Antibiofilm activity of propolis extracts

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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-five 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 *S. epidermidis*) 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 API-Staph (Biomérieux) or 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. aureus* ATCC 25923 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:

$$OD = (\sum OR1, OR2, OR3)/3 - (\sum NC1, NC2, NC3)/3)$$

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 TSBg) 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. Non-inoculated TSBg was used as sterility control. After incubation for 24 h at 37°C, plates were washed and stained as for the biofilm production assay. 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 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 (**Fig. 1**).

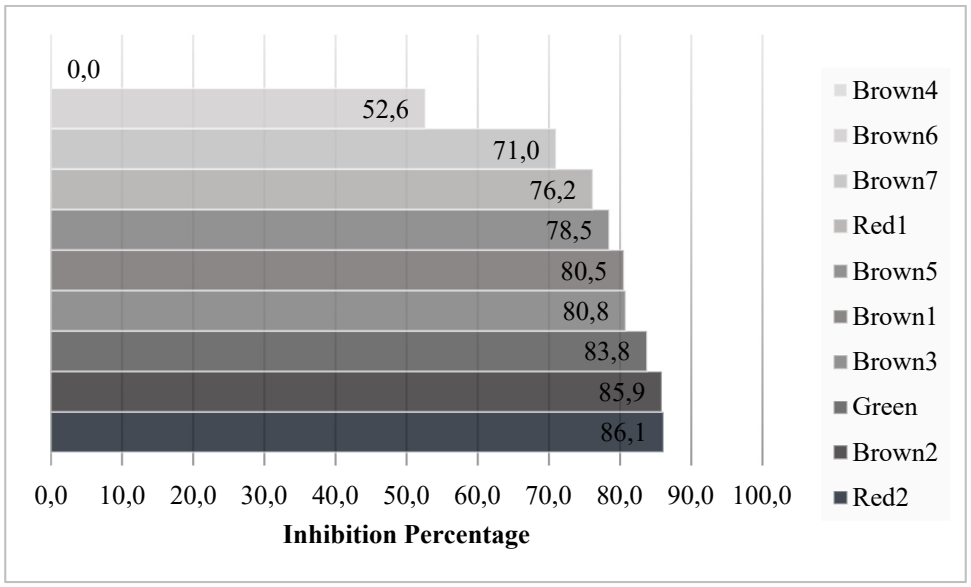

Fig. 1 Mean inhibition percentage for each propolis ethanol extract.

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 1**).

Table 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	77.9
% of Inhibition	88.8	73.6	100.0	0.0	85.4	65.1	56.9	76.4	68.3
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	83.0
% of Inhibition	85.7	59.3	80.3	0.0	56.8	62.0	85.5	54.0	60.5
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	83.2
% of Inhibition	85.8	92.1	81.0	80.3	91.0	95.1	98.5	71.7	86.9
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	83.4
% of Inhibition	82.6	97.1	98.9	0.0	72.9	96.3	68.2	62.8	72.4
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	80.2
% of Inhibition	88.6	86.4	79.9	0.0	85.9	79.0	91.6	62.1	71.7
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	96.4

% of Inhibition	78.5	91.5	99.5	82.9	83.3	69.9	83.8	77.8	83.4
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	55.9
% of Inhibition	84.2	48.0	90.7	0.0	64.5	89.8	0.0	50.5	53.5
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	55.2
% of Inhibition	54.4	85.2	81.1	0.0	47.2	0.0	40.0	40.6	43.6
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	68.7
% of Inhibition	78.4	34.5	61.2	0.0	53.9	63.5	75.9	67.2	54.3

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% (**Fig. 2**).

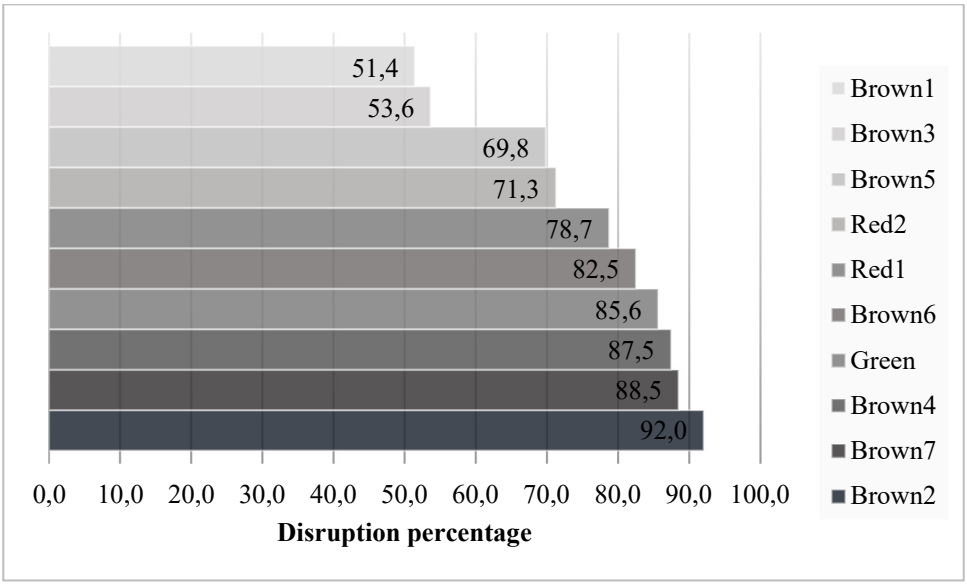

Fig. 2 Mean activity percentage on biofilm disruption for each propolis ethanol extract.

The percentage of biofilm disruption ranged from 0 to 100% (**Table 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.*

Table 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	91.7
% of Disruption	93.4	23.3	58.6	100.0	95.5	100.0	100.0	68.9	80.0
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	63.3
% of Disruption	70.7	54.9	98.0	0.0	97.1	79.3	100.0	96.3	74.5
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	93.1
% of Disruption	62.8	74.2	85.6	73.1	66.9	100.0	98.7	90.1	81.4
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	68.6
% of Disruption	32.0	86.2	83.6	0.0	65.9	87.2	70.3	36.8	57.8
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	66.3
% of Disruption	94.4	52.2	81.3	0.0	91.5	0.0	100.0	100.0	64.9
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	86.1
% of Disruption	40.9	81.4	75.9	29.1	71.0	62.4	84.3	45.6	61.3
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	39.6
% of Disruption	84.1	100.0	0.0	0.0	100.0	100.0	73.6	95.9	69.2
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	57.8
% of Disruption	52.7	43.2	97.7	0.0	98.2	0.0	98.9	64.0	56.8
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	60.9
% of Disruption	70.8	41.3	99.8	0.0	99.1	0.0	100.0	91.5	62.8
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	78.7
% of Disruption	88.3	69.2	100.0	76.8	92.5	0.0	100.0	87.4	76.8

Antibiofilm activity is summarised in **Fig. 3**.

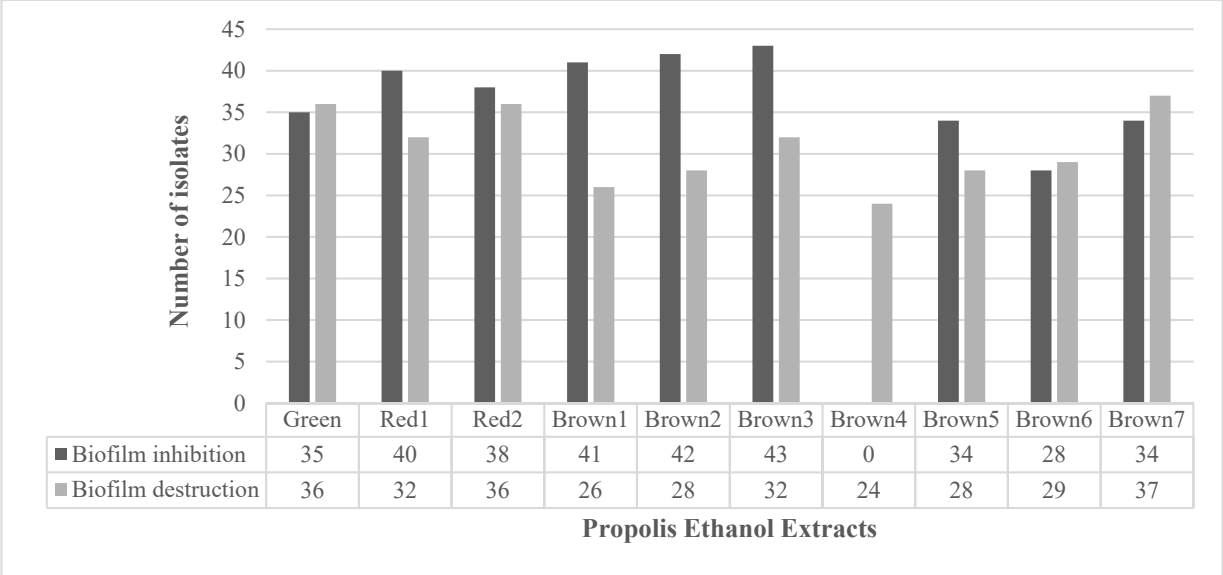

Fig. 3 PEE effect on biofilm formation and on established biofilms: number of isolates affected by each PEE.

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.

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 (QS) 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, Guimar??es 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.