005.01.006.
48. Merino, Nekane, Alejandro Toledo-Arana, Marta Vergara-Irigaray, Jaione Valle, Cristina Solano, Enrique Calvo, Juan Antonio Lopez, Timothy J. Foster, José R. Penadés, and Iñigo Lasa. 2009. "Protein A-Mediated Multicellular Behavior in *Staphylococcus aureus*." *Journal of Bacteriology* 191 (3): 832–43. doi:10.1128/JB.01222-08.
49. Mirolyubov, M. G., and A. A. Barskov. 1980. "Propolis and Mastitis." *Veterinariya, Moscow* 2: 45–46. doi:http://dx.doi.org/10.1136/adc.2006.104778.
50. Montenegro, G, RC Peña, G Avila, and BN Timmermann. 2001. "Botanical Origin and Seasonal Production of Propolis in Hives of Central Chile." *Boletim de Botânica Da Universidade de São Paulo*. http://www.jstor.org/stable/42871506.
51. Moura, F.F. 2000. "Determinação Das Propriedades Físicoquímicas E Biológicas Da Própolis Produzida

Por Apis Mellifera Na Região Nordeste Do Brasil." Universidade Estadual de Campinas. Campina, Brasil.
52. National Mastitis Council (U.S.). 2004. *Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality*. 4th ed. Verona, Wis. : NMC, 2004.
53. Oliveira, M., R. Bexiga, S. F. Nunes, C. Carneiro, L. M. Cavaco, F. Bernardo, and C. L. Vilela. 2006. "Biofilm-Forming Ability Profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* Mastitis Isolates." *Veterinary Microbiology* 118 (1–2): 133–40. doi:10.1016/j.vetmic.2006.07.008.
54. Onni, Toniangelo, Giovanna Sanna, Jesper Larsen, and Sebastiana Tola. 2011. "Antimicrobial Susceptibilities and Population Structure of *Staphylococcus epidermidis* Associated with Ovine Mastitis." *Veterinary Microbiology* 148 (1): 45–50. doi:10.1016/j.vetmic.2010.07.024.
55. Oravcová, M, L Mačuhová, and V Tančin. 2018. "The Relationship between Somatic Cells and Milk Traits , and Their Variation in Dairy Sheep Breeds in Slovakia." *Journal of Animal and Feed Sciences* 27: 97–104. doi:10.22358/jafs/90015/2018.
56. Park, Yong K., Severino M. Alencar, and Claudio L. Aguiar. 2002. "Botanical Origin and Chemical Composition of Brazilian Propolis." *Journal of Agricultural and Food Chemistry* 50 (9): 2502–6. doi:10.1021/jf011432b.
57. Park, Yong Kun, and Masaharu Ikegaki. 1998. "Preparation of Water and Ethanolic Extracts of Propolis and Evaluation of the Preparations." *Bioscience, Biotechnology, and Biochemistry* 62 (11): 2230–32. doi:10.1271/bbb.62.2230.
58. Paschino, Authors Pietro, Giuseppe Massimo Vacca, Luisa Dettori, and Michele Pazzola. 2018. "Estimation of Somatic Cells' Limit in Milk from Sarda Ewes: An Approach Based on the Effect on Milk Traits and Coagulation Properties." *Small Ruminant Research*. Elsevier B.V. doi:10.1016/j.smallrumres.2018.10.010.
59. Pereyra, Elizabet A L, Florencia Picech, Mar??a S. Renna, Celina Baravalle, Carolina S. Andreotti, Romina Russi, Luis F. Calvinho, Cristina Diez, and Bibiana E. Dallard. 2016. "Detection of *Staphylococcus aureus* Adhesion and Biofilm-Producing Genes and Their Expression during Internalization in Bovine Mammary Epithelial Cells." *Veterinary Microbiology* 183. Elsevier B.V.: 69–77. doi:10.1016/j.vetmic.2015.12.002.
60. Queiroga, M.C., N. Andrade, and M. Laranjo. 2018. "Antimicrobial Action of Propolis Extracts against Staphylococci." In *Understanding Microbial Pathogens: Current Knowledge and Educational Ideas on Antimicrobial Research*, edited by A. Méndez-Vilas. Badajoz: Formatex Research Center.
61. Queiroga, Maria Cristina, Elsa L. Duartea, and Marta Laranjo. 2018. "Sheep Mastitis *Staphylococcus epidermidis* Biofilm Effects on Cell Adhesion and Inflammatory Changes." *Small Ruminant Research* 168 (September): 6–11. doi:10.1016/j.smallrumres.2018.09.009.
62. Raynal-Ljutovac, K., A. Pirisi, R. de Crémoux, and C. Gonzalo. 2007. "Somatic Cells of Goat and Sheep Milk: Analytical, Sanitary, Productive and Technological Aspects." *Small Ruminant Research* 68 (1–2): 126–44. doi:10.1016/j.smallrumres.2006.09.012.
63. Rovai, Maristela, Gerardo Caja, Joan Miquel Quevedo, Carmen L. Manuelian, Jordi Saldo, Ahmed A.K. Salama, Paloma Torre, Ines Arana, Xavier Such, and Gabriel Leitner. 2015a. "Effect of Subclinical Intrammary Infection on Milk Quality in Dairy Sheep: II. Matured-Pressed Cheese (Manchego) Produced from Milk of Uninfected and Infected Glands and from Their Blends." *Small Ruminant Research* 125 (April). Elsevier B.V.: 127–36. doi:10.1016/j.smallrumres.2015.02.019.
64. Rovai, Maristela, Natalia Rusek, Gerardo Caja, Jordi Saldo, and Gabriel Leitner. 2015b. "Effect of Subclinical Intramammary Infection on Milk Quality in Dairy Sheep: I. Fresh-Soft Cheese Produced from Milk of Uninfected and Infected Glands and from Their Blends." *Small Ruminant Research* 125. Elsevier B.V.: 127–36. doi:10.1016/j.smallrumres.2015.02.019.
65. Runyoro, Deborah K.B., Olipa D. Ngassapa, and Alex Kamugisha. 2017. "Antimicrobial Activity of Propolis from Tabora and Iringa Regions, Tanzania and Synergism with Gentamicin." *Journal of Applied Pharmaceutical Science* 7 (1): 171–76. doi:10.7324/JAPS.2017.70124.
66. Santana, Henrique Freitas, Ana Andréa Teixeira Barbosa, Sukarno Olavo Ferreira, and Hilário Cuquetto Mantovani. 2012. "Bactericidal Activity of Ethanolic Extracts of Propolis against *Staphylococcus aureus* Isolated from Mastitic Cows." *World Journal of Microbiology and Biotechnology* 28 (2): 485–91. doi:10.1007/s11274-011-0839-7.
67. Silva, Joaquim Fernando Mendes, Maria Clara de Souza, Samara Ramalho Matta, Marisol Ribeiro Andrade, and Flavia Vila Nova Vidal. 2006. "Correlation Analysis between Phenolic Levels of Brazilian Propolis Extracts and Their Antimicrobial and Antioxidant Activities." *Food Chemistry* 99 (3): 431–35. doi:10.1016/j.foodchem.2005.07.055.
68. Simojoki, H., P. Hyvönen, C. Plumed Ferrer, S. Taponen, and S. Pyörälä. 2012. "Is the Biofilm Formation and Slime Producing Ability of Coagulase-Negative Staphylococci Associated with the Persistence and

Severity of Intramammary Infection?" *Veterinary Microbiology* 158 (3–4): 344–52. doi:10.1016/j.vetmic.2012.02.031.

69. Šiugždaitė, Jūratė, Aušra Gabinaitienė, and Remigijus Šiugžda. 2016. "ANTIMICROBIAL RESISTANCE OF PATHOGENS FROM EWES SUBCLINICAL." *Veterinarija Ir Zootechnika (Vet Med Zoot)* 73 (95): 122–27.
70. Slinkard, Karen, and Vernon L Singleton. 1977. "Total Phenol Analysis: Automation and Comparison with Manual Methods." *American Journal of Enology and Viticulture* 28 (1): 49 LP-55. http://www.ajevonline.org/content/28/1/49.abstract.
71. Tremblay, Yannick D N, Daphnée Lamarche, Pauline Chever, Denis Haine, Serge Messier, and Mario Jacques. 2013. "Characterization of the Ability of Coagulase-Negative Staphylococci Isolated from the Milk of Canadian Farms to Form Biofilms." *Journal of Dairy Science* 96 (1). Elsevier: 234–46. doi:10.3168/jds.2012-5795.
72. Trentin, Danielle S., Denise B. Silva, Matheus W. Amaral, Karine R. Zimmer, Márcia V. Silva, Norberto P. Lopes, Raquel B. Giordani, and Alexandre J. Macedo. 2013. "Tannins Possessing Bacteriostatic Effect Impair Pseudomonas Aeruginosa Adhesion and Biofilm Formation." *PLoS ONE* 8 (6). doi:10.1371/journal.pone.0066257.
73. Uzel, Ataç, Kadriye Sorkun, Özant Önçağ, Dilşah Çoğulu, Ömür Gençay, and Bekir Salih. 2005. "Chemical Compositions and Antimicrobial Activities of Four Different Anatolian Propolis Samples." *Microbiological Research* 160 (2): 189–95. doi:10.1016/j.micres.2005.01.002.
74. Vasileiou, N.G.C., D.C. Chatzopoulos, D.A. Gougoulis, S. Sarrou, A.I. Katsafadou, V. Spyrou, V.S. Mavrogianni, E. Petinaki, and G.C. Fthenakis. 2018. "Slime-Producing Staphylococci as Causal Agents of Subclinical Mastitis in Sheep." *Veterinary Microbiology* 224 (August). Elsevier: 93–99. doi:10.1016/j.vetmic.2018.08.022.
75. Veh, K.A., R.C. C Klein, C. Ster, G. Keefe, P. Lacasse, D. Scholl, J-P J.-P. Roy, et al. 2015. "Genotypic and Phenotypic Characterization of *Staphylococcus aureus* Causing Persistent and Nonpersistent Subclinical Bovine Intramammary Infections during Lactation or the Dry Period." *Journal of Dairy Science* 98 (1): 155–68. doi:10.3168/jds.2014-8044.
76. Veloz, Jorge Jesús, Nicolás Saavedra, Alexis Lillo, Marysol Alvear, Leticia Barrientos, and Luis A. Salazar. 2015. "Antibiofilm Activity of Chilean Propolis on *Streptococcus mutans* Is Influenced by the Year of Collection." *BioMed Research International* 2015. doi:10.1155/2015/291351.
77. Vermerris, W.Nicholson, R. 2006. *Phenolic Compound Biochemistry*. Springer, The Netherlands.
78. Virdis, Salvatore, Christian Scarano, Francesca Cossu, Vincenzo Spanu, Carlo Spanu, and Enrico Pietro Luigi De Santis. 2010. "Antibiotic Resistance in *Staphylococcus aureus* and Coagulase Negative Staphylococci Isolated from Goats with Subclinical Mastitis." *Veterinary Medicine International* 2010: 517060. doi:10.4061/2010/517060.
79. Volpi, Nicola, and Gianluca Bergonzini. 2006. "Analysis of Flavonoids from Propolis by on-Line HPLC-Electrospray Mass Spectrometry." *Journal of Pharmaceutical and Biomedical Analysis* 42 (3): 354–61. doi:10.1016/j.jpba.2006.04.017.
80. Wang, Kai, Xiao-Lu Jin, Xiao-Ge Shen, Li-Ping Sun, Li-Ming Wu, Jiang-Qin Wei, Maria Cristina Marcucci, Fu-Liang Hu, and Jian-Xin Liu. 2016. "Effects of Chinese Propolis in Protecting Bovine Mammary Epithelial Cells against Mastitis Pathogens-Induced Cell Damage." *Mediators of Inflammation*, 1–12. doi:10.1155/2016/8028291.
81. Wojtyczka, Robert D., Małgorzata Kępa, Danuta Idzik, Robert Kubina, Agata Kabała-Dzik, Arkadiusz Dziedzic, and Tomasz J. Wąsik. 2013. "In Vitro Antimicrobial Activity of Ethanolic Extract of Polish Propolis against Biofilm Forming *Staphylococcus epidermidis* Strains." *Evidence-Based Complementary and Alternative Medicine* 2013. doi:10.1155/2013/590703.
82. Woolhouse, Mark, Melissa Ward, Bram Van Bunnik, and Jeremy Farrar. 2015. "Antimicrobial Resistance in Humans, Livestock and the Wider Environment." *Philosophical Transactions of the Royal Society B: Biological Sciences* 370 (1670). doi:10.1098/rstb.2014.0083.
83. Zaccaria, Vincenzo, Valeria Curti, Arianna Di Lorenzo, Alessandra Baldi, Cristina Maccario, Sabrina Sommatis, Roberto Mocchi, and Maria Daglia. 2017. "Effect of Green and Brown Propolis Extracts on the Expression Levels of microRNAs, mRNAs and Proteins, Related to Oxidative Stress and Inflammation." *Nutrients* 9 (1090): 1–17. doi:10.3390/nu9101090.
84. Zuniga, Eveline, Priscilla A. Melville, André B S Saidenberg, Marco A. Laes, Fernanda F. Gonsales, Sandra R S Salaberry, Fabio Gregori, et al. 2015. "Occurrence of Genes Coding for MSCRAMM and Biofilm-Associated Protein Bap in *Staphylococcus* spp. Isolated from Bovine Subclinical Mastitis and Relationship with Somatic Cell Counts." *Microbial Pathogenesis* 89: 1–6. doi:10.1016/j.micpath.2015.08.014.

Chapter 5

5. Antimicrobial action of propolis extracts against staphylococci

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Antimicrobial action of propolis extracts against staphylococci

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Abstract

One Health is a worldwide strategy of healthcare for humans, animals, and the environment. Antimicrobial resistance is a serious global problem, recently recognised by the World Health Organization. Prudent, responsible use of antimicrobials should be a concern for both human and veterinary doctors. Concerning animal health and its repercussion in human health, antimicrobials and disinfectants have been widely used for the control of mastitis. This practice induces a selective pressure for resistant bacterial strains, which is deleterious for public health associated with milk consumption. Antimicrobial resistance genes have been detected in pathogens associated to small ruminants' mastitis. These genes may be transferred to the indigenous microbiota of humans. The presence of disinfectant resistance genes has also been reported in staphylococci from both ovine and caprine milk. Propolis is a resinous substance produced by honeybees using different types of plants. It is used to seals holes and cracks in the beehive, contributes to an aseptic internal environment, maintains the hive's internal temperature, and prevents predators from entering the beehive. Propolis has been used as a natural medicine for its antiseptic, antimicrobial, antioxidant, anti-inflammatory, and other immunomodulatory properties. The aim of the present study was to investigate the *in vitro* activity of propolis ethanol extracts (PEE) against staphylococci isolated from mastitic milk of sheep and goats. Antimicrobial susceptibility was assessed for 16 antimicrobials (ampicillin, gentamicin, lincomycin, trimethoprim/sulfamethoxazole, penicillin, streptomycin, tetracycline, cloxacillin, neomycin, cefazolin, cefoperazone, cephalexin, amoxicillin+clavulanic acid, oxacillin, ceftriaxone, and ciprofloxacin) by the disk

diffusion method. Ten PEE from propolis samples collected both in Portugal (three brown), and Brazil (one green, two red, and four brown) were evaluated for their antimicrobial action against 146 staphylococci: 35 *S. aureus*, and 104 coagulase-negative staphylococci isolates, together with seven reference strains. Antimicrobial activity of PEE was assessed on polystyrene flatbottomed microtiter plates in triplicate by the microdilutions methodology for concentrations between 0.0065 and 214 mg/mL. All staphylococci isolates revealed susceptibility to all but one of the studied PEE. Minimal bactericidal concentration for most isolates was 3.34 mg/mL. According to our results, propolis may be an important alternative to the use of antimicrobials, with remarkable advantages for public health.

Keywords: *Staphylococcus*; antimicrobial resistance; One Health

1. Introduction

One Health is a worldwide strategy of healthcare for humans, animals, and the environment, shared by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organisation for Animal Health (OIE). Addressing the rising threat of antimicrobial resistance (AMR) requires a holistic and multisectoral approach, because antimicrobials used to treat various infectious diseases in animals may be the same or similar to those used for humans. Resistant bacteria arising in humans, animals or the environment may spread from one to the other, and from one country to another. According to the WHO, antimicrobial resistance is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals, and antimalarials) from working against it. As a result, standard treatments become ineffective, infections persist, and may spread to others.

Antimicrobials and disinfectants have been widely used for the control of mastitis in small ruminants, a practice with severe consequences for human health. The massive use of antimicrobials induces a selective pressure for resistant bacterial strains, which is deleterious for public health associated with milk consumption.

Mastitis, the inflammation of the mammary gland, which is mostly caused by bacteria, is highly prevalent in dairy herds [1]. *Staphylococcus* spp. are the main aetiological

agents of mastitis in small ruminants [2, 3]. The prophylaxis and treatment of mastitis is currently mostly dependent on the use of antimicrobials, which exert pressure selection over resistant and multi-resistant strains [4-6]. A recent study showed a noticeable increase in antimicrobial resistance, over a period of ten years, in *Staphylococcus aureus* isolates from goats and sheep milk [7].

These resistant bacteria may worsen the problem of mastitis, but in addition, they may be threatening for human health. Their access to the consumer through milk and dairy products may be responsible for the transfer of resistance genes to the microorganisms of the indigenous microbiota in the gut of humans [8].

The search for control measures and antimicrobial alternatives to decrease intramammary infections are needed to reduce losses in the dairy sector; to increase the motivation and profitability of farmers and, last, but not least, to protect public health.

One possible alternative is propolis, a natural antimicrobial. Propolis is a resinous mass produced by *Apis mellifera* bees by manipulating resins harvested in several plants with their salivary gland secretions, which they use to close the hive to difficult the access of invaders. Its biological properties are related to the chemical composition, which differs in its structure and concentration depending on the region of production, availability of sources for resin harvesting, genetic variability of the queen bee and technique used for production [9].

The biological activity of propolis has been used in traditional medicine since ancient times. Different solvents have already been tested for extracting propolis components and to produce extracts [10, 11]. Propolis has been shown to possess several properties, such as: antioxidant action, anti-inflammatory [12], antiulcerogenic [13], antitumoral [14], antidiabetogenic [15], antiatherogenic and anti-angiogenic [16], immunomodulatory [17], antifungal [18, 19], antiviral [20], namely for human immunodeficiency virus (anti-HIV) [21], anti-bacterial [22, 23] and acting versus some virulence factors, such as antibiofilm [24, 25] and antimotility [26, 27].

The aim of the present study was to evaluate the *in vitro* activity of propolis ethanol extracts (PEE) against staphylococci isolated from mastitic milk of sheep and goats.

2. Materials and methods

2.1 Staphylococci isolates

Thirty-five *Staphylococcus aureus* and 104 coagulase-negative staphylococci (CNS) were isolated from small ruminant milk samples. Seven references strains were also used: five *S. aureus* (ATCC 25923, ATCC 29213, COL, FRI 472, and FRI 913) and two *S. epidermidis* (ATCC 12228, and ATCC 35984).

2.2 Antimicrobial Sensitivity Test (AST) - disk diffusion method

The AST followed the performance standard M02-A11 CLSI [28] using the Kirby Bauer diffusion method. Bacterial cultures were suspended in sterile saline solution whose turbidity was adjusted at 0.5 of the MacFarland scale ($1x10^8$ cfu/mL) and confirmed in turbidimeter (DensiChek, bioMérieux). The suspension was inoculated onto the surface of Muller-Hinton agar (MHA) plates (Oxoid, CM0337) and after 5 minutes at room temperature, antimicrobial disks were applied with the aid of a disk dispenser (Oxoid, ST 6090). The plates were incubated at 37 ºC for approximately 24 hours and the inhibition zone diameter was measured. The list of 16 antimicrobials studied, used in intramammary antibiotics preparations, are grouped according to their chemical structure and their mode of action in **Table 5.1**.

Table 5.1. Antibacterial agents, their classes, and their corresponding mode of action.

Antimicrobials	Class	Mode of action
Penicillin	*β*-LACTAMS	Cell wall inhibitors
Ampicillin	Semisynthetic penicillins	
Cloxacillin		
Amoxicillin + Clavulanic Acid		
Oxacillin		
Cephalexin	Cephalosporins	
Cefazolin	*Generation I*•	
Ceftriaxone	*Generation III*•	
Cefoperazone		
Streptomycin	Aminoglycosides	Protein synthesis inhibitors
Gentamycin		
Neomycin		
Lincomycin	Lincosamides	
Tetracycline	Tetracyclines	
Ciprofloxacin	Fluoroquinolones	DNA replication inhibitor
Cotrimoxazole (Sulfamides + Trimethoprim)	Chemotherapeutic agents	Competitive inhibitor

2.3 Propolis Ethanol Extracts (PEE)

Ten propolis samples, seven from Brazil (Green, Red1, Red2, Brown1, Brown2, Brown3, and Brown4), and three from Portugal (Brown5, Brown6, and Brown7) were collected from apiaries located in different regions.

Propolis ethanol extracts were prepared according to the official standards for extracts production in Normative Instruction Nº 3, 19/01/2001, published by the Department of Inspection of Animal Products of the Ministry of Agriculture, Livestock and Food Supply, Brazil [29]. Cold maceration of 300 g of raw propolis in 700 mL of 70% ethanol was performed, resulting in a 30% PEE. The preparations were kept at room temperature, protected from light, for 45 days. After this period, extracts were filtered with a sterile funnel and filter paper. Extracts were kept under refrigeration at 4 °C, in amber bottles, until use [46].

2.4 In vitro antimicrobial activity of propolis extracts

In vitro antimicrobial activity of propolis ethanol extracts was performed according to the CLSI protocol M07-A9 [30]. A total of 150 µL of 30% PEE were added to 150 µL of Mueller-Hinton Broth (MHB) (Oxoid, CM0405), in polystyrene flat-bottomed 96-well microtiter plates, in triplicate, by the microdilutions methodology for concentrations between 0.0065 and 214 mg/mL. Ethanol (70%) was used as a control, to ensure that the resulting antimicrobial action was not due to the ethanol used for extract production.

A suspension with a turbidity equivalent to a 0.5 McFarland standard ($1x10^8$ cfu/mL) was made for each bacterial culture using a turbidimeter (DensiChek, bioMérieux), and was further diluted to reach a concentration of $5x10^5$ cfu/mL after added to extract. Microplates were incubated at 37°C for approximately 24 hours. In order to determine the minimum bactericidal concentration (MBC), a 96-pin microplate replicator (Boekel Scientific) was used to inoculate approximately 10 µl of each dilution onto a 150 mm diameter Petri dish with Mueller-Hinton Agar (MHA) (**Fig. 1**). Analyses were performed in triplicate, and Petri dishes incubated for 24 hours at 37 ºC. The MBC is defined as the lowest dilution that inactivated the inoculum visibly.

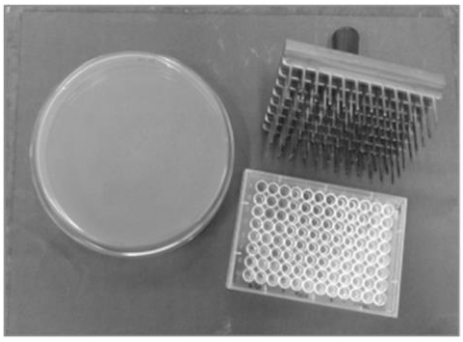


Figure 5.1. Minimum Bactericidal Concentration (MBC) methodology: polystyrene flat-bottomed 96-well microtiter plates, 96-pin microplate replicator; and 150 mm diameter Petri dish.

3. Results

3.1 Antimicrobial susceptibility test of staphylococci

Staphylococci isolates and reference strains have shown distinct resistance rates to the tested antimicrobials. Thirty eight percent of all 146 staphylococci were non-susceptible to streptomycin, 30% to penicillin, 27% to ampicillin and lincomycin, 16% to cloxacillin and oxacillin and 13% to tetracycline. Less than 10% of all staphylococci were found to be resistant to all other antimicrobials (**Fig. 2**). Nevertheless, at least one resistant *Staphylococcus* to each antimicrobial was found.

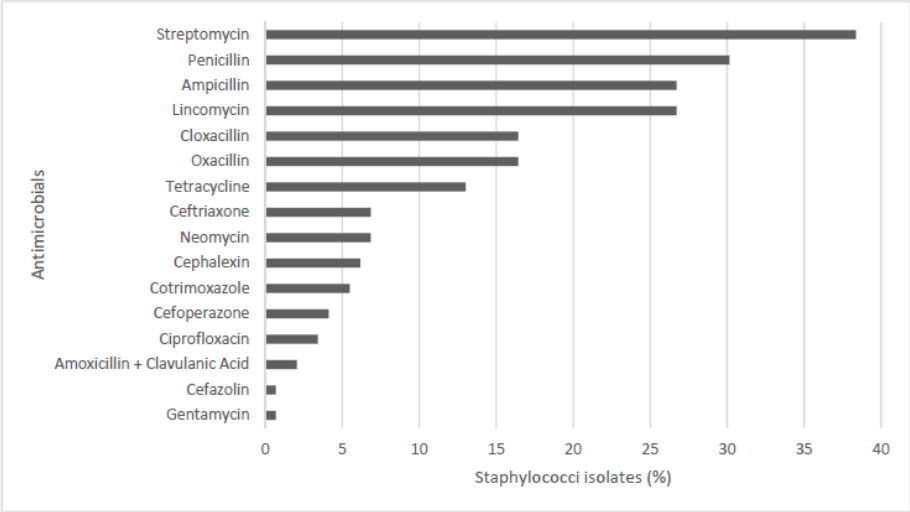


Figure 5.2. Antimicrobial resistance of 146 staphylococci isolates and reference strains.

3.2 Antimicrobial activity of PEE against staphylococci

Bacterial activity is shown in **Fig. 3**. Identifications per column are shown from left to right: 1st, 2nd and 3rd are triplicates of dilutions of Brown1 extract; 4th, 5th and 6th are triplicates of dilutions of the Red2 extract; 7th, 8th and 9th are triplicates of Brown3 dilutions; 10th is ethanol control (70% ethanol dilutions with inoculum); 11th negative control (MHB culture medium only) and 12th positive control (culture medium with inoculum). Different dilutions of each PEE are positioned in different rows.

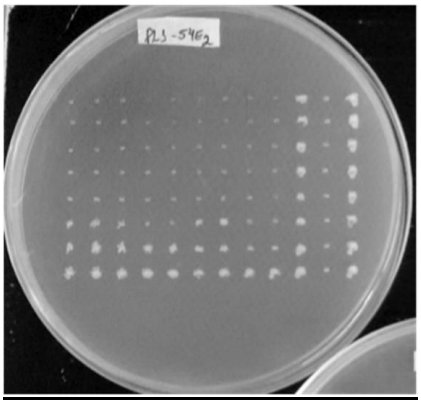
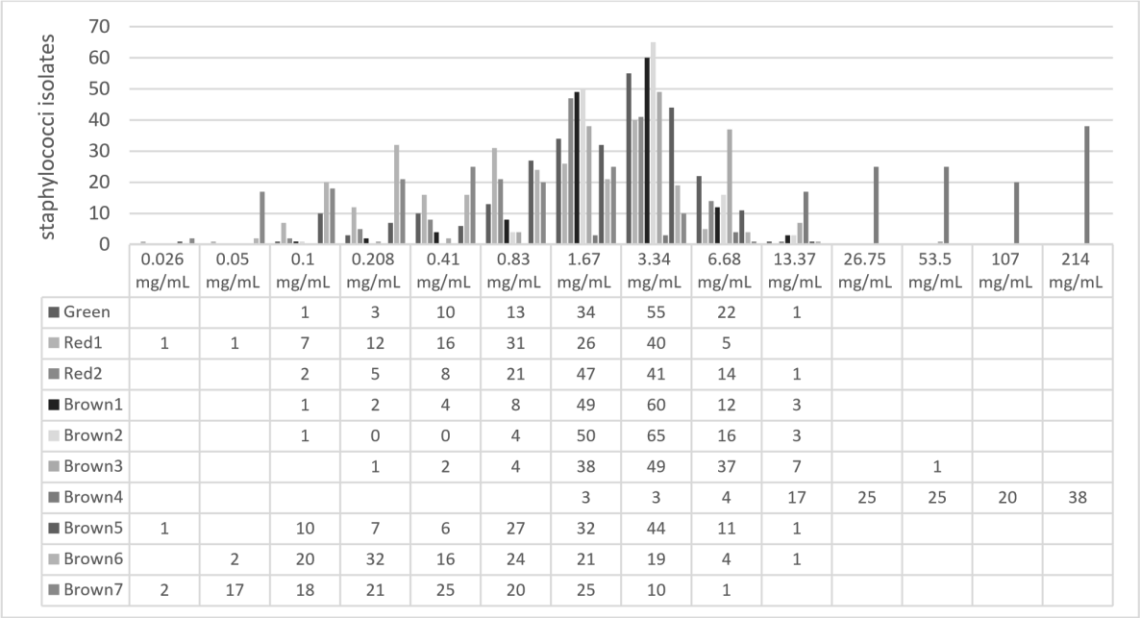


Fig. 5.3 Minimum Bactericidal Concentration (MBC): Petri dish showing the growth of a bacterial isolate in the presence of Brown1, Red2 and Brown3 PEE.

All 139 staphylococci isolates revealed susceptibility to all but one of the studied PEE, in concentrations ranging between 0.026 and 13.37 mg/mL. Furthermore, the inhibitory activity was bactericidal. Most PEE minimal bactericidal concentration for most isolates was

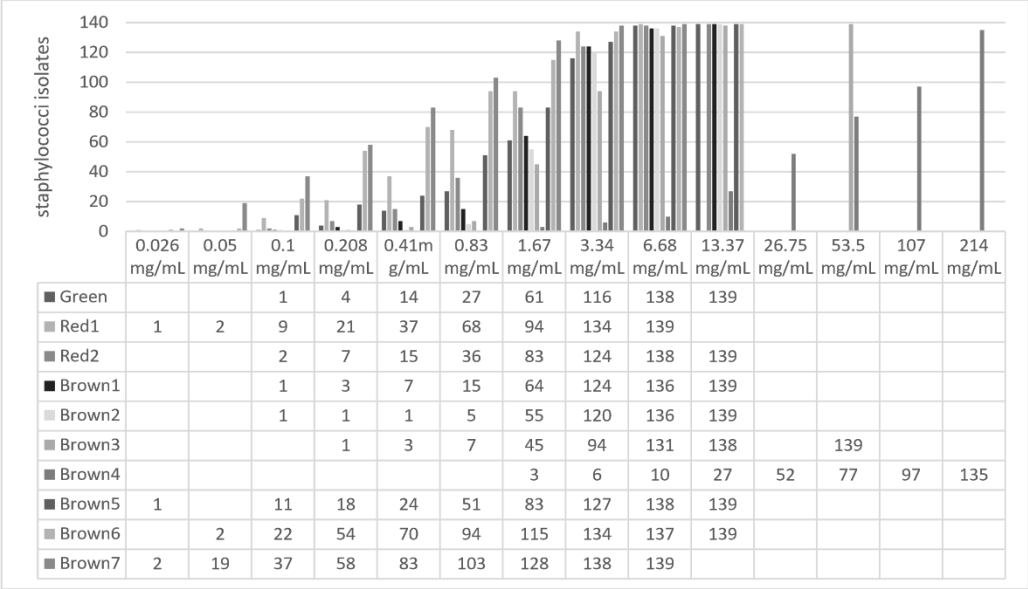
3.34 mg/mL. Brown4 was the only PEE that showed less antimicrobial activity against the studied isolates (**Figure 5.4**).

Figure 5.4. Minimum inhibitory concentration of 10 PEE against 139 staphylococci isolates.



	0.026 mg/mL	0.05 mg/mL	0.1 mg/mL	0.208 mg/mL	0.41 mg/mL	0.83 mg/mL	1.67 mg/mL	3.34 mg/mL	6.68 mg/mL	13.37 mg/mL	26.75 mg/mL	53.5 mg/mL	107 mg/mL	214 mg/mL
Green			1	3	10	13	34	55	22	1				
Red1	1	1	7	12	16	31	26	40	5					
Red2			2	5	8	21	47	41	14	1				
Brown1			1	2	4	8	49	60	12	3				
Brown2			1	0	0	4	50	65	16	3				
Brown3				1	2	4	38	49	37	7		1		
Brown4							3	3	4	17	25	25	20	38
Brown5	1		10	7	6	27	32	44	11	1				
Brown6		2	20	32	16	24	21	19	4	1				
Brown7	2	17	18	21	25	20	25	10	1					

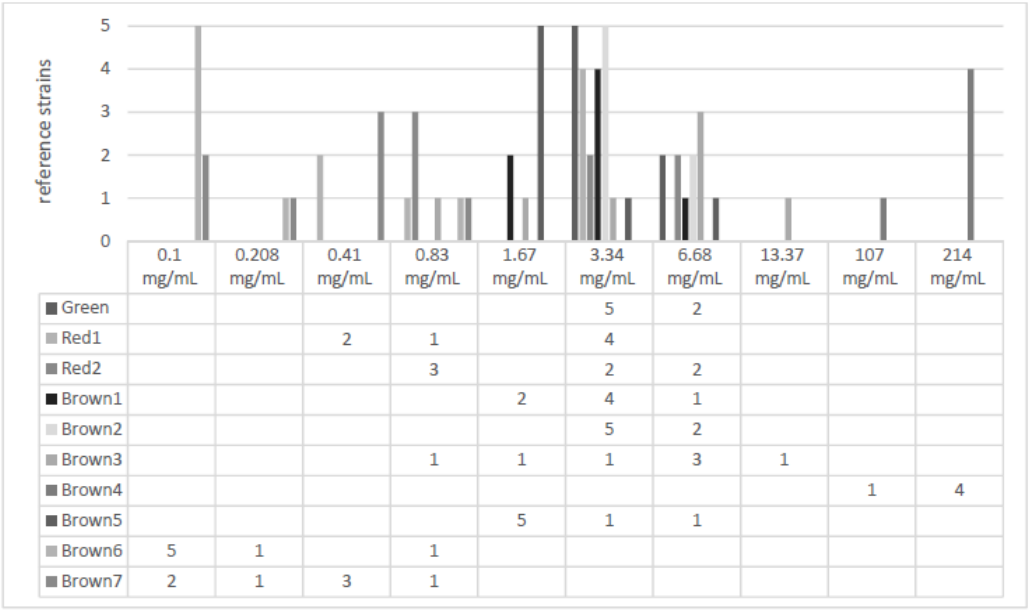
All but one isolate were inhibited with concentrations between 0.208 and 13.37 mg/mL of the nine PEE. The concentration of 13.37 mg/mL, of these nine PEE, is adequate to inhibit *Staphylococcus* isolates. Red1 and Brown7 PEE were bactericidal to all isolates at the concentration of 6.68 mg/mL. Red1, Brown5, 6 and 7 extracts showed bactericidal activity with the lowest concentrations. This last one, Brown7, collected in PT, showed to inhibit more isolates at all concentrations (**Figure 5.5**), showing the best performance. Following best performance was for Brown6, then Red1 followed by Brown5. Brown1 and Brown2 PEE showed quite similar antimicrobial activity. Green PEE showed an inhibitory activity not much different from Brown1, Brown2 and Brown3 PEE.



	0.026 mg/mL	0.05 mg/mL	0.1 mg/mL	0.208 mg/mL	0.41m g/mL	0.83 mg/mL	1.67 mg/mL	3.34 mg/mL	6.68 mg/mL	13.37 mg/mL	26.75 mg/mL	53.5 mg/mL	107 mg/mL	214 mg/mL
Green			1	4	14	27	61	116	138	139				
Red1	1	2	9	21	37	68	94	134	139					
Red2			2	7	15	36	83	124	138	139				
Brown1			1	3	7	15	64	124	136	139				
Brown2			1	1	1	5	55	120	136	139				
Brown3				1	3	7	45	94	131	138		139		
Brown4							3	6	10	27	52	77	97	135
Brown5	1		11	18	24	51	83	127	138	139				
Brown6		2	22	54	70	94	115	134	137	139				
Brown7	2	19	37	58	83	103	128	138	139					

Figure 5.5. Antimicrobial activity of 10 PEE against 139 staphylococci isolates.

Regarding the antimicrobial activity of the PEE against the studied reference strains (**Figure 5.6**), all strains showed sensitivity to the same nine PEE, in concentrations between 0.1 and 13.37 mg/mL, and 5 *S. aureus* (71.4%) revealed sensitivity to Brown4 extract, between 107 and 214 mg/mL concentrations. Two reference strains (*S. epidermidis* ATCC 12228 and 35984) showed Brown4 resistance, which was the one that showed less antimicrobial activity against all the strains tested. PEE Brown6 and 7 showed antimicrobial activity at lower concentrations.



	0.1 mg/mL	0.208 mg/mL	0.41 mg/mL	0.83 mg/mL	1.67 mg/mL	3.34 mg/mL	6.68 mg/mL	13.37 mg/mL	107 mg/mL	214 mg/mL
Green						5	2			
Red1			2	1		4				
Red2				3		2	2			
Brown1					2	4	1			
Brown2						5	2			
Brown3				1	1	1	3	1		
Brown4									1	4
Brown5					5	1	1			
Brown6	5	1		1						
Brown7	2	1	3	1						

Figure 5.6. Minimum inhibitory concentration of 10 PEE against reference strains.

4. Discussion

In this study, resistance rate was higher to streptomycin (38%), followed by penicillin (30%), ampicillin and lincomycin (27%). Although antimicrobial resistance is not particularly high for all tested antimicrobials, it is noteworthy that resistant isolates were found to all antimicrobials and that 16% isolates showed resistance to cloxacillin and oxacillin, which is mostly relevant, as methicillin resistant staphylococci are a real scourge for public health. Staphylococci isolated from the milk of small ruminant with mastitis with low resistance patterns have been mentioned by other researchers [4, 31]. Nevertheless, some reports indicate *S. aureus* from ewes' subclinical mastitis with a high rate of resistance to ampicillin, cefalotin, cephalexin, gentamicin, streptomycin, erythromycin, oxytetracycline and sulphonamide with a percentage from 50.0 to 100.0 [32]. Additionally, Onni *et al.* [33] reported 38% *S. epidermidis* associated with ovine mastitis (n = 50) resistant to penicillin.

Except for Brown4 PEE, all the other PEE showed inhibitory activity against all staphylococci isolates. Furthermore, all ten PEE are bactericidal, which is a very important feature of propolis. For example, essentials oils (EO) of Aromatic and Medicinal Plants (AMP) have been tested for their antimicrobial effect against staphylococci, and they were found to be only bacteriostatic [34].

Due to bacterial resistance to antimicrobials, researchers are exploring the natural components of propolis for their antimicrobial properties [35]. Plant compounds have been shown not to be prone to develop acquired bacterial resistance even after prolonged exposure [36].

The antimicrobial activity of propolis has been addressed before by other authors [22-24, 37-39]. The antibacterial activity of PEE over the bacterial cell wall was confirmed through atomic force microscopy (AFM) images. Furthermore, it is possible to differentiate the degenerative ability of propolis against Gram-negative and Gram-positive bacteria. In fact, PEE were found to be more effective against the Gram-positive *S. aureus* [40].

Propolis extracts can be used alone or in combination with antimicrobials [35]. This mixture represents a synergy that potentiates the antimicrobial power of antibiotics against various microorganisms. Minimal inhibitory concentrations (MIC) of penicillin G, doxycycline, streptomycin, cloxacillin, chloramphenicol, cephradine, ampicillin, and

polymyxin B were established, in the absence of PEE, against *S. aureus*. When PEE was added at concentrations of up to 600 µg/ml to antimicrobial solutions, a high synergistic effect was observed in the anti-bacterial activity of streptomycin and cloxacillin, and a moderate synergistic effect with other antibiotics, but no action was noticed when at the junction with ampicillin [41]. On the contrary, the good performance of a mixture of propolis with ampicillin, was observed by Ismael et al. [42], *in vivo*, when treating sheep and goats affected with *Listeria monocytogenes*. The animals treated with this mixture showed the best result, compared to the mixture of cefotaxime antibiotics, cefotaxime alternative with gentamicin and trimethoprim-sulfadimethoxine combination. The author also noted that treatments with combinations of propolis and antibiotics were more effective than those treatments with only antibiotics. Better synergistic results between PEE and antimicrobials were observed with chloramphenicol, gentamicin, netilmicin, tetracycline and clindamycin, antibiotics (causers interference in protein synthesis of bacteria), than those antibiotics that show other forms of action in the inactivation of the bacterium [43].

Resistant and multi-resistant microorganisms to antibiotics were submitted to analysis with sub-inhibitory concentrations of PEE together with antimicrobials. The results of the synergistic action demonstrated the potential of propolis to improve the action of certain antimicrobials, which had been previously undetected [44]. Scazzocchio *et al.* [45] also evaluated sub-inhibitory concentrations of PEE added to different antimicrobials. These showed divergent activities against *S. aureus*. When sub-inhibitory concentrations were added to ampicillin, gentamicin and streptomycin showed high antimicrobial potency against this strain, when added to chloramphenicol, ceftriaxone and vancomycin showed moderate activity and when added to erythromycin did not show antimicrobial action.

Our results have clearly demonstrated that propolis may be highly efficient as antimicrobial for staphylococci. Its use alone or in combination with antimicrobials may be an important alternative for the control of small ruminant mastitis, with remarkable advantages for public health.

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References

1. Radostits, O.M., C.C. Gay, K.W. Hinchcliff, and P.D. Constable, An Overview on the Epidemiology and Diagnosis of Bovine Mastitis, in Veterinary Medicine: A text book of the disease of cattle, horses, sheep, pigs and goats. . 2007, Elsevier: London. p. 674-762.
2. Queiroga, M.C., Prevalence and aetiology of sheep mastitis in Alentejo region of Portugal. Small Ruminant Research, 2017. 153: p. 123-130.
3. Peixoto, R.d.M., R.A. Mota, and M.M.d. Costa, Mastite em pequenos ruminantes no Brasil. Pesquisa Veterinária Brasileira, 2010. 30: p. 754-762.
4. Martins, K.B., P.Y. Faccioli, M.F. Bonesso, S. Fernandes, A.A. Oliveira, A. Dantas, et al., Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. Journal of Dairy Science, 2017. 100(3): p. 2184-2195.
5. Virdis, S., C. Scarano, F. Cossu, V. Spanu, C. Spanu, and E.P.L. De Santis, Antibiotic Resistance in *Staphylococcus aureus* and Coagulase Negative Staphylococci Isolated from Goats with Subclinical Mastitis. Veterinary medicine international, 2010. 2010: p. 517060-517060.
6. Contreras, A., D. Sierra, A. Sánchez, J.C. Corrales, J.C. Marco, M.J. Paape, et al., Mastitis in small ruminants. Small Ruminant Research, 2007. 68(1-2): p. 145-153.
7. Andrade, N., M. Costa, M. Laranjo, and C. Queiroga. Controlo de mastites em pequenos ruminantes: utilização de própolis como alternativa a antimicrobianos. in X Jornadas Hospital Veterinário da Muralha. 2017. Évora, Portugal.
8. Lee, J.H., Methicillin (Oxacillin) - Resistant *Staphylococcus aureus* Strains Isolated from Major Food Animals and Their Potential Transmission to Humans. Applied and Environmental Microbiology, 2003. 69(11): p. 6489-6494.
9. Toreti, V.C., H.H. Sato, G.M. Pastore, and Y.K. Park, Recent Progress of Propolis for Its Biological and Chemical Compositions and Its Botanical Origin. Evidence-Based Complementary and Alternative Medicine, 2013. 2013: p. 13.
10. Park, Y.K. and M. Ikegaki, Preparation of Water and Ethanolic Extracts of Propolis and Evaluation of the Preparations. Bioscience, Biotechnology, and Biochemistry, 1998. 62(11): p. 2230-2232.
11. Pinto, M.S., J.E.d. Faria, D. Message, S.T.A. Cassini, C.S. Pereira, and M.M. Gioso, Efeito de extratos de própolis verde sobre bactérias patogênicas isoladas do leite de vacas com mastite. Brazilian Journal of Veterinary Research and Animal Science, 2001. 38: p. 278-283.
12. Yildiz, O., F. Karahalil, Z. Can, H. Sahin, and S. Kolayli, Total monoamine oxidase (MAO) inhibition by chestnut honey, pollen and propolis. Journal of Enzyme Inhibition and Medicinal Chemistry, 2014. 29(5): p. 690-694.
13. Barros, M.P.d., M. Lemos, E.L. Maistro, M.F. Leite, J.P.B. Sousa, J.K. Bastos, et al., Evaluation of antiulcer activity of the main phenolic acids found in Brazilian Green Propolis. Journal of Ethnopharmacology, 2008. 120(3): p. 372-377.

14. Silva Frozza, C.O., C.S.C. Garcia, G. Gambato, M.D.O. de Souza, M. Salvador, S. Moura, et al., Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. Food and Chemical Toxicology, 2013. 52: p. 137-142.

15. Pacheco, A., J.B. Daleprane, V.S. Freitas, S. Ferderbar, S. Hirabara, A. Cuevas, et al., Efecto del Propóleos Chileno sobre el Metabolismo de Glucosa en Ratones Diabéticos. International Journal of Morphology, 2011. 29: p. 754-761.

16. Daleprane, J.B., V. da Silva Freitas, A. Pacheco, M. Rudnicki, L.A. Faine, F.A. Dörr, et al., Anti-atherogenic and anti-angiogenic activities of polyphenols from propolis. The Journal of Nutritional Biochemistry, 2012. 23(6): p. 557-566.

17. Castaldo, S. and F. Capasso, Propolis, an old remedy used in modern medicine. Fitoterapia, 2002. 73(SUPPL. 1): p. 1-6.

18. Mendonça, L.S.d., F.M.R.d. Mendonça, Y.L.F.M.d. Araújo, E.D.d. Araújo, S.A. Ramalho, N. Narain, et al., Chemical markers and antifungal activity of red propolis from Sergipe, Brazil. Food Science and Technology, 2015. 35: p. 291-298.

19. Agüero, M.B., L. Svetaz, M. Sánchez, L. Luna, B. Lima, M.L. López, et al., Argentinean Andean propolis associated with the medicinal plant Larrea nitida Cav. (Zygophyllaceae). HPLC–MS and GC–MS characterization and antifungal activity. Food and Chemical Toxicology, 2011. 49(9): p. 1970-1978.

20. Nolkemper, S., J. Reichling, K.H. Sensch, and P. Schnitzler, Mechanism of herpes simplex virus type 2 suppression by propolis extracts. Phytomedicine, 2010. 17(2): p. 132-138.

21. Gekker, G., S. Hu, M. Spivak, J.R. Lokensgard, and P.K. Peterson, Anti-HIV-1 activity of propolis in CD4(+) lymphocyte and microglial cell cultures. Journal of ethnopharmacology, 2005. 102(2): p. 158-163.

22. Lu, L.-C., Y.-W. Chen, and C.-C. Chou, Antibacterial activity of propolis against *Staphylococcus aureus*. International Journal of Food Microbiology, 2005. 102(2): p. 213-220.

23. Santana, H.F., A.A.T. Barbosa, S.O. Ferreira, and H.C. Mantovani, Bactericidal activity of ethanolic extracts of propolis against *Staphylococcus aureus* isolated from mastitic cows. World Journal of Microbiology and Biotechnology, 2012. 28(2): p. 485-491.

24. Wojtyczka, R.D., M. Kępa, D. Idzik, R. Kubina, A. Kabała-Dzik, A. Dziedzic, et al., In vitro antimicrobial activity of ethanolic extract of polish propolis against biofilm forming *Staphylococcus epidermidis* strains. Evidence-based Complementary and Alternative Medicine, 2013. 2013.

25. Doganli, G.A., Phenolic content and antibiofilm activity of propolis against clinical MSSA strains. Records of Natural Products, 2016. 10(5): p. 617-627.

26. Josenhans, C. and S. Suerbaum, The role of motility as a virulence factor in bacteria. International Journal of Medical Microbiology, 2002. 291(8): p. 605-614.

27. Mirzoeva, O.K., R.N. Grishanin, and P.C. Calder, Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. Microbiological Research, 1997. 152(3): p. 239-246.

28. CLSI, *Performance Standards for Antimicrobial Disk Susceptibility Tests*, in *Approved Standard-Eleventh Edition*. 2012, Clinical and Laboratory Standards Institute (CLSI).

29. Brasil, Regulamentos Técnicos de Identidade e Qualidade de apitoxina, cera de abelha, geléia real, geléia real liofilizada, pólen apícola, própolis e extrato de própolis. 2001.

30. CLSI, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, in *Approved Standard-Ninth Edition*. 2012, Clinical and Laboratory Standards Institute (CLSI).

31. Vyletelova, M., Vyzkumny Ustav pro Chov Skotu Rapotin, Vikyrovice (Czech Republic), O. Hanus, Vyzkumny Ustav pro Chov Skotu Rapotin, Vikyrovice (Czech Republic), R. Karpiskova, Veterinarni a Farmaceuticka Univ., Brno (Czech Republic), and Z. Stastkova, Veterinarni a Farmaceuticka Univ., Brno (Czech Republic), Occurrence and antimicrobial sensitivity in staphylococci isolated from goat, sheep and cow's milk. may2011. v. 59.

32. Šiugždaite, J.G.A.Š.R., Antimicrobial resistance of pathogens from ewes subclinical mastitis. Veterinarija ir Zootechnika, 2016. 73: p. 122-122.

33. Onni, T., G. Sanna, J. Larsen, and S. Tola, Antimicrobial susceptibilities and population structure of *Staphylococcus epidermidis* associated with ovine mastitis. Veterinary Microbiology, 2011. 148(1): p. 45-50.

34. Queiroga, M.C., M. Pinto-Coelho, S. Arantes, M.E. Potes, and M.R. Martins, Antimicrobial activity of essential oils of Lamiaceae aromatic spices towards sheep mastitis-causing *Staphylococcus aureus* and *Staphylococcus epidermidis*. Journal of Essential Oil Bearing Plants, 2018.

35. Afrouzan, H., A. Tahghighi, S. Zakeri, and A. Es-haghi, Chemical Composition and Antimicrobial Activities of Iranian Propolis. Iranian Biomedical Journal, 2018. 22(1): p. 50-65.

36. Ohno, T., M. Kita, Y. Yamaoka, S. Imamura, T. Yamamoto, S. Mitsufuji, et al., Antimicrobial Activity of Essential Oils against Helicobacter pylori. Helicobacter, 2003. 8(3): p. 207-215.

37. Lightfoot, D.A., P. Ristivojević, I. Dimkić, J. Trifković, T. Berić, I. Vovk, et al., Antimicrobial Activity of Serbian Propolis Evaluated by Means of MIC, HPTLC, Bioautography and Chemometrics. Plos One, 2016. 11(6): p. e0157097.

38. Sforcin, J.M., A. Fernandes, C.A.M. Lopes, V. Bankova, and S.R.C. Funari, Seasonal effect on Brazilian propolis antibacterial activity. Journal of Ethnopharmacology, 2000. 73(1): p. 243-249.

39. Silva, W.E.d.L., W.l.D. Ferrari Junior, P.R.d. Rosa, R.d.M.P. Peixoto, J.A.B. Tenóriio, T.M.S.d. Silva, et al., In vitro activ ity of propolis: Synergism in combi nation with antibiotics against *Staphylococcus* spp. African Journal of Agricultural Research, 2015. 9(1): p. 1-5.

40. Campos, J.V.d., O. Benedito, G. Assis, and R.B. Filho. Análise por AFM da ação de extratos de própolis verde sobre as morfologias de bactérias *Staphylococcos aureus* e *Escherichia coli*. 2017.

41. Krol, W., S. Scheller, J. Shani, G. Pietsz, and Z. Czuba, Synergistic effect of ethanolic extract of propolis and antibiotics on the growth of *Staphylococcus aureus*. Arzneimittelforschung, 1993. 43(5): p. 607-9.

42. Ismael, A.B., E.-S.E.-S. Masoud, and S.M. El-Nabtityc, Potential Clinical Role of Propolis in Treatment of Clinical Ovine and Caprine Listeriosis. Vet. Med. J., 2009. 57(4): p. 11-22.

43. Fernandes Júnior, A., E.C. Balestrin, J.E.C. Betoni, R.d.O. Orsi, M.d.L.R.d.S.d. Cunha, and A.C. Montelli, Propolis: anti*Staphylococcus aureus* activity and synergism with antimicrobial drugs. Memórias do Instituto Oswaldo Cruz, 2005. 100: p. 563566.

44. Stepanović, S., N. Antić, I. Dakić, and M. Švabić-Vlahović, In vitro antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. Microbiological Research, 2003. 158(4): p. 353-357.

45. Scazzocchio, F., F.D. D'Auria, D. Alessandrini, and F. Pantanella, Multifactorial aspects of antimicrobial activity of propolis. Microbiological Research, 2006. 161(4): p. 327-333.

46. Silva, E.C.A. (2003). Preparo do extrato de própolis legal. Revista Mensagem Doce – APACAME.

Chapter 6

6. Antibiofilm activity of propolis extracts

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Antibiofilm activity of propolis extracts

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Abstract

Propolis is a resinous substance produced by honeybees with plant ingredients, which is used to fill gaps in the hive to prevent the entry of undesirable visitors and to provide better thermal insulation. It has been used for centuries to treat different pathologies and more recently became very popular in Europe due to its antibacterial activity. Biofilm is a multi-layered cluster of bacteria embedded in an extracellular polysaccharide matrix, which is known to increase bacterial ability to colonise inert materials and to protect bacteria from body defence mechanisms and antimicrobials, contributing to the establishment of persistent infections. Bacteria of the genus *Staphylococcus* are responsible for a plethora of infections in humans and animals and are the main etiological agents of mastitis in ruminants. The production of biofilm by these bacteria increases their resistance to antimicrobials, greatly hindering the treatment of infections. This study aims to investigate the in vitro activity of propolis ethanol extracts (PEE) against biofilms produced by staphylococci isolated from the milk of small ruminants with mastitis. The inhibitory action on biofilm formation and the PEE ability to eliminate established biofilms were evaluated. Ten PEE were produced from seven samples of propolis harvested in several regions in Brazil (one green, two red and four brown) and three samples collected in different regions in Portugal (all brown). These PEE were assessed for biofilm formation inhibition and biofilm disruption ability on 45 biofilm producing *Staphylococcus* isolates (26 *S. aureus*, seven *S. chromogenes*, four *S. warneri*, three *S. auricularis*, two *S. simulans*, one *S. caprae*, one *S. capitis*, and one *S.*

epidermidis) on polystyrene flat-bottom microtiter plates. All PEE showed antibiofilm activity against some *Staphylococcus* isolates. Generally, PEE are more effective in inhibiting biofilm formation, than in destroying the formed biofilm. According to these results, propolis deserves to be considered for the control of infections caused by biofilm producing staphylococci.

Keywords: *Staphylococcus*; propolis; biofilm; mastitis.

1. Introduction

Propolis is a resinous mass, rich in flavonoids and other phenolic compounds, produced by honeybees *Apis mellifera* for the protection of the honeycomb. Propolis may display different colours due to the biodiversity of plants used for its production. It has earned the attention of many researchers due to its antimicrobial action (1–3), but also due to its synergistic effect in combination with antibiotics (4). Besides antimicrobial activity, propolis has other biological properties, like antiviral, antifungal, antitumor, anti-inflammatory and antioxidant ones, and is used in traditional medicine since Antiquity (5–7). Recently antibiofilm activity of propolis has been described (3,8) and anti-quorum sensing was also stated (9).

Inflammation of the mammary gland, known as mastitis, is a serious problem for milk producers, both bovine and small ruminant, as it is responsible for lowering milk yield and quality (10). *Staphylococcus* spp. are recognized worldwide as a frequent cause of intramammary infections in small ruminants (11–19). Some of these bacteria also produce enterotoxins accountable for public health threats (20–22). Treatment and control of mastitis due to *Staphylococcus* spp. is challenging due to antimicrobial resistance (22–24) and bacterial ability to produce biofilm (26,27). Biofilm hampers mastitis control as it increases microbial survival and contribute to pathogens' persistence in the farm (25). Biofilm production has been considered responsible for antimicrobial resistance and for persistent mastitis (28–33). Its major components are an exopolysaccharide matrix (slime), proteins and environmental DNA (eDNA) along with the bacterial cells (34).

Propolis may replace or reduce the use of antibiotics in veterinary medicine due to its antimicrobial action, but also due to its antibiofilm activity. This study aims at investigating the *in vitro* antibiofilm activity of propolis ethanol extracts (PEE) against

biofilm produced by staphylococci isolated from the milk of small ruminants with mastitis. The study includes the assessment of PEE inhibitory action on biofilm formation and PEE ability to eliminate established biofilms.

2. Materials and methods

2.1 Propolis ethanol extracts

Ten different batches of raw propolis, seven from Brazil (Green, Red1, Red2, Brown1, Brown2, Brown3 and Brown4) and three from Portugal (Brown5, Brown6 and Brown7) were collected in apiaries located in different regions with different climates and vegetation (data not shown). Propolis ethanol extracts were prepared as follows (35). Cold maceration of 300 g of raw propolis in 700 mL of 70% ethanol was performed, resulting in 30% PEE. The preparations were kept at room temperature, protected from light, for 45 days. After this period, extracts were filtered through a sterile funnel and filter paper. Extracts were kept refrigerated at 4°C, in amber bottles, until use.

2.2 Bacterial isolates

Forty-four biofilm producing *Staphylococcus* field isolates (26 *S. aureus*, seven *S. chromogenes*, four *S. warneri*, three *S. auricularis*, two *S. simulans*, one *S. caprae*, one *S. capitis*) collected from different mammary glands of sheep and goats, belonging to different flocks, with clinical and subclinical mastitis, were included in this study. Milk samples were aseptically collected and immediately refrigerated until processed, within no more than 12 hours. Bacteriological analyses were undertaken according to the National Mastitis Council methodology (36) and isolates were identified to the species level using Vitek 2 Compac (Biomérieux).

2.3 Biofilm production

Biofilm production was assessed according to Merino et al. (37) with some modifications. Bacteria were grown overnight in Trypticase Soy Broth (Oxoid, CM0129) with 1% glucose (TSBg) at 37°C. Bacterial cultures were diluted in sterile TSBg until the turbidity reached 0.5 of the MacFarland scale (approximately 1 x 10^8 CFU/mL), according to the turbidimeter reading (DensiChek, bioMérieux), and again diluted 1:20 to reach 5 x 10^6 CFU/mL (38). One hundred microliters of these suspensions were added to 100 µL of

sterile TSBg in flat bottom sterile 96-well polystyrene microtiter plates. A non-biofilm producer *Staphylococcus epidermidis* ATCC 12228 was used as negative control, S. *epidermidis* ATCC 35984 as positive control and non-inoculated TSBg as sterility control. Plates were incubated overnight at 37°C. The wells were gently washed three times with 200 µL of distilled water, dried in an inverted position, and stained with 100 µL 0.25% gentian violet for 3 min at room temperature. Wells were rinsed again, 200 µL of alcohol-acetone (80:20) were added and the optical density was read at 620 nm in an ELISA plate reader (BioRad). Each assay was performed in triplicate and repeated three times. Results were recorded as follows: optical readings (OR) average value of the triplicates, subtracting the average value for the negative control (NC) in the same microplate according to the following formula: OD = (∑ OR1, OR2, OR3)/3 - (∑ NC1, NC2, NC3)/3)

No production of biofilm (isolate OD ≤ negative control OD), weak biofilm formation (negative control OD < isolate OD ≤ 2 X negative control OD), moderate biofilm formation (2 X negative control OD < isolate OD ≤ 4 X negative control OD) and strong biofilm formation (isolate OD > 4 X negative control OD).

2.4 Effect on biofilm formation

Isolates were grown overnight at 37°C in 3 ml TSBg and 5 x 10^6 CFU/mL suspensions were prepared. One hundred microliters of these suspensions were added to 100 µL of half minimum bactericidal concentration (1/2 MBC) from each PEE (diluted in Mueller Hinton broth) into each well of 96- well polystyrene flat-bottomed microtiter plates. The bactericidal activity of the extracts towards the different isolates was previously determined (see Chapter “Antimicrobial action of propolis extracts against staphylococci” in this book) and half MBC was used in this assay, so that bacteria wouldn’t be inactivated. TSBg along with 0.5 CBM of each extract, previously determined for each isolate, were used as a sterility control. After incubation for 24 h at 37°C, plates were washed and stained as for the biofilm production assay. Absorbance was settled at 620 nm and the biofilm density was determined as described previously. Each assay was performed in triplicate and repeated three times. These results were compared with the results of the biofilm formation assay and the PEE inhibitory action on the biofilm formation was established as a percentage of inhibition.

2.5 Effect on established biofilms

To evaluate the PEE ability to eliminate established biofilms, the same isolates were grown as biofilms using polystyrene flat-bottomed microtiter plates. After 24 h of incubation at 37°C, the wells were washed three times with distilled water and 200 µL of the corresponding half MBC of PEE were added. Non-inoculated MBC of each PEE for each isolate and TSBg were used as sterility controls. After incubation on the regular conditions, microplates were again washed and stained following the described procedure. Absorbance was determined at 620 nm and the formation of biofilm was determined as described previously. Each assay was performed in triplicate and repeated three times. These results were compared with the results of the biofilm formation assay and the PEE activity on the biofilm disruption was established as a percentage.

3. Results

3.1 PEE effect on biofilm formation

Nine PEE showed the ability to inhibit or stop biofilm-formation in 74.4% of the isolates analysed. The mean inhibition for each PEE, varied between 52.6 and 86.1%. Brown1, 2 and 3 Brazilian PEE showed higher activity compared to the Brown5, 6 and 7 Portuguese PEE, but Brown4 Brazilian PEE showed no activity against any of the tested isolates (**Figure 6.1**).

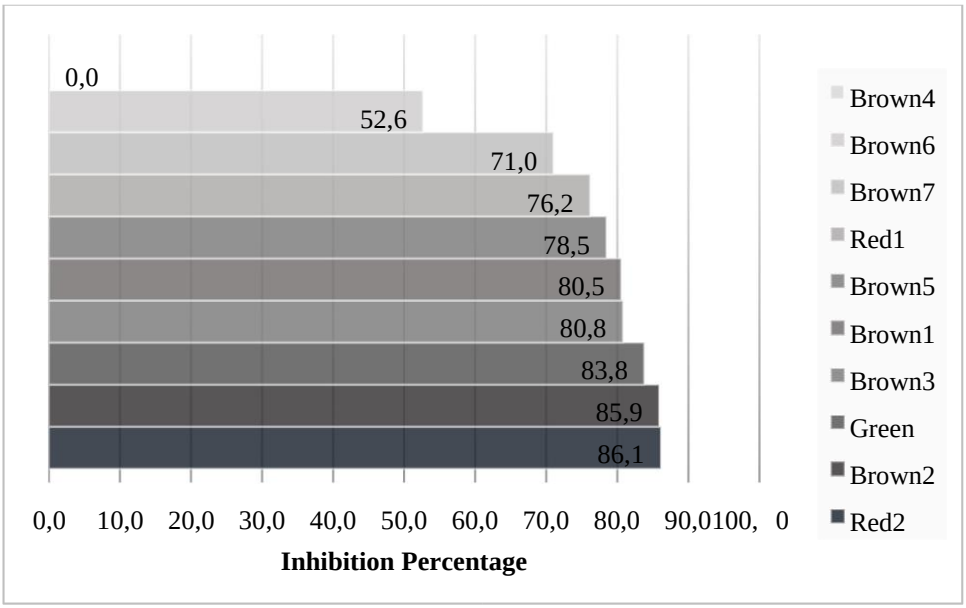

Figure 6.1. Mean inhibition percentage for each propolis ethanol extract.

Twenty-six *S. aureus* isolates were biofilm producers and only Brown2 PEE showed to inhibit biofilm formation of all isolates. The percentage of biofilm inhibition for each *S. aureus* isolate ranged from 54.4 to 88.8%. Biofilm of *S. epidermidis* ATCC 35984, used as a positive control, was susceptible to eight PEE and its percentage of inhibition varied between 62 and 96.3% (**Table 6.1**).

Table 6.1. PEE effect on biofilm formation: number of isolates, and respective percentage, and mean inhibition percentage by species for each PEE.

Isolates/Species	*S. aureus*	*S. auricularis*	*S. caprae*	*S. capitis*	*S. chromogenes*	*S. epidermidis*	*S. simulans*	*S. warneri*	Total N/Mean %
N	**26**	**3**	**1**	**1**	**7**	**1**	**2**	**4**	**45**
Inhibited by PEE Green (N)	20	3	1	0	5	1	2	3	35
Inhibited isolates (%)	76.9	100.0	100.0	0.0	71.4	100.0	100.0	75.0	80.6
% of Inhibition	88.8	73.6	100.0	0.0	85.4	65.1	56.9	76.4	83.8
Inhibited by PEE Red1 (N)	24	3	1	0	5	1	2	4	40
Inhibited isolates (%)	92.3	100.0	100.0	0.0	71.4	100.0	100.0	100.0	91.8
% of Inhibition	85.7	59.3	80.3	0.0	56.8	62.0	85.5	54.0	76.2
Inhibited by PEE Red2 (N)	23	2	1	1	6	1	1	3	38
Inhibited isolates (%)	88.5	66.7	100.0	100.0	85.7	100.0	50.0	75.0	85.7
% of Inhibition	85.8	92.1	81.0	80.3	91.0	95.1	98.5	71.7	86.1
Inhibited by PEE Brown1 (N)	24	3	1	0	7	1	2	3	41
Inhibited isolates (%)	92.3	100.0	100.0	0.0	100.0	100.0	100.0	75.0	93.7
% of Inhibition	82.6	97.1	98.9	0.0	72.9	96.3	68.2	62.8	80.5
Inhibited by PEE Brown2 (N)	26	2	1	0	7	1	2	3	42
Inhibited isolates (%)	100.0	66.7	100.0	0.0	100.0	100.0	100.0	75.0	96.6
% of Inhibition	88.6	86.4	79.9	0.0	85.9	79.0	91.6	62.1	85.9
Inhibited by PEE Brown3 (N)	25	3	1	1	7	1	2	3	43
Inhibited isolates (%)	96.2	100.0	100.0	100.0	100.0	100.0	100.0	75.0	96.0
% of Inhibition	78.5	91.5	99.5	82.9	83.3	69.9	83.8	77.8	80.8
Inhibited by PEE Brown4 (N)	0	0	0	0	0	0	0	0	0
Inhibited isolates (%)	0	0	0	0	0	0	0	0	0.0
% of Inhibition	0	0	0	0	0	0	0	0	0.0
Inhibited by PEE Brown5 (N)	23	1	1	0	7	1	0	1	34
Inhibited isolates (%)	88.5	33.3	100.0	0.0	100.0	100.0	0.0	25.0	88.1
% of Inhibition	84.2	48.0	90.7	0.0	64.5	89.8	0.0	50.5	78.5
Inhibited by PEE Brown6 (N)	16	1	1	0	5	0	2	3	28
Inhibited isolates (%)	61.5	33.3	100.0	0.0	71.4	0.0	100.0	75.0	67.8
% of Inhibition	54.4	85.2	81.1	0.0	47.2	0.0	40.0	40.6	52.6
Inhibited by PEE Brown7 (N)	21	1	1	0	6	1	2	2	34
Inhibited isolates (%)	80.8	33.3	100.0	0.0	85.7	100.0	100.0	50.0	80.7
% of Inhibition	78.4	34.5	61.2	0.0	53.9	63.5	75.9	67.2	71.0

3.2 PEE effect on established biofilms

All ten PEE showed to partially or totally destroy the biofilm produced by 45 staphylococci isolates. The mean PEE activity percentage on biofilm disruption varied between 51.4 and 92.0% (**Figure 6.2**).

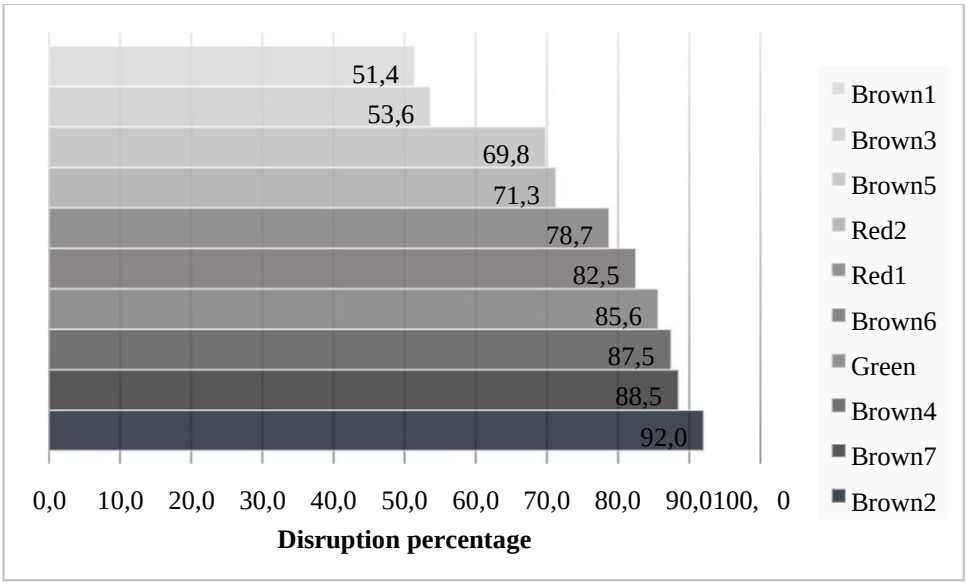

Figure 6.2. Mean activity percentage on biofilm disruption for each propolis ethanol extract.

The percentage of biofilm disruption ranged from 0 to 100% (**Table 6.2**). Propolis Ethanol Extract Brown 2 showed biofilm disruption activity against 88.4% of *S. aureus* isolates but did not affect biofilm *from S. epidermidis* and *S. capitis.*

Generally, PEE are more effective in inhibiting biofilm formation, than in destroying the formed biofilm, with the exception of Green, Brown4, Brown6, and Brown 7. PEE Brown1, Brown2 and Brown3 were able to inhibit more than 40 out of 45 isolates (88.9%). However, these were not the best PEE in disrupting the established biofilm. Brown7, Green and Red were the most effective PEE in destroying biofilm. Brown4, while completely unable to inhibit biofilm formation, was still able to disrupt the established biofilm in more than 50% of the isolates. Antibiofilm activity is summarised in Figure 6.3.

Table 6.2. PEE effect on established biofilms: number of isolates, and respective percentage, and mean activity percentage on biofilm disruption by species for each PEE.

Isolates/Species	*S. aureus*	*S. auricularis*	*S. caprae*	*S. capitis*	*S. chromogenes*	*S. epidermidis*	*S. simulans*	*S. warneri*	Total N/Mean %
N	**26**	**3**	**1**	**1**	**7**	**1**	**2**	**4**	**45**
Affected by PEE Green (N)	19	3	1	1	6	1	2	3	36
Affected isolates (%)	73.0	100.0	100.0	100.0	85.7	100.0	100.0	75.0	81.3
% of Disruption	93.4	23.3	58.6	100.0	95.5	100.0	100.0	68.9	85.6
Affected by PEE Red1 (N)	20	1	1	0	5	1	1	3	32
Affected isolates (%)	76.9	33.3	100.0	0.0	71.4	100.0	50.0	75.0	75.1
% of Disruption	70.7	54.9	98.0	0.0	97.1	79.3	100.0	96.3	78.7
Affected by PEE Red2 (N)	19	3	1	1	5	1	2	4	36
Affected isolates (%)	73.0	100.0	100.0	100.0	71.4	100.0	100.0	100.0	81.8
% of Disruption	62.8	74.2	85.6	73.1	66.9	100.0	98.7	90.1	71.3
Affected by PEE Brown1 (N)	12	2	1	0	6	1	2	2	26
Affected isolates (%)	46.1	66.6	100.0	0.0	85.7	100.0	100.0	50.0	65.4
% of Disruption	32.0	86.2	83.6	0.0	65.9	87.2	70.3	36.8	51.4
Affected by PEE Brown2 (N)	23	2	1	0	7	0	2	3	38
Affected isolates (%)	88.4	66.6	100.0	0.0	100.0	0.0	100.0	75.0	89.2
% of Disruption	94.4	52.2	81.3	0.0	91.5	0.0	100.0	100.0	92.0
Affected by PEE Brown3 (N)	16	2	1	1	6	1	2	3	32
Affected isolates (%)	61.5	66.6	100.0	100.0	85.7	100.0	100.0	75.0	73.6
% of Disruption	40.9	81.4	75.9	29.1	71.0	62.4	84.3	45.6	53.6
Affected by PEE Brown4 (N)	17	1	0	0	3	1	1	1	24
Affected isolates (%)	65.3	33.3	0.0	0.0	42.8	100.0	50.0	25.0	60.3
% of Disruption	84.1	100.0	0.0	0.0	100.0	100.0	73.6	95.9	87.5
Affected by PEE Brown5 (N)	14	1	1	0	7	0	2	3	28
Affected isolates (%)	53.8	33.3	100.0	0.0	100.0	0.0	100.0	75.0	71.8
% of Disruption	52.7	43.2	97.7	0.0	98.2	0.0	98.9	64.0	69.8
Affected by PEE Brown6 (N)	14	1	1	0	7	0	2	4	29
Affected isolates (%)	53.8	33.3	100.0	0.0	100.0	0.0	100.0	100.0	75.4
% of Disruption	70.8	41.3	99.8	0.0	99.1	0.0	100.0	91.5	82.5
Affected by PEE Brown7 (N)	20	2	1	1	6	0	2	4	36
Affected isolates (%)	76.9	66.6	100.0	100.0	85.7	0.0	100.0	100.0	82.9
% of Disruption	88.3	69.2	100.0	76.8	92.5	0.0	100.0	87.4	88.5

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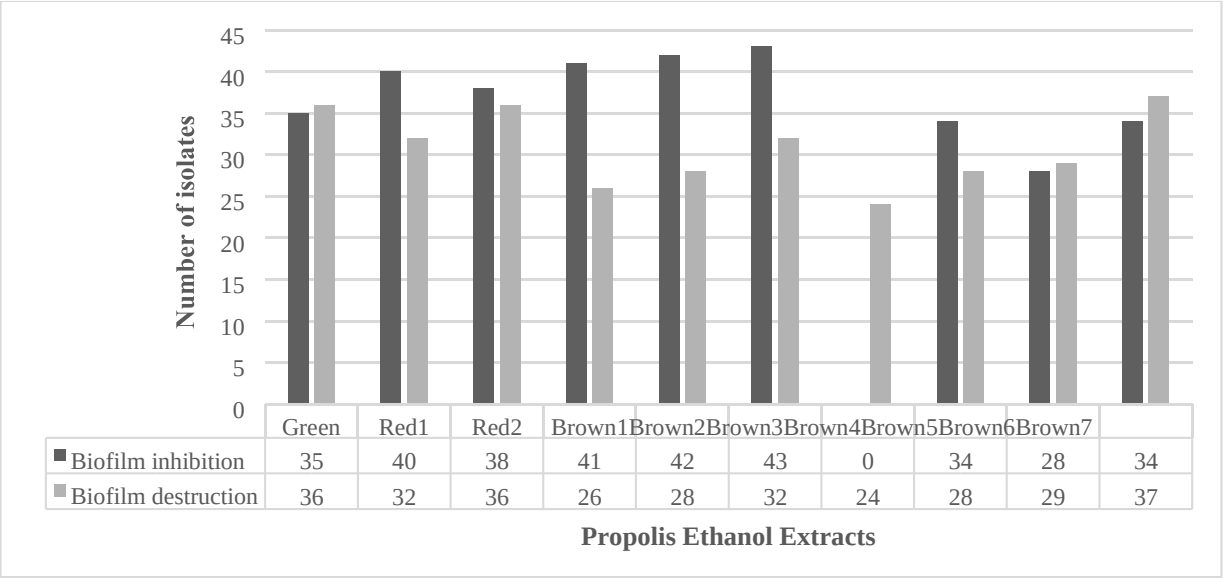

Figure 6.3. PEE effect on biofilm formation and on established biofilms: number of isolates affected by each PEE.

4. Discussion

Nine PEE showed ability to prevent biofilm formation and all the tested PEE revealed biofilm disruption aptitude. It should be noted that the concentration of PEE used in these trials was half the minimum bactericidal concentration for each bacterial isolate. The dosage of PEE to be used in clinical situations should be at least the MBC, therefore twice the amount used here, hence the antibiofilm activity should be better than the one here presented.

The need to control biofilm associated with mammary infection seems to be of major importance. As revised by Melchior *et al.* (30) several researchers have shown that bacteria growing in a biofilm can become 10 to 1000 times more resistant to antimicrobials than planktonic growing bacteria of the same strain. Concerning the pathology of the mammary gland, although some authors refer that the *in vitro* biofilm-forming ability of a given strain was not related to its clinical origin, considering cows with persistent and non-persistent intramammary infection (39), others mention that *S. aureus* strains persisting in the bovine mammary gland through the dry period produced significantly more biofilm *in vitro* than strains that do not persist after calving (33). Furthermore, mice inoculated with a bovine mastitis strong biofilm forming *S. aureus*

isolate produced marked acute mastitis lesions while the damage was significantly less severe in mammary glands of mice infected with a weak biofilm-forming *S. aureus* strain (40). Results from a study on staphylococci causing subclinical mastitis in sheep confirmed the significance of slime producing strains in the aetiology of this affection, with 80.4% of all cases of staphylococcal subclinical mastitis caused by slime producing strains (26).

A key aspect to keep in mind is to ensure that the PEE has antimicrobial activity. Since an action on the disassemble of biofilm without the concomitant bacterial inhibition would be responsible for the release of living bacteria that would colonise additional body parts (34). All ten PEE are bactericidal in appropriate concentrations (see Chapter "Antimicrobial action of propolis extracts against staphylococci" in this book).

Other studies have shown that propolis extracts were able to inhibit *Pseudomonas aeruginosa* biofilm formation (41) and stated an inhibitory effect of propolis extracts on biofilm formation by *Streptococcus mutans* (42) with some propolis components displaying a potent inhibition of glucosyltransferase activity, which is an enzyme that catalyses the formation of biofilm (43). Biofilm formation ability by *Staphylococcus epidermidis* strains in the presence of PEE was significantly inhibited by incubation time and was observed after 12 and 24 hours of incubation (3).

Furthermore, some investigators showed quorum sensing inhibitory activity of distinct propolis samples (9,44). Quorum sensing is the phenomenon through which bacteria, within a biofilm, use signalling molecules, autoinducers, that when accumulate to a threshold concentration activate a transcriptional regulator, which in turn regulates the expression of various genes, including virulence factors and thus is one of the essential factors that regulate bacterial virulence and pathogenicity (45,46). This plays an important role in biofilm development, resistance, and virulence. Therefore, the interruption of QS can be an effective strategy to control disease-causing pathogens.

Biofilm formation is accompanied by significant genetic and subsequent physiological changes in the microorganisms resulting in a loss of sensitivity to virtually all classes of antibiotics (30). As mostly studied in *S. aureus* and to some degree in *S. epidermidis*, quorum sensing is achieved by activating the accessory gene regulator (*agr*) that results in the production of a regulatory mRNA molecule termed RNAIII, which activates multiple toxin genes (47).

Biofilm production ability can greatly hinder conventional antimicrobial therapy suggesting the need of alternative control approaches such as the use of propolis. According to our results some PEE have antibiofilm activity against small ruminants' mastitis causing *Staphylococcus* spp. biofilm and might, therefore, be a promising approach for disease control either as alternative to antibiotics or in combination with antibiotics taking advance of synergistic effect (48).

Studies to isolate and identify the specific propolis compounds responsible for the bioactivity are needed.

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References

1. Lu L-C, Chen Y-W, Chou C-C. Antibacterial activity of propolis against *Staphylococcus aureus*. Int J Food Microbiol [Internet]. 2005 [cited 2017 May 24];102(2):213–20. Available from: http://www.sciencedirect.com/science/article/pii/S0168160505000589
2. Santana HF, Barbosa AAT, Ferreira SO, Mantovani HC. Bactericidal activity of ethanolic extracts of propolis against *Staphylococcus aureus* isolated from mastitic cows. World J Microbiol Biotechnol [Internet]. 2012;28(2):485–91. Available from: http://dx.doi.org/10.1007/s11274-011-0839-7
3. Wojtyczka RD, Kępa M, Idzik D, Kubina R, Kabała-Dzik A, Dziedzic A, et al. In vitro antimicrobial activity of ethanolic extract of polish propolis against biofilm forming *Staphylococcus epidermidis* strains. Evidence-based Complement Altern Med. 2013;2013.
4. Silva WE de L, Ferrari Junior W lson D, Rosa PR da, Peixoto R de MP, Tenóriio JAB, Silva TMS da, et al. In vitro activ ity of propolis: Synergism in combi nation with antibiotics against *Staphylococcus* spp. African J Agric Res [Internet]. 2015;9(1):1–5. Available from: http://www.academicjournals.org/journal/AJAR/article-abstract/375F3A640396
5. Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R, Popov S. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J Ethnopharmacol [Internet]. 1999 [cited 2017 May 24];64(3):235–40. Available from: http://www.sciencedirect.com/science/article/pii/S0378874198001317
6. Banskota AH, Tezuka Y, Adnyana IK, Midorikawa K, Matsushige K, Message D, et al. Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China. J Ethnopharmacol [Internet]. 2000 [cited 2017 May 24];72(1):239–46. Available from: http://www.sciencedirect.com/science/article/pii/S037887410000252X
7. Marcucci MC, Ferreres F, Garcı C. Phenolic compounds from Brazilian propolis with pharmacological activities. 2001;74:105– 12.
8. Doganli GA. Phenolic content and antibiofilm activity of propolis against clinical MSSA strains. Rec Nat Prod. 2016;10(5):617– 27.
9. Kasote D, Ahmad A, Chen W, Combrinck S, Viljoen A. Phytochemistry Letters HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis. Phytochem Lett [Internet]. 2014;11:6–11. Available from: http://dx.doi.org/10.1016/j.phytol.2014.08.017
10. Leitner G, Chaffer M, Shamay A, Shapiro F, Merin U, Ezra E, et al. Changes in Milk Composition as Affected by Subclinical Mastitis in Sheep. J Dairy Sci. 2004;87(1):46–52.
11. Bergonier, D.; Lagriffoul, G.; Concordet, D.; Barillet, F. and Berthelot X. Subclinical mastitis in dairy sheep: Aetiology and diagnosis using somatic cell counts. In: Proceedings of the 4th IDF International Mastitis Conference, Maastricht, The Netherlands, June 2005. 2005. p. 921–2.
12. Cuccuru C, Meloni M, Sala E, Scaccabarozzi L, Locatelli C, Moroni P, et al. Effects of intramammary infections on somatic cell score and milk yield in Sarda sheep. N Z Vet J [Internet]. 2011 May;59(3):128–31. Available from: http://www.tandfonline.com/doi/abs/10.1080/00480169.2011.562862

13. Dore S, Liciardi M, Amatiste S, Bergagna S, Bolzoni G, Caligiuri V, et al. Survey on small ruminant bacterial mastitis in Italy, 2013-2014. Small Rumin Res [Internet]. Elsevier B.V.; 2016;141:91–3. Available from: http://dx.doi.org/10.1016/j.smallrumres.2016.07.010

14. Fthenakis GC, Leontides L, Skoufos J, Taitzoglou IA, Tzora A. Case report: High prevalence rate of ovine mastitis, caused by coagulase-negative staphylococci and predisposed by increased gossypol consumption. Small Rumin Res. 2004;52(1–2):185–9.

15. Hariharan H, Donachie W, Macaldowie C, Keefe G. Bacteriology and somatic cell counts in milk samples from ewes on a Scottish farm. Can J Vet Res. 2004;68(3):188–92.

16. Kiossis E, Brozos CN, Petridou E, Boscos C. Program for the control of subclinical mastitis in dairy Chios breed ewes during lactation. Small Rumin Res [Internet]. 2007 Nov;73(1–3):194–9. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0921448807000387

17. Leitner G, Chaffer M, Zamir S, Mor T, Glickman A, Winkler M, et al. Udder disease etiology, milk somatic cell counts and NAGase activity in Israeli Assaf sheep throughout lactation. Small Rumin Res. 2001;39(2):107–12.

18. Queiroga MC. Prevalence and aetiology of sheep mastitis in Alentejo region of Portugal. Small Rumin Res [Internet]. 2017 Aug;153:123–30. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0921448817301621

19. Spanu C, Berger YM, Thomas DL, Ruegg PL. Impact of intramammary antimicrobial dry treatment and teat sanitation on somatic cell count and intramammary infection in dairy ewes. Small Rumin Res [Internet]. Elsevier B.V.; 2011;97(1–3):139–45. Available from: http://dx.doi.org/10.1016/j.smallrumres.2011.03.005

20. Hummerjohann J, Naskova J, Baumgartner A, Graber HU. Enterotoxin-producing *Staphylococcus aureus* genotype B as a major contaminant in Swiss raw milk cheese. J Dairy Sci [Internet]. 2014 [cited 2017 Jun 14];97(3):1305–12. Available from: http://www.sciencedirect.com/science/article/pii/S0022030214000502

21. Salaberry SRS, Saidenberg ABS, Zuniga E, Melville PA, Santos FGB, Guimarães EC, et al. Virulence factors genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis. Microb Pathog. 2015;85:35–9.

22. Martins KB, Faccioli PY, Bonesso MF, Fernandes S, Oliveira AA, Dantas A, et al. Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. J Dairy Sci [Internet]. American Dairy Science Association; 2017;100(3):2184–95. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030217300413

23. Šiugždaitė J, Gabinaitienė A, Šiugžda R. ANTIMICROBIAL RESISTANCE OF PATHOGENS FROM EWES SUBCLINICAL. Vet ir Zootech (Vet Med Zoot). 2016;73(95):122–7.

24. Virdis S, Scarano C, Cossu F, Spanu V, Spanu C, De Santis EPL. Antibiotic Resistance in *Staphylococcus aureus* and Coagulase Negative Staphylococci Isolated from Goats with Subclinical Mastitis. Vet Med Int. 2010;2010:517060.

25. Gomes F, Saavedra MJ, Henriques M. Bovine mastitis disease/pathogenicity: evidence of the potential role of microbial biofilms. Pathog Dis [Internet]. 2016;(January):1–19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26772653

26. Vasileiou NGC, Chatzopoulos DC, Gougoulis DA, Sarrou S, Katsafadou AI, Spyrou V, et al. Slime-producing staphylococci as causal agents of subclinical mastitis in sheep. Vet Microbiol [Internet]. Elsevier; 2018;224(August):93–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0378113518308952

27. Queiroga MC, Duartea EL, Laranjo M. Sheep mastitis *Staphylococcus epidermidis* biofilm effects on cell adhesion and inflammatory changes. Small Rumin Res [Internet]. 2018;168(September):6–11. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0921448818305789

28. Cucarella C, Tormo MA, Úbeda C, Trotonda MP, Monzón M, Peris C, et al. Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*. Infect Immun. 2004;72(4):2177–85.

29. Fontaine MC, Smith DGE. Microbial biofilms: Does breaking the microbes' community spirit hold the key to beating persistent mastitis? Vet J. 2006;171(3):387–8.

30. Melchior MB, Vaarkamp H, Fink-Gremmels J. Biofilms: A role in recurrent mastitis infections? Vet J. 2006;171(3):398–407.

31. Tremblay YDN, Lamarche D, Chever P, Haine D, Messier S, Jacques M. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. J Dairy Sci [Internet]. Elsevier; 2013;96(1):234–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23141829

32. Vanderhaeghen W, Piepers S, Leroy F, Van Coillie E, Haesebrouck F, De Vliegher S. Invited review: Effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. J Dairy Sci [Internet]. Elsevier; 2014;97(9):5275–93. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030214004317

33. Veh KA, Klein RCC, Ster C, Keefe G, Lacasse P, Scholl D, et al. Genotypic and phenotypic characterization of *Staphylococcus aureus* causing persistent and nonpersistent subclinical bovine intramammary infections during lactation or the dry period. J Dairy Sci [Internet]. 2015;98(1):155–68. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25468698%5Cnhttp://linkinghub.elsevier.com/retrieve/pii/S0022030214007280

34. Buttner H, Dietrich M, Rohde H. Structural basis of *Staphylococcus epidermidis* biofilm formation: mechanisms and molecular interactions. Front Cell Infect Microbiol [Internet]. 2015;5(February):1–15. Available from: http://journal.frontiersin.org/Article/10.3389/fcimb.2015.00014/abstract

35. Brasil. Regulamentos Técnicos de Identidade e Qualidade de apitoxina, cera de abelha, geléia real, geléia real liofilizada, pólen apícola, própolis e extrato de própolis. In: Instrução Normativa n3. 2001.

36. National Mastitis Council (U.S.). Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. 4th ed. Verona, Wis. : NMC, 2004.; 2004. 47 p.

37. Merino N, Toledo-Arana A, Vergara-Irigaray M, Valle J, Solano C, Calvo E, et al. Protein A-mediated multicellular behavior in *Staphylococcus aureus*. J Bacteriol. 2009;191(3):832–43.

38. CLSI. M07-A9—Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. Clinical and Laboratory Standards Institute (CLSI)–approved standards. 2012;

39. Pereyra EAL, Picech F, Renna MS, Baravalle C, Andreotti CS, Russi R, et al. Detection of *Staphylococcus aureus* adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells. Vet Microbiol [Internet]. Elsevier B.V.; 2016;183:69–77. Available from: http://dx.doi.org/10.1016/j.vetmic.2015.12.002

40. Gogoi-Tiwari J, Williams V, Waryah CB, Costantino P, Al-Salami H, Mathavan S, et al. Mammary Gland Pathology Subsequent to Acute Infection with Strong versus Weak Biofilm Forming *Staphylococcus aureus* Bovine Mastitis Isolates: A Pilot Study Using Non-Invasive Mouse Mastitis Model. PLoS One [Internet]. 2017;12(1):e0170668. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28129375%0Ahttp://dx.plos.org/10.1371/journal.pone.0170668

41. De Marco S, Piccioni M, Pagiotti R, Pietrella D. Antibiofilm and Antioxidant Activity of Propolis and Bud Poplar Resins versus Pseudomonas aeruginosa. Evidence-based Complement Altern Med. 2017;2017.

42. Veloz JJ, Saavedra N, Lillo A, Alvear M, Barrientos L, Salazar LA. Antibiofilm Activity of Chilean Propolis on *Streptococcus mutans.* Is Influenced by the Year of Collection. Biomed Res Int. 2015;2015.

43. Koo H, Rosalen PL, Cury JA, Park YK, Bowen WH. Effects of Compounds Found in Propolis on *Streptococcus mutans* Growth and on Glucosyltransferase Activity. 2002;46(5):1302–9.

44. Savka MA, Dailey L, Popova M, Mihaylova R, Merritt B, Masek M, et al. Chemical Composition and Disruption of Quorum Sensing Signaling in Geographically Diverse United States Propolis. 2015;2015.

45. Rutherford ST, Bassler BL. Bacterial quorum sensing: Its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med. 2012;2(11):1–25.

46. Kong KF, Vuong C, Otto M. *Staphylococcus* quorum sensing in biofilm formation and infection. Int J Med Microbiol. 2006;296(2–3):133–9.

47. Novick RP, Geisinger E. Quorum Sensing in Staphylococci. Annu Rev Genet [Internet]. 2008;42(1):541–64. Available from: http://www.annualreviews.org/doi/10.1146/annurev.genet.42.110807.091640

48. Król W, Scheller S, Shani J, Pietsz G, Czuba Z. Synergistic effect of ethanol extract of propolis and antibiotics on the growth of *Staphylococcus aureus.* Vol. 43, Arzneimittel-Forschung. 1993. 607-609 p.

Chapter 7

7. General discussion and future perspectives

7.1 – General discussion

7.1.1 – *Staphylococcus* identification

With the aim of assessing propolis as an alternative to traditional antimicrobials for the control of small ruminant mastitis, we collected milk samples from 70 sheep and 258 goats with mastitis. Our collection of 137 staphylococci isolates included *S. aureus* and 13 CNS species, 12 identified in goats and seven in sheep milk samples. Six *Staphylococcus* species were isolated from both animal species. *Staphylococcus caprae*, *S. warneri*, *S. capitis*, *S. hominis*, *S hyicus* and *S. equorum* were found only in goat's milk. *S. caprae* was mostly found in goat's milk, as was reported by other authors (Bergonier and Berthelot, 2003; Peixoto et al., 2010; Gosselin et al., 2018), despite having also been isolated from sheep's milk (Martins et al., 2017). The other species, on the other hand, are commonly found in sheep's milk (Zafalon et al., 2018; Vasileiou et al., 2018). An isolate of the species *S. rostri* was found in sheep's milk in this work. As far as we know, this species had only

been once isolated from the milk of a sheep with mastitis (Persson et al., 2017). No data were found in the literature related to goat's mastitis, *S.rostri* being more commonly found in cow's milk (Jenkins et al., 2019; Wuytack et al., 2020).

The difference in the number of isolates in the different chapters, as in chapters 4, 5 and 6, is due to the fact of some isolates had not been sequenced in the year of publication of the articles. In chapter 3 there is the correct quantity of all isolates analyzed in this study.

Biochemical identification with the automated compact system VITEK 2 (bioMérieux, F) showed excellent to very good results for most isolates and 16S rRNA gene sequencing was only necessary for four isolates. Nevertheless, the presence of *coa* and *nuc* genes was investigated in all staphylococci isolates, as these are genes frequently assessed to differentiate *S. aureus* from CNS. Our isolates were found to be polymorphic regarding the *coa* gene, showing fragments between 400 and 900 bp in length. Likewise, this gene was also amplified from *S. aureus* of small ruminants and humans isolates with variations from 300 to 800 bp (Vimercati et al., 2006; Mahmoudi et al., 2017). Soltan Dallal et al. (2016), found polymorphism between 500 and 1000 bp of *S. aureus* isolated from food samples. The *coa* gene had been reported as polymorphic before and these polymorphisms are known to be strain-specific (Soltan Dallal et al., 2016).

The *nuc* gene was detected in 48.9% of the analysed staphylococci isolates. All 35 *S. aureus* carried the *nuc* gene (100%). However, this gene was also detected in 22.6% of CNS. Although this gene is often used to identify *S.aureus*, this is not a reliable technique according to our results. These findings agree with those of other authors which detected this gene in other species of *Staphylococcus*, both CPS and CNS (Hirotaki et al., 2011; Silva et al., 2003). Moreover, Leeuwen et al. (2008) did not detect it in MRSA.

The *nuc* gene is an important virulence factor for bacterial survival (Olson et al., 2013), as it defeats the host defences (Berends et al., 2010) and it is an indicator of biofilm dispersion (Mann et al., 2009). In the present study we found an association between *Staphylococcus* species and the presence of the *nuc* gene. This gene was detected in more than 50% of *S. warneri* (4/7), *S. lentus* (3/5), *S. auricularis* (3/4), and *S. hyicus* (3/3) isolates. This feature may eventually affect the pathogenesis of mastitis due to those pathogens.

7.1.2 – Biofilm production and biofilm-associated genes

To detect whether the isolates were capable of forming biofilm, the polystyrene microtiter plates method was used, according to Merino et al. (2009). This is considered one of the most effective phenotypic methods (Stiefel et al., 2016; Salina et al., 2020), showing a 96.7% agreement with molecular methods (Melo et al., 2012). Of the biofilm-forming isolates, 59% were S *aureus* and 41% CNS. Ebrahimi et al. (2014) also found a higher percentage of phenotypic biofilm formation to *S. aureus* than CNS in sheep isolates.

Considering biofilm as a relevant virulence factor for mastitis, our studies disclosed an association between mammary gland inflammatory intensity and biofilm formation. However, no association was found between this feature and bacterial susceptibility to PEE (Chapter 4). Other authors have mentioned *S. aureus* ability to produce biofilm as a virulence factor affecting mastitis pathogenesis (Felipe et al., 2017).

Regarding biofilm associated genes, the *bap* gene was only present in CNS, while *ica*A and *ica*D genes were mainly detected in *S. aureus*. Other studies showed that the *bap* gene is more often detected in CNS species than CPS (Salaberry et al., 2015; Martins et al., 2017). In the present study the isolates carrying the *bap* gene did not harbour the *ica* operon genes. Likewise, Szweda et al. (2012) reported no isolate carrying both the *bap* and *ica* operon genes. However, in bovine mastitis, 56% of CPS harboured *ica*A, *ica*D and *bap* genes together, unlike CNS (Salina et al., 2020).

Some isolates studied, including *S. aureus* and CNS, did not evidence any of the three biofilm-associated genes. The same absence of *bap*, *ica*A and *ica*D genes in biofilm-producing *S. aureus* was reported (Khoramian et al., 2015; Vitale et al., 2019; Torres et al., 2019). The *nuc* gene was detected in 53.4% biofilm-producing isolates, while in only about 35% of the non-producers, although we did not find any association between the presence of this gene and biofilm production. We might wonder about the role of the Nuc thermonuclease in staphylococci biofilm formation. Nevertheless Mann et al. (2009) suggested its role as a promoter of biofilm dispersal. Investigating biofilm formation mechanisms in strains that do not harbour *bap* or *ica* genes is important to have a better understanding of the resources used by bacteria in the production of this virulence factor.

7.1.3 – Antimicrobial susceptibility and resistance genes

The detection of staphylococci susceptibility profile to antimicrobials available for mastitis control was evaluated. Some staphylococci isolates were multidrug resistant. This constitutes a potential risk for consumers (Omoshaba et al., 2020). Antimicrobials resistance acquired by staphylococci is a reality, as well as its transfer to human microbiota. In addition, these microorganisms can reach the human population through a variety of routes, including though food of animal origin (Kuile et al., 2016). *Staphylococcus* isolated from bovine milk samples, carrying the *mec*A gene and phenotypic resistant were transmitted to humans (Lee, 2003). *Bla*Z gene was found in *Staphylococcus* isolated from goat cheese (Aragão et al., 2019), as well as *mec*A gene was detected in *Staphylococcus* isolated from Kurd cheeses samples (Hachiya et al., 2017).

Non-susceptibility of the isolates to β-lactam antimicrobials was mostly observed. Other studies describe less efficacy of these antimicrobials over the years (Virdis et al., 2010; França et al., 2012; Santos et al., 2020), highlighting the possibility of resistance acquisition by this genus. The resistance is individual and develops through mutations and rearrangements in the staphylococcal genome or also through the acquisition of resistance determinants. The increased antibiotic pressure, together with genetic variants, contributes to this virulence factor (Vasileiou et al., 2019). Accordingly, *bla*Z was the antimicrobial resistance gene mostly detected in this work. Some of these isolates showed susceptibility to some or all β-lactam antimicrobial analysed, however one must be aware that caring the *bla*Z gene demands to consider the isolate resistant to all penicillins and semi-synthetic penicillins. Similar results were found by Ferreira et al. (2017). The most reliable way to detect if the isolate produces β-lactamase is through the detection of *bla*Z gene (Pitkälä et al., 2007). In the present study some penicillin-susceptible isolates harboured the *bla*Z gene. Some *Staphylococcus* may present this gene and not show resistance phenotypically (CLSI, 2016). El Feghaly et al. (2012) also stated that commonly used phenotypic sensitivity methods may not be reliable.

The presence of the *mec*A and *mec*C genes was not confirmed in isolates non-susceptible to oxacillin. Although in most studies all *mec*A-positive isolates showed oxacillin resistance (Obaidat et al., 2017), some reports mention oxacillin-resistant isolates not carrying the *mec*A gene Shah et al. (2017). Other resistance mechanisms are possible, as

the presence of the recently described *mec*B gene (Becker et al., 2018) or overproduction of β-lactamase, modified penicillin-binding proteins, distinct SCCmec elements, as well as putative *mec*A mutations (Xu et al., 2008; Paterson et al., 2014).

Curiously, *mec*A-positive S. *aureus* showing susceptibility to oxacillin, which were classified as oxacillin-susceptible *mec*A-positive *Staphylococcus aureus* (OS-MRSA) was mentioned by Pu et al. (2014). Goering et al. (2019), demonstrated that oxacillin susceptibility was associated with mutations in the *mec*A gene and that subinhibitory exposure of the isolate to oxacillin restored its resistance.

Regarding tetracycline resistance, the few *S. aureus* isolates assessed harboured either the *tet*K or the *tet*M gene. However, conclusions cannot be drawn due to the few samples genotypically analysed. Liu et al. (2017), observed correlations between *S. aureus* resistant phenotypes and genotypes for tetracycline. In the present study one CNS isolate phenotypically resistant to this antimicrobial did no carry either *tet*K or *tet*M gene. Other authors reported non-susceptible S. intermedius not carrying *tet*K, *tet*L, *tet*M, and *tet*O genes (El-Razik et al., 2017). However, Mama et al. (2019), found these genes in tetracycline-resistant CNS.

Gentamicin and cefazolin inhibited the growth of all tested isolates. Gentamicin is an aminoglycoside, generally used for Gram negative bacteria, however it showed an excellent performance against different species of *Staphylococcus* in this study. Other authors reported very good results with staphylococci isolates from buffalo, goat and sheep mastitis (Santos et al., 2020). Cefazolin is a first generation cephalosporin, although it is not reserved for human use, such as those of third and higher generations, it should not be a molecule of first choice. Kumari et al. (2020) referred 10% of cefazoline-resistant *S. aureus.*

7.1.4 – Antimicrobial and antibiofilm activity of Propolis

Seven propolis batches from Brazil and three from Portugal were used to produce propolis ethanol extracts, which were characterized by UPLC-QTOF MS^E. We used UPLC-Photo Diode Array (PDA) to detect phenolic profile and UPLC-QTOF-MS / MS to identify different compounds. As expected, diverse PEE showed differences in composition. This is due to the vegetable biodiversity (Tolêdo et al., 2011) found in each country, also resulting

in the propolis differences colour. The variety of individual compounds found allowed to separate the PEE into 6 groups in this work, green (Group I), red (Group II) and brown (Group III, IV from Brazil and Group V and VI from Portugal). Flavonoids (chalcones, flavanones, flavanonols, flavones, flavonols, isodhydroflavones, isoflavans), non-flavonoid phenolics and triterpenes were the constituents identified.

Green propolis is known, in different countries, for diverse activities due to its peculiar components (Machado et al., 2012; Szliszka et al., 2012; Chen et al., 2018). All Green PEE individual components were exclusive, in this study. This may be due to the components of its main botanical source, Asteraceae *Baccharis dracunculifolia* (Park et al., 2002), which were also found in this PEE.

Red coloured propolis is found in several states from Brazil, but its constituents are not always the same as those found in its probable botanical source *Dalbergia ecastophyllum*, according to López et al. (2013). The constituents found in this vegetable by Silva et al. (2007), in the same Brazilian state, differ from those detected in this study as well as from other supposed sources *Schinus terebinthifolius* (Kassem et al., 2004) and *Rhizophora mangle* (Kandil et al., 2004). The compounds detected in our Red PEE possibly come from sources not yet known.

Substantial differences were found in different groups of Brown PEE. The of group III had a greater number of constituents and showed none in common with group IV, which had fewer compounds. This may be due to the biodiversity found in Brazil. On the other hand, groups V and VI PEE, produced with propolis from Portugal, showed three similar components (pinobanksin, chrysin and acacetin). Interestingly, no work was found mentioning these three compounds in the botanical source indicated here by beekeepers as the vegetable sources surrounding the apiaries. However, pinobanksin and chrysin are found in species of the genus *Pinus* (Nisula, 2018), a plant commonly found in Portugal (Ratola et al., 2010), which may be the main botanical source of these components.

Regarding the antimicrobial activity, all 10 PEE showed actions against the analysed staphylococci. Green and red PEE inhibited most bacteria at a concentration of 3.34 mg/mL. Similar results were also found for MRSA and MSSA by Veiga et al. (2017) and Bueno-Silva et al. (2017) for the same types and sources of PEE, respectively.

Among the Brown coloured PEE, Brown7 (PT) inhibited the isolates in the lowest concentrations (0.026 mg/mL), whereas PEE Brown4 (BR) was not inhibitory or only in much higher concentrations. Brown7 propolis was harvested in the South of Portugal and the PEE contains the flavonoids pinobanksin, chrysin, acacetin, apigenin, pinocembrin and kaempferol-dimethyl-ether in its composition. According to Falcão et al. (2013), the propolis from this region are rich in kaempferol derivatives similar to *Cistus ladanifer* exudates, a shrub commonly found in the Mediterranean area. Velikova et al. (2000) also found pinobanksin, chrysin and pinocembrin in propolis samples from Mediterranean region and these showed better action on bacteria than on fungi. Kasote et al. (2015) referred pinobanksin, detected in South African propolis, active against Gram-positive bacteria.

Pinobanksin derivatives are components presents in propolis that keeps the hive free from intruders (Alotaibi et al., 2019). This bioflavonoid was also detected in group V (PT) and II (BR) PEE, which showed antimicrobial activities similar to Brown7. Castaldo and Capasso (2002) believe that pinobanksin is one of the main propolis compounds with antimicrobial properties, in addition to pinocembrin and galangin. Boisard et al. (2015), on the other hand, observed antimicrobial effect against MRSA and MSSA due to the synergism of some French propolis compounds, including pinobanksin.

Concerning antibiofilm activity, Brown2, from Brazil, showed to inhibit biofilm formation with the lowest PEE concentration. Regarding Brown4 PEE, chapter4 and chapter6 denote slight discrepancies in results because chapter6 shows percentage averages and chapter4 shows results of statistical analyses.

Brown4 PEE did not show any inhibitory effect on the biofilm formation, but presented compounds similar to Brown3, mostly triterpenes derivates. Silva et al., (2019) refers that triterpenes and some derivatives show antibiofilm activity against *S. aureus* and *S. epidermidis*, stating that the ideal antibiofilm compound must lack antimicrobial activity. In the present study, the presence of triterpenes negatively influences the antimicrobial activity, however Catteau et al. (2018) summarizes the activity of some triterpenoid derivatives as anti-staphylococcal and as modifiers of resistance agents when combined with antibiotics.

Brown7 (PT) inhibited biofilm formation even though significantly different from the PEE that showed the best action. This PEE components act more in growth inhibition of the of the bacteria than in the antibiofilm action, however it showed the two activities. Meto et al. (2020), also found the antimicrobial and antibiofilm actions, only for Gram-negative bacteria, of a sample of propolis from Albania containing several components similar to those found in Brown7, such as pinocembrin, apigenin, chrysin and pinobanksin.

Regarding the PEE disruption of the formed biofilm, Brown2 (BR) and Brown7 (PT) showed the best results, although not owning common components.

In our study, all flavonoids, detected in any PEE, positively influenced bactericidal activity, however some (apigenin, pinocembrin and kaempferol-dimethyl-ether) did not influence the inhibition of biofilm formation, while others (pinobanksin, chrysin, acacetin) contributed to decreased biofilm inhibition. Apigenin, pinocembrin and kaempferol-dimethyl-ether, on the other hand, increased the disruption of the formed biofilm and pinobanksin, chrysin, acacetin did not influence either actions. Thus, suggesting that the antibiofilm action is given to the synergy of several compounds.

We confirm that each PEE can have different components and that it is probably their synergy that causes the two activities antimicrobial and antibiofilm, as generally each component is not able to achieve both activities.

With this work we demonstrated the antimicrobial and antibiofilm activity of PEE against *Staphylococcus* spp., showing a promising contribution for the control of mastitis in goats and sheep. The use of PEE, in replace or combined with antimicrobials, may help to decrease the selection pressure for resistant and multi-resistant staphylococci while fighting small ruminant mastitis.

7.2 – Perspectives for future studies

Finding antimicrobials to control mastitis is a challenge difficult to overcome. Bacteria are able to acquire antimicrobial resistance genes at every opportunity. Natural alternative products with antimicrobial and antibiofilm activity such as propolis may be an interesting and promising option to consider.

The present study has provided useful insights on the potential of propolis against mastitis pathogens. The individual antimicrobial and antibiofilm activity against staphylococci must be evaluated for the different propolis components. Furthermore, more studies to determine the concentration of individual PEE compound are needed to better correlate these with the biological activities of propolis. Moreover, propolis extracts were found to be efficient bactericides and contribute to biofilm inhibition or destruction that may be helpful in the control of mastitis.

The use of propolis alone or in combination with antimicrobials may be an important alternative for the control of small ruminant mastitis, with remarkable advantages for public health and contributing to the reduction of antimicrobial residues in the environment. These results should however be complemented with *in vivo* studies.

References

1. Alotaibi, A., Ebiloma, G.U., Williams, R., Alenezi, S., Donachie, A.M., Guillaume, S., Igoli, J.O., Fearnley, J., Koning, H.P. and Watson, D.G. (2019). European propolis is highly active against trypanosomatids including *Crithidia fasciculata*. *Sci. Rep*. **9**: 1–10. doi:10.1038/s41598-019-47840-y
2. Aragão, B.B., Trajano, S.C., Silva, J.G., Silva, B.P., Oliveira, R.P., Pinheiro Junior, J.W., Peixoto, R.M., Mota, R.A. (2019). Short communication: High frequency of β-lactam-resistant *Staphylococcus aureus* in artisanal coalho cheese made from goat milk produced in northeastern Brazil. *Dairy Sci*. **102**: 6923–6927.doi: 10.3168/jds.2018-16162
3. Becker, K., van Alen, S., Idelevich, E.A., Schleimer, N., Seggewiß, J., Mellmann, A., Kaspar, U., Peters, G. (2018). Plasmid-encoded transferable mecb-mediated methicillin resistance in *Staphylococcus aureus*. *Emerg. Infect. Dis.* **24**: 242–248. doi:10.3201/eid2402.171074
4. Berends, E.T.M., Horswill, A.R., Haste, N.M., Monestier, M., Nizet, V. and Von Köckritz-Blickwede, M. (2010). Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J. Innate Immun*. **2**: 576–586. doi:10.1159/000319909
5. Bergonier, D. and Berthelot, X. (2003). New advances in epizootiology and control of ewe mastitis. *Livest. Prod. Sci*. **79**: 1–16. doi:10.1016/S0301-6226(02)00145-8
6. Boisard, S., Le Ray, A.M., Landreau, A., Kempf, M., Cassisa, V., Flurin, C. and Richomme, P. (2015). "Antifungal and Antibacterial Metabolites from a French Poplar Type Propolis", *Evidence-Based Complementary and Alternative Medicine*,. Article ID 319240, 10 pages. https://doi.org/10.1155/2015/319240
7. Bueno-Silva, B., Marsola, A., Ikegaki, M., Alencar, S.M. and Rosalen, P.L. (2017). The effect of seasons on Brazilian red propolis and its botanical source: chemical composition and antibacterial activity. *Nat. Prod. Res*. **31**: 1318–1324. doi:10.1080/14786419.2016.1239088
8. Castaldo, S. and Capasso, F. (2002). Propolis, an old remedy used in modern medicine. *Fitoterapia* **73**: 1–6. doi:10.1016/S0367-326X(02)00185-5
9. Catteau, L., Zhu, L., Bambeke, F.V. and Quetin-Leclercq, J. (2018). Natural and hemi-synthetic pentacyclic triterpenes as antimicrobials and resistance modifying agents against *Staphylococcus aureus*: a review. *Phytochem. Rev*. **17**: 1129–1163. doi:10.1007/s11101-018-9564-2
10. Chen, Y.W., Ye, S.R., Ting, C., Yu, Y.H. (2018). Antibacterial activity of propolis from Taiwanese green propolis. *J. Food Drug Anal*. **26**: 761–768. doi:10.1016/j.jfda.2017.10.002
11. CLSI, 2016. M100S, 26th ed. Performance Standards for Antimicrobial Susceptibility Testing, CLSI document M100S.
12. Ebrahimi, A., Soleimani, F., Moatamedi, A., Shams, N. and Lotfalian, S. (2014). Study on some characteristics of Staphylococci isolated from sheep sub clinical mastitis milk in Shahrekord. *Iran. Biol. J. Microorg*. **2**: 57–62.
13. El Feghaly, R.E., Stamm, J.E., Fritz, S.A. and Burnham, C.A.D. (2012). Presence of the *bla*Z beta-lactamase gene in isolates of *Staphylococcus aureus* that appear penicillin susceptible by conventional phenotypic methods. *Diagn. Microbiol. Infect. Dis*. **74**: 388–393. doi:10.1016/j.diagmicrobio.2012.07.013
14. El-Razik, K.A.A., Arafa, A.A., Hedia, R.H., Ibrahim, E.S. (2017). Tetracycline resistance phenotypes and genotypes of coagulase-negative staphylococcal isolates from bubaline mastitis in Egypt. Vet. World. **10**: 702–710. doi:10.14202/vetworld.2017.702-710
15. Falcão, S.I., Tomás, A., Vale, N., Gomes, P., Freire, C. and Vilas-Boas, M. (2013). Phenolic quantification and botanical origin of Portuguese propolis. *Ind. Crops Prod*. **49**: 805–812. doi:10.1016/j.indcrop.2013.07.021
16. Felipe, V., Morgante, C.A., Somale, P.S., Varroni, F., Zingaretti, M.L., Bachetti, R.A., Correa, S.G. and Porporatto, C. (2017). Evaluation of the biofilm forming ability and its associated genes in *Staphylococcus* species isolates from bovine mastitis in Argentinean dairy farms. *Microb. Pathog*. **104**: 278–286. doi:10.1016/j.micpath.2017.01.047
17. Ferreira, A.M., Martins, K.B., Silva, V.R., Mondelli, A.L. and Cunha, M.L.R.S. (2017). Correlation of phenotypic tests with the presence of the *bla*Z gene for detection of beta-lactamase. *Brazilian J. Microbiol*. **48**: 159–166. doi:10.1016/j.bjm.2016.10.011
18. França, C.A., Peixoto, R.M, Cavalcante, M.B, Melo, N.F, Oliveira, C.J.B, Veschi, J.A., Mota, R.A. and Costa, M.M. (2012). Antimicrobial resistance of *Staphylococcus* spp. from small ruminant mastitis in Brazil.

Pesq. Vet. Bras. **32**: 747–753. doi:10.1590/S0100-736X2012000800012

19. Goering, R. V., Swartzendruber, E.A., Obradovich, A.E., Tickler, I.A. and Tenover, F.C. (2019). Emergence of Oxacillin Resistance in Stealth Methicillin Resistant *Staphylococcus aureus* Due to *mec*A Sequence Instability **63**:1–5. https://doi.org/10.1128/AAC.00558-19
20. Gosselin, V.B., Lovstad, J., Dufour, S., Adkins, P.R.F. and Middleton, J.R. (2018). Use of MALDI-TOF to characterize staphylococcal intramammary infections in dairy goats. *J. Dairy Sci*. **101**: 6262–6270. doi:10.3168/jds.2017-14224
21. Hachiya, J.O., Rossi, G.A.M., Ribeiro, L.F., Sato, R.A., Silva, H.O., Vidal, A.M.C., Amaral, L.A. (2017). Methicillin-resistant *Staphylococcus* spp. isolated from curd cheese "requeijão" and "especialidade láctea type requeijão" sold in Brazil. *Ciência Rural, Santa Maria*. **47**: 07. Doi: 10.1590/0103-8478cr20170008
22. Hirotaki, S., Sasaki, T., Kuwahara-Arai, K. and Hiramatsu, K. (2011). Rapid and accurate identification of human-associated staphylococci by use of multiplex PCR. *J. Clin. Microbiol*. **49**: 3627–3631. doi:10.1128/JCM.00488-11
23. Jenkins, S.N., Okello, E., Rossitto, P. V., Lehenbauer, T.W., Champagne, J., Penedo, M.C.T., Arruda, A.G., Godden, S., Rapnicki, P., Gorden, P.J., Timms, L.L. and Aly, S.S. (2019). Molecular epidemiology of coagulase-negative *Staphylococcus* species isolated at different lactation stages from dairy cattle in the United States. *PeerJ*. **7**:e6749. doi:10.7717/peerj.6749
24. Kandil, F.E., Grace, M.H., Seigler, D.S. and Cheeseman, J.M. (2004). Polyphenolics in *Rhizophora mangle* L. leaves and their changes during leaf development and senescence. *Trees - Struct. Funct.* **18**: 518–528. doi:10.1007/s00468-004-0337-8
25. Kasote, D., Ahmad, A., Chen, W., Combrinck, S. and Viljoen, A. (2015). HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis. *Phytochem*. *Lett*. **11**: 326–331. doi:10.1016/j.phytol.2014.08.017
26. Kassem, M.E.S., El-Desoky, S.K. and Sharaf, M. (2004). Biphenyl esters and biflavonoids from the fruits of *Schinus terebenthefolus*. *Chem. Nat. Compd*. **40**: 447–450. doi:10.1007/s10600-005-0008-z
27. Khoramian, B., Jabalameli, F,. Niasari-Naslaji, A., Taherikalani, M. and Emaneini, M. (2015). Comparison of virulence factors and biofilm formation among *Staphylococcus aureus* strains isolated from human and bovine infections. *Microbial Pathogenesis*. doi: 10.1016/j.micpath.2015.08.007
28. Kuile, B.H.T., Kraupner, N., Brul, S. (2016). The risk of low concentrations of antibiotics in agriculture for resistance in human health care. *FEMS Microbiol. Lett*. **363**: 1–7. doi:10.1093/femsle/fnw210
29. Kumari, S., Kumar, S., Prasad, A., Sahay, S., Kumar, R., Minj, N. and Ahmad, T. (2020). A study on antibiotic sensitivity test of methicillin resistant and non methicillin resistant *Staphylococcus aureus* from mastitic milk. *Journal of Entomology and Zoology Studies*. **8**: 77–79.
30. Lee, J.H. (2003). Methicillin (Oxacillin)-Resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl. environ. microbiol*. **69**: 6489–6494. doi: 10.1128/AEM.69.11.6489–6494.2003
31. Leeuwen, W.V., Roorda, L., Hendriks, W., Francois, P. and Schrenzel, J. (2008). A nuc-deficient meticillin-resistant *Staphylococcus aureus* strain. *FEMS Immunol. Med. Microbiol*. **54**: 157. doi:10.1111/j.1574-695X.2008.00478.x
32. Liu, H., Li, S., Meng, L., Dong, L., Zhao, S., Lan, X., Wang, J. and Zheng, N. (2017). Prevalence, antimicrobial susceptibility, and molecular characterization of *Staphylococcus aureus* isolated from dairy herds in northern China. *J. Dairy Sci*. **100**: 8796–8803. doi:10.3168/jds.2017-13370
33. López, B.G.C., Schmidt, E.M., Eberlin, M.N. and Sawaya, A.C.H.F. (2013). Phytochemical markers of different types of red propolis. *Food Chem*. **146**: 174–180. doi:10.1016/j.foodchem.2013.09.063
34. Machado, J.L., Assunção, A.K.M., Silva, M.C.P., Reis, A.S., Costa, G.C., Arruda, D.D.S., Rocha, B.A., Vaz, M.M.D.O.L.L., Paes, A.M.D.A., Guerra, R.N.M., Berretta, A.A., Nascimento, F.R.F. (2012). Brazilian green propolis: Anti-inflammatory property by an immunomodulatory activity. Evidence-based Complement. *Altern. Med*. doi:10.1155/2012/157652
35. Mahmoudi, H., Arabestani, M.R., Mousavi, S.F. and Alikhani, M.Y. (2017). Molecular analysis of the coagulase gene in clinical and nasal carrier isolates of methicillin-resistant *Staphylococcus aureus* by restriction fragment length polymorphism. *J. Glob. Antimicrob. Resist*. **8**: 41–45. doi:10.1016/j.jgar.2016.10.007
36. Mama, O.M., Gómez-Sanz, E., Ruiz-Ripa, L., Gómez, P. and Torres, C. (2019). Diversity of staphylococcal species in food producing animals in Spain, with detection of PVL-positive MRSA ST8 (USA300). *Vet. Microbiol*. **233**: 5–10. doi:10.1016/j.vetmic.2019.04.013
37. Mann, E.E., Rice, K.C., Boles, B.R., Endres, J.L., Ranjit, D., Chandramohan, L., Tsang, L.H., Smeltzer, M.S.,

Horswill, A.R. and Bayles, K.W. (2009). Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. *PLoS One.* **4**. doi:10.1371/journal.pone.0005822

38. Martins, K.B., Faccioli, P.Y., Bonesso, M.F., Fernandes, S., Oliveira, A.A., Dantas, A., Zafalon, L.F. and Cunha, M.L.R.S. (2017). Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. *J. Dairy Sci*. **100**: 2184–2195. doi:10.3168/jds.2016-11583
39. Melo, P.D.C., Ferreira, L.M. and Nader-filho, A. (2012). Phenotypic and molecular analysis of biofilm production by *Staphylococcus aureus* strains isolated of bovine mastitis. *Biosci. J., Uberlândia* **28**: 94–99.
40. Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, H., Lopez, J.A., Foster, T.J., Penadés J.R. and Lasa, I. (2009). Protein A-Mediated Multicellular Behavior in *Staphylococcus aureus*. *Journal of Bacteriology.* **191**: 832–843. doi:10.1128/JB.01222-08
41. Meto, A., Colombari, B., Meto, A., Boaretto, G., Pinetti, D., Marchetti, L., Benvenuti, S., Pellati, F. and Blasi, E. (2020). Propolis affects pseudomonas aeruginosa growth, biofilm formation, eDNA release and phenazine production: Potential involvement of polyphenols. *Microorganisms.* **8**:243. doi:10.3390/microorganisms8020243
42. Nisula, L. (2018). *Wood extractives in conifers: a study of stemwood and knots of industrially important species*. Åbo: Åbo Akademi University Press. ISBN 978-951-765-891-1.
43. Obaidat, M.M., Salman, A.E. and Roess, A.A. (2017). High prevalence and antimicrobial resistance of *mec*A *Staphylococcus aureus* in dairy cattle, sheep, and goat bulk tank milk in Jordan. *Trop. Anim. Health Prod*. **50**: 405–412. doi:10.1007/s11250-017-1449-7
44. Olson, M.E., Nygaard, T.K., Ackermann, L., Watkins, R.L., Zurek, O.W., Pallister, K.B., Griffith, S., Kiedrowski, M.R., Flack, C.E., Kavanaugh, J.S., Kreiswirth, B.N., Horswill, A.R. and Voyich, J.M. (2013). *Staphylococcus aureus* nuclease is an SaeRS-dependent virulence factor. *Infect.Immun*. **81**: 1316–1324. doi:10.1128/IAI.01242-12
45. Omoshaba, E.O., Ojo, O.E., Oyekunle, M.A., Sonibare, A.O., Adebayo, A.O. (2020). Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from raw milk and nasal swabs of small ruminants in Abeokuta, Nigeria. Trop. Anim. Health Prod. **52**: 2599–2608. doi:10.1007/s11250-020-02301-x
46. Park, Y.K., Alencar, S.M. and Aguiar, C.L. (2002). Botanical origin and chemical composition of Brazilian propolis. *J. Agric. Food Chem*. **50**: 2502–2506. doi:10.1021/jf011432b
47. Paterson, G.K., Harrison, E.M., Holmes, M.A. (2014). The emergence of *mec*C methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. **22**: 42–47. doi:10.1016/j.tim.2013.11.003
48. Peixoto, R.M., França, C.A., Souza Júnior, A.F., Veschi, J.L.A. Costa, M.M. (2010). Etiologia e perfil de sensibilidade antimicrobiana dos isolados bacterianos da mastite em pequenos ruminantes e concordância de técnicas empregadas no diagnóstico. *Pesqui. Vet. Bras*. **30**: 735–740. doi:10.1590/S0100-736X2010000900005
49. Persson, Y., Nyman, A.K., Söderquist, L., Tomic, N. and Waller, K.P. (2017). Intramammary infections and somatic cell counts in meat and pelt producing ewes with clinically healthy udders. *Small Rumin. Res*. **156**: 66–72. doi:10.1016/j.smallrumres.2017.09.012
50. Pitkälä, A., Salmikivi, L., Bredbacka, P., Myllyniemi, A.L. and Koskinen, M.T. (2007). Comparison of tests for detection of β-lactamase-producing staphylococci. *J. Clin. Microbiol*. **45**: 2031–2033. Doi:10.1128/JCM.00621-07
51. Pu, W.X., Su, Y., Li, J.X., Li, C.H., Yang, Z.Q., Deng, H.P. and Ni, C.X. (2014). High incidence of oxacillin-susceptible *mec*A-positive *Staphylococcus aureus* (OS-MRSA) associated with bovine mastitis in China. *PloS One* **9**. Doi:10.1371/journal.pone.0088134
52. Ratola, N., Amigo, J.M. and Alves, A. (2010). Levels and sources of PAHs in selected sites from Portugal: Biomonitoring with *pinus pinea* and *pinus pinaster* needles. *Arch. Environ. Contam. Toxicol*. **58**: 631–647. Doi:10.1007/s00244-009-9462-0
53. Salaberry, S.R.S., Saidenberg, A.B.S., Zuniga, E., Melville, P.A., Santos, F.G.B., Guimarães, E.C., Gregori, F. and Benites, N.R. (2015). Virulence factors genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis. *Microb. Pathog*. **85**: 35–39. Doi:10.1016/j.micpath.2015.05.007
54. Salina, A., Pereira, V.B.R. and Menozzi, B.D. (2020). Detection of *ica*A, *ica*D, and *bap* genes and biofilm production in *Staphylococcus aureus* and non-*aureus* staphylococci isolated from subclinical and clinical bovine mastitis Arq. Bras. Med. Vet. Zootec. **72**: 1034–1038.
55. Santos, A.D.S., Lima, D.C.V., Abad, A.C.A., Oliveira, P.R.F., Silva, J.G., Moura, G.S., Silva, A.T.F., Amorim, V.D.S., Costa, M.M. and Mota, R.A. (2020). Antimicrobial resistance profile of non-*aureus* Staphylococci isolates from buffalo, goat and sheep mastitis in the Northeast region of Brazil. *J. Dairy Res*. **87**: 290–294. Doi:10.1017/S0022029920000771

56. Shah, D.A., Wasim, S. and Abdullah, F.E. (2017). Comparison of Oxacillin and Cefoxitin for the detection of *mec*A gene to determine Methicillin resistance in coagulase negative staphylococci (CoNs). *J. Coll. Physicians* Surg. Pakistan **27**: 520–522. PMID: 28903850.
57. Silva, B.B., Rosalen, P.L., Cury, J.A., Ikegaki, M., Souza, V.C., Esteves, A. and Alencar, S.M. (2007). Chemical composition and botanical origin of red propolis, a new type of Brazilian propolis. Evidence-based Complement. *Altern. Med*. **5**, 313–316. doi:10.1093/ecam/nem059
58. Silva, G.N.S., Primon-Barros, M., Macedo, A.J. and Gnoatto, S.C.B. (2019). Triterpene derivatives as relevant scaffold for new antibiofilm drugs. *Biomolecules* **9**: 1–11. doi:10.3390/biom9020058
59. Silva, W.P., Silva, J.A., Macedo, M.R.P., Araújo, M.R., Mata, M.M., Gandra, E.A. (2003). Identification of *Staphylococcus aureus, S. intermedius* and *S. hyicus* by PCR amplification of *coa* and *nuc* genes. *Brazilian J. Microbiol*. **34**: 125–127. doi:10.1590/S1517-83822003000500043
60. Soltan Dallal, M.M., Khoramizadeh, M.R., Amiri, S.A., Saboor Yaraghi, A.A. and Fard, R.M.N. (2016). Coagulase gene polymorphism of *Staphylococcus aureus* isolates: A study on dairy food products and other foods in Tehran, Iran. *Food Sci. Hum. Wellness* **5**: 186–190. doi:10.1016/j.fshw.2016.09.004
61. Stiefel, P., Rosenberg, U., Schneider, J., Mauerhofer, S., Maniura-Weber, K. and Ren, Q. (2016). Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. *Appl. Microbiol. Biotechnol*. **100**: 4135–4145. doi:10.1007/s00253-016-7396-9
62. Szliszka, E., Zydowicz, G., Mizgala, E., Krol, W. (2012). Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) sensitizes LNCaP prostate cancer cells to TRAIL-induced apoptosis 818–828. doi:10.3892/ijo.2012.1527
63. Szweda, P., Schielmann, M., Milewski, S., Frankowska, A. and Jakubczak, A. (2012). Biofilm production and presence of ica and bap genes in *Staphylococcus aureus* strains isolated from cows with mastitis in the Eastern Poland. *Polish J. Microbiol*. **61**: 65–69. doi:10.33073/pjm-2012-009
64. Tolêdo, D.P., Ferreira, R.M.V.C., Jacovine, L.A.G. (2011). Brazilian agroforestry systems. In "Short rotation forestry and Agroforestry: an exchange of experience between CDM countries and Europe". *Benwood*. 109-114
65. Torres G., Vargas, K., Sánchez-Jiménez, M., Reyes-Velez, J. and Olivera-Angel, M. (2019). Genotypic and phenotypic characterization of biofilm production by *Staphylococcus aureus* strains isolated from bovine intramammary infections in Colombian dairy farms. *Heliyon.* **5**. E02535. doi.org/10.1016/j.heliyon.2019.e02535
66. Vasileiou, N.G.C., Chatzopoulos, D.C., Gougoulis, D.A., Sarrou, S., Katsafadou, A.I., Spyrou, V., Mavrogianni, V.S., Petinaki, E. and Fthenakis, G.C. (2018). Slime-producing staphylococci as causal agents of subclinical mastitis in sheep. *Vet. Microbiol*. **224**: 93–99. doi:10.1016/j.vetmic.2018.08.022
67. Vasileiou, N.G.C., Sarrou, S., Papagiannitsis, C., Chatzopoulos, D.C., Malli, E., Mavrogianni, V.S., Petinaki, E., Fthenakis, G.C. (2019). Antimicrobial Agent Susceptibility and Typing of Staphylococcal Isolates from Subclinical Mastitis in Ewes. *Microb. Drug Resist*. **25**: 1099–1110. doi:10.1089/mdr.2019.0009
68. Veiga, R.S., Mendonça, S., Mendes, P.B., Paulino, N., Mimica, M.J., Lagareiro Netto, A.A., Lira, I.S., López, B.G.C., Negrão, V. and Marcucci, M.C. (2017). Artepillin C and phenolic compounds responsible for antimicrobial and antioxidant activity of green propolis and *Baccharis dracunculifolia* DC. *J. Appl. Microbiol*. **122**: 911–920. doi:10.1111/jam.13400
69. Velikova, M., Bankova, V., Sorkun, K., Houcine, S., Tsvetkova, I. and Kujumgiev, A. (2000). Propolis from the Mediterranean region: Chemical composition and antimicrobial activity. *Zeitschrift fur Naturforsch. - Sect. C J. Biosci*. **55**: 790–793. doi:10.1515/znc-2000-9-1019
70. Vimercati, C., Cremonesi, P., Castiglioni, B., Pisoni, G., Boettcher, P.J., Stella, A., Vicenzoni, G. and Moroni, P. (2006). Molecular typing of *Staphylococcus aureus* isolated from cows, goats and sheep with intramammary infections on the basis of gene polymorphisms and toxins genes. J. *Vet. Med. Ser. B Infect. Dis. Vet. Public Heal.* **53**: 423–428. doi:10.1111/j.1439-0450.2006.00980.x
71. Virdis, S., Scarano, C., Cossu, F., Spanu, V., Spanu, C. and Santis, E.P.L. (2010). Antibiotic resistance in *Staphylococcus aureus* and coagulase negative staphylococci isolated from goats with subclinical mastitis. *Vet. Med. Int*. doi:10.4061/2010/517060
72. Vitale, M., Galluzzo, P., Buffa, P.G., Carlino E., Spezia, O. and Alduina, R. (2019). Comparison of Antibiotic Resistance Profile and Biofilm Production of *Staphylococcus aureus* Isolates Derived from Human Specimens and Animal-Derived Samples. *Antibiotics*, **8**: 97. doi:10.3390/antibiotics8030097
73. Wuytack, A., Visscher, A., Piepers, S., Haesebrouck, F. andVliegher, S. (2020). Fecal non-*aureus* Staphylococci are a potential cause of bovine intramammary infection. *Vet. Res*. **51**: 1–13. doi:10.1186/s13567-020-00761-5
74. Xu, Z., Shi, L., Alam, M.J., Li, L., Yamasaki, S. (2008). Integron-bearing methicillin-resistant coagulase-negative staphylococci in South China, 2001-2004. *FEMS Microbiol. Lett*. **278**: 223–230.

doi:10.1111/j.1574-6968.2007.00994.x

75. Zafalon, L.F., Cunha, M.L.R.S., Brandão, H.M., Mosqueira, V.C.F., Santana, R.C.M., Júnior, W.B., Martins, K.B. and Pilon, L.E. (2018). Relationship between virulence factor genes in coagulase-negative *Staphylococcus* spp. and failure of antimicrobial treatment of subclinical mastitis in sheep. *Pesqui. Vet. Bras*. **38**: 579–585

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