Effect of an Italian propolis on the growth of *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* in milk and whey cheese

Francesca Pedonese, Giada Verani, Beatrice Torracca, Barbara Turchi, Antonio Felicioli, Roberta Nuvoloni

Department of Veterinary Sciences, University of Pisa, Italy

Abstract

Propolis antimicrobial activity has been limitedly studied in food, particularly in dairy products. We studied the antimicrobial activity of an alcoholic extract of an Italian propolis in sterile skim milk, pasteurized cow's milk, and cow's and goat's whey cheese (ricotta). Following the determination of the minimal inhibitory concentration on Gram+ and Gram- bacteria, the extract was employed at 2 and 5% (P2, P5), using controls with the same ethanol concentrations (E2, E5) and without any addition. In milk trials, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Pseudomonas fluorescens* were tested. P2 and P5 samples registered significant decreases of Gram+ bacteria in skim milk. The same was true for P5 in cows' milk, but only with *S. aureus* for P2. Ricotta was inoculated with *L. monocytogenes*, *S. aureus* and *B. cereus* and stored at 8.5°C. In cow's milk ricotta, *L. monocytogenes* counts in P5 were always lower than control during the storage time, significantly so from the 14th day. In goat's ricotta, *L. monocytogenes* counts in P5 were at least one logarithm lower than E5, whereas the extract didn't show a significant effect on *S. aureus* and *B. cereus*. The antimicrobial activity of propolis, particularly on *L. monocytogenes*, could be employed in ready-to-eat refrigerated dairy products.

Introduction

Among beehive products, propolis is a complex material, collected by honeybees from plant buds and exudates and enriched by beeswax and bee secretions (Zabaiou *et al.*, 2017). It has been used in folk medicine for centuries and its beneficial effects are well known (Sforcin and Bankova, 2011) and attributed to a variety of active compounds, including flavonoids (Huang *et al.*, 2014).

A promising area of propolis use involves its application as a preservative in different foods, especially fruit juices, fruits and vegetables, due to its antimicrobial and antioxidative properties (Bankova *et al.*, 2016). Though the antimicrobial effect of propolis has been extensively studied (Banskota *et al.*, 2001; De Vecchi and Drago, 2007), knowledge about its antimicrobial activity in food of animal origin is quite limited and mostly focused on meat and fish products, where beneficial antimicrobial effects (mainly decrease of mesophilic and psychrotrophic counts) are reported in beef patties, sausages, filleted and minced fish meat (Pobiega *et al.*, 2019). There are only few studies about milk and/or dairy products and they are focused on some specific applications of the propolis antimicrobial properties (shelf-life of cheese: Metwalli, 2011; *L. monocytogenes* in refrigerated milk: Thamnopoulos *et al.*, 2018). With the aim to increase knowledge about practical effects of propolis in milk and dairy products, we evaluated the antimicrobial activity of an Italian propolis against food microorganisms in milk at *optimum* growth temperature and investigated the antimicrobial effects on *L. monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* in a refrigerated ready-to-eat dairy product (ricotta), commonly consumed and potentially exposed to thermal abuse.

Materials and Methods

Propolis and ethanolic extract preparation

Propolis was collected in Val di Cecina (Tuscany, 50-450 m above sea level) in a single harvesting season. Pollen analysis of propolis was carried out following Ricciardelli D'Albore (1979). Raw propolis was stored at -20°C, finely ground, extracted with 70% ethanol (10 g in 100 ml) and repeatedly submitted to refrigerated centrifugation (9000 rpm, 15 min) and filtration phases, after being frozen at -20°C, to remove insoluble waxes. On the obtained ethanolic extract of propolis (EEP), flavonoids quantification and determination of dry residue content were performed according to Popova *et al.* (2004) and Baldini *et al.* (1996), respectively.

Bacterial cultures

Overall, 7 different microorganisms of relevance for food safety and quality were studied, 4 Gram+ (*L. monocytogenes* ATCC 7644, *S. aureus* ATCC 25923, *S. aureus* ATCC 35556, high biofilm-producer, *B. cereus* DSV12, a wild strain of food origin) and 3 Gram- (*Salmonella enterica* serovar Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922, *Pseudomonas fluorescens* ATCC 13525). For milk and whey cheese trials, those showing the highest *in vitro* susceptibility to EEP in preliminary tests were chosen.

Antimicrobial disk susceptibility test

The test was made according to CLSI (2013), using Tryptone Soy Agar (TSA, Oxoid, Basingstoke, UK), a bacterial *inoculum* of 0.5 McFarland turbidity and sterile filter paper disks with 10 µL each of EEP, 70% ethanol and dimethyl sulfoxide (DMSO); the inhibition zone diameters were determined after 24 hours of incubation at 25°C (pseudomonads) and 37°C (other bacteria).

Minimal inhibitory concentration and minimal bactericidal concentration determination assay

MIC values (the lowest concentration that inhibits visible microbial growth) were determined for EEP and 70% ethanol following Wiegand *et al.* (2008) with minor modifications. The assay was performed in microtiter plates using 10 µL of bacterial *inoculum* and 190 µL of each dilution. EEP was diluted in DMSO (1:3) and two-fold dilutions from 1/8 to 1/16,384 were prepared in Tryptone Soy Broth (Oxoid). For

Correspondence: Francesca Pedonese, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124 Pisa, Italy.
Tel.: +39.050-2216707 - Fax: +39-050-2210654
E-mail: francesca.pedonese@unipi.it.

Key words: Propolis, *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus,* Dairy.

Conflict of interest: The authors declare no potential conflict of interest.

Funding: this research was funded by Pisa University grants (Fondi di Ateneo).

Received for publication: 17 January 2019.
Revision received: 6 August 2019.
Accepted for publication: 28 August 2019.

Italian Journal of Food Safety 2019; 8:8036
doi:10.4081/ijfs.2019.8036

the bacterial *inoculum* an overnight broth culture of each microorganism, spectrophotometrically adjusted at about 1.5×10^8 cfu/mL, was used. The microplates were incubated at 25°C (pseudomonads) and 37°C (other bacteria) for 24 hours. For MBC assay, a loopful from MIC and higher dilutions wells was inoculated onto TSA, with the same incubation, and the lowest concentration with no growth was considered as the MBC value. MIC/MBC assays were made in triplicate.

Quantification of EEP and ethanol effect on bacterial growth in milk

Bacterial growth was tested in sterilized skim milk (Skim Milk Powder, Oxoid) and in cow's milk, pasteurized at 63°C for 30 minutes. Total bacterial counts were determined in Plate Count Agar (Oxoid) at 30°C for 72 hours before and after milk pasteurization. An overnight culture of each microorganism (the Gram+ ones and *P. fluorescens*) was inoculated at 1% in each type of milk with 2% EEP (P2), 5% EEP (P5), 2% ethanol (E2), 5% ethanol (E5) and in milk alone (control). After 24 hours at 37°C (25°C for *P. fluorescens*), bacterial counts were determined on the following media (Oxoid): Listeria Selective Agar Base with Oxford Supplement for *L. monocytogenes* (Heo *et al.*, 2014), Baird Parker Agar with Egg Yolk-Tellurite for coagulase-positive staphylococci (UNI, 2004), Mannitol Egg Yolk Polymixin Agar for *B. cereus* (FDA BAM, 2017), Pseudomonas Agar Base with CFC Supplement for *P. fluorescens* (Chiesa *et al.*, 2014).

Quantification of EEP and ethanol effect on bacterial growth in whey cheese

Bacterial growth was tested in an industrial cow's whey cheese and in an artisanal goat's one, purchased at the beginning of shelf-life, at retail level and directly from the producer, respectively, and immediately inoculated. P2, P5, E2, E5 and control samples were prepared and inoculated with a pool *inoculum* of 4 chosen microorganisms (the Gram+ ones). Before mixing, the concentration of each microorganism was spectrophotometrically adjusted in saline solution at about 1.5×10^8 cfu/mL. The final *inoculum* was used at 1% in ricotta. Control samples without *inocula* were tested to exclude the presence of *L. monocytogenes*, *S. aureus* and *B. cereus*. Ricotta samples were stored at 8.5±0.5°C for 28 and 14 days, for cow's and goat's product, respec-

Table 1. Minimal inhibitory concentration (MIC) and Minimal Bactericidal Concentration (MBC) values (mg/mL) of propolis ethanolic extract and ethanol against the tested microorganisms.

	MIC		MBC	
	EEP	Ethanol	EEP	Ethanol
L.m. 7644	1.78	13.91	7.11	55.63
S.a. 25923	0.89	13.91	3.55	55.63
S.a. 35556	1.78	13.91	3.55	55.63
B.c. DSV12	0.89	13.91	3.55	55.63
S.T. 14028	3.55	13.91	28.44	55.63
E.c. 25922	3.55	13.91	7.11	55.63
P.f. 13525	1.78	13.91	28.44	55.63

EEP: ethanolic extract of propolis. *L.m.: Listeria monocytogenes*; *S.a.: Staphylococcus aureus*; *B.c.: Bacillus cereus*; S.T.: *Salmonella enterica* serovar Typhimurium; *E.c.: Escherichia coli; P.f.: Pseudomonas fluorescens*. Results are the mode of three independent trials.

Table 2. Growth of the tested microorganisms in milk with different percentages of propolis ethanolic extract and ethanol.

	P2	P5	E2	E5	C
Skim milk					
L.m. 7644	6.40±0.48^{b} (-1.87)	3.92±0.14^{c} (-4.35)	8.04±0.19^{a} (-0.23)	7.50±0.34a^{a} (-0.77)	8.27±0.07^{a}
S.a. 25923	5.98±0.50^{b} (-1.98)	5.31±0.26^{b} (-2.65)	7.84±0.16^{a} (-0.12)	7.46±0.07^{a} (-0.50)	7.96±0.08^{a}
S.a. 35556	5.74±0.33^{b} (-2.63)	5.33±0.42^{b} (-3.04)	8.19±0.05^{a} (-0.18)	8.09±0.13^{a} (-0.28)	8.37±0.08^{a}
B.c. DSV12	3.31±0.35^{b} (-2.72)	2.94±0.32^{b} (-3.09)	6.57±0.92^{a} (+0.54)	6.86±0.24^{a} (+0.83)	6.03±0.44^{a}
P.f. 13525	6.78±0.11^{c} (-1.09)	6.55±0.07^{c} (-1.32)	7.45±0.41^{ab} (-0.42)	6.95±0.20^{bc} (-0.92)	7.87±0.18^{a}
Pasteurized cow's milk					
L.m. 7644	5.59±0.31^{a} (-0.47)	4.66±0.22^{b} (-1.40)	5.79±0.45^{a} (-0.27)	5.82±0.14^{a} (-0.24)	6.06±0.31^{a}
S.a. 25923	6.30±0.16^{c} (-1.12)	5.09±0.13^{d} (-2.33)	7.10±0.31^{ab} (-0.32)	6.62±0.27^{bc} (-0.80)	7.42±0.21^{a}
S.a. 35556	7.04±0.35^{a} (-0.67)	4.49±0.09^{b} (-3.22)	7.44±0.43^{a} (-0.27)	7.13±0.36^{a} (-0.58)	7.71±0.32^{a}
B.c. DSV12	6.21±0.27a^{a} (0.24)	3.12±0.56^{b} (-2.85)	5.75±0.28a^{a} (-0.22)	6.25±0.21a^{a} (0.28)	5.97±0.47^{a}
P.f. 13525	7.32±0.20 (0.17)	6.98±0.24 (-0.17)	7.18±0.36 (0.03)	6.89±0.42 (-0.26)	7.15±0.16

P2: milk with 2% propolis ethanolic extract; P5: milk with 5% propolis ethanolic extract; E2: milk with 2% ethanol (70%); E5: milk with 5% ethanol (70%); C: control (milk). *L.m.: Listeria monocytogenes; S.a.: Staphylococcus aureus; B.c.: Bacillus cereus; P.f.: Pseudomonas fluorescens*. Results are mean values of three independent trials ± standard deviation. In brackets: difference in bacterial counts in comparison with the corresponding control. Values are expressed in log cfu/mL. ^{abc}Different letters in the same row denote significant differences ($P<0.05$).

tively, based on their shelf-life and analyzed immediately after inoculation (t0) and weekly (t7, t14, t21, t28) to quantify the different microorganisms, as described for milk tests.

Statistical analysis

For each microorganism and substrate (milk and whey cheeses) one-way ANOVA and Tukey HSD test for *post-hoc* comparisons were performed (R software v. 3.5.0, R Foundation for Statistical Computing, Vienna, Austria), to evaluate the differences in bacterial counts at each time, considering the sample type (P2, P5, E2, E5, C) as factor. Differences were considered statistically significant with P value <0.05.

Table 3. Growth of the tested microorganisms in cow's and goat's whey cheese with different percentages of propolis ethanolic extract and ethanol during storage.

		P2	P5	E2	E5	C
Cow's whey cheese						
L.m. 7644	t0	5.48±0.43 (-0.11)	5.58±0.28 (-0.01)	5.59±0.34 (0.00)	5.50±0.32 (-0.09)	5.59±0.27
	t7	6.53±1.66 (-0.67)	5.38±0.36 (-1.82)	6.06±0.40 (-1.14)	5.39±0.92 (-1.81)	7.20±0.89
	t14	7.30±0.74^{a} (0.00)	5.02±0.24^{b} (-2.28)	7.32±0.02^{a} (0.02)	5.79±0.12^{ab} (-1.51)	7.30±0.74^{a}
	t21	7.12±0.91^{ab} (-1.01)	4.87±0.21^{b} (-3.26)	7.67±0.19^{a} (-0.46)	6.45±1.06^{ab} (-1.68)	8.13±0.10^{a}
	t28	7.71±0.08^{a} (-0.51)	4.53±0.48^{b} (-3.69)	7.99±0.17^{a} (-0.23)	6.86±0.96^{a} (-1.36)	8.22±0.18^{a}
S.a. 25923/35556	t0	5.41±0.08 (0.04)	5.36±0.04 (-0.01)	5.43±0.01 (0.06)	5.25±0.10 (-0.12)	5.37±0.10
	t7	4.75±0.20 (-0.10)	4.56±0.45 (-0.29)	4.97±0.38 (0.12)	4.88±0.21 (0.03)	4.85±0.16
	t14	4.38±0.51 (0.00)	3.81±1.11 (-0.57)	4.81±0.32 (0.43)	4.58±0.03 (0.20)	4.38±0.35
	t21	4.27±0.65 (-0.32)	3.52±1.10 (-1.07)	4.73±0.12 (0.14)	4.38±0.03 (-0.21)	4.59±0.15
	t28	4.29±0.91 (0.18)	3.34±0.80 (-0.77)	4.63±0.21 (0.52)	4.12±0.08 (0.01)	4.11±0.38
B.c. DSV12	t0	3.71±0.70 (-0.04)	3.68±0.68 (-0.07)	3.73±0.64 (-0.02)	3.61±0.73 (-0.14)	3.75±0.65
	t7	1.57±0.98 (-1.66)	1.33±0.58 (-1.90)	3.37±0.58 (0.14)	2.71±0.98 (-0.52)	3.23±0.46
	t14	1.35±0.49 (-0.95)	1.35±0.49 (-0.95)	2.44±1.05 (0.14)	2.00±0.43 (-0.30)	2.30±0.85
	t21	1.00±0.00 (-0.85)	1.00±0.00 (-0.85)	1.35 ± 0.49 (-0.50)	1.00±0.00 (-0.85)	1.85±0.21
	t28	1.00±0.00 (-0.57)	1.00±0.00 (-0.57)	1.23±0.40 (-0.33)	1.57±0.51 (0.00)	1.57±0.51
Goat's whey cheese						
L.m. 7644	t0	5.37±0.18 (-0.09)	5.40±0.21 (-0.06)	5.41±0.25 (-0.05)	5.45±0.22 (-0.01)	5.46±0.29
	t7	7.00±0.11^{a} (-0.10)	5.44±0.28^{b} (-1.66)	6.90±0.24^{a} (-0.21)	6.86±0.31^{a} (-0.24)	7.10±0.08^{a}
	t14	7.01±0.15^{ab} (-0.15)	5.70±0.72^{b} (-1.46)	7.06±0.14^{ab} (-0.10)	6.97±0.18^{ab} (-0.19)	7.16±0.14^{a}
S.a. 25923/35556	t0	4.98±0.28 (0.06)	5.00±0.28 (0.08)	5.07±0.39 (0.15)	4.96±0.30 (0.04)	4.92±0.23
	t7	5.10±0.19 (-0.08)	4.95±0.30 (-0.23)	5.25±0.31 (0.07)	5.11±0.10 (-0.07)	5.18±0.03
	t14	5.08±0.08 (0.05)	4.87±0.24 (-0.16)	5.16±0.23 (0.13)	5.18±0.07 (0.15)	5.03±0.02
B.c. DSV12	t0	3.86±0.25 (0.02)	3.91±0.24 (0.07)	3.83±0.32 (-0.01)	3.65±0.36 (-0.19)	3.84±0.26
	t7	3.04±0.80 (0.03)	2.27±0.38 (-0.74)	3.07±0.75 (0.06)	3.12±0.81 (0.11)	3.01±0.38
	t14	1.00±0.00 (-0.35)	1.00±0.00 (-0.35)	1.35±0.49 (0.00)	1.00±0.00 (-0.35)	1.35±0.49

P2: milk with 2% propolis ethanolic extract; P5: milk with 5% propolis ethanolic extract; E2: milk with 2% ethanol (70%); E5: milk with 5% ethanol (70%); C: control (milk). *L.m.: Listeria monocytogenes; S.a.: Staphylococcus aureus; B.c.: Bacillus cereus.* Results are mean values of two independent trials ± standard deviation. In brackets: difference in bacterial counts in comparison with the corresponding control. Values are expressed in log cfu/g. ^{abc}Different letters in the same row denote significant differences (P<0.05).

Results

Characterization of propolis and EEP

Pollen analysis of propolis revealed a wide variety of pollen types. They were identified, according to Louveaux *et al.* (1978), as belonging to *Castanea* (27% of total pollen), *Hedera* (10%), and *Coronilla*/*Hippocrepis*, *Olea* f., *Pinus* f., *Trifolium* gr., *Quercus* gr., all in percentages of 4.5-7%, with various *Compositae*, *Umbelliferae* and *Graminaceae* in lower quantities. A dry residue content of 8.44% and a flavonoids content of 2.3% (w/w) was found in EEP.

Antimicrobial activity of EEP

Gram+ bacteria showed EEP inhibition zone diameters of 12 mm (*L. monocytogenes*), 13 mm (*B. cereus*) and 19-20 mm (*S. aureus*), higher than those of Gram- (10-11 mm). Ethanol and DMSO did not inhibit any of the strains, except for a slight inhibition recorded for ethanol against *P. fluorescens* (7 mm) and *E. coli* (8 mm). As for EEP MIC (Table 1), values of 0.89-1.78 mg/mL were obtained for Gram+ bacteria, and in the range 1.78 (*P. fluorescens*)-3.55 mg/mL for Gram-; ethanol values were ever 13.91 mg/mL, showing that the predominant effect was exerted by propolis compounds. EEP MBC values for Gram+ were 3.55-7.11 mg/mL, and 7.11-28.44 mg/mL for Gram-, with ethanol values of 55.63 mg/mL.

Effect of EEP and ethanol on bacterial growth in milk

As shown in Table 2, P2 and P5 determined in skim milk significant differences in counts in comparison with control for all microorganisms. However, for *P. fluorescens*, a lower EEP effect was noted, together with a significant inhibiting effect of E5. As for the pasteurized cow's milk, total bacterial count was 3.80 log cfu/mL in raw milk and below 2.18 log cfu/mL after the pasteurization. Its results were similar to skim milk, although counts significantly decreased only in P5 for all microorganisms, with the exception of *P. fluorescens*, and in P2 just in the case of *S. aureus* 25923; apart from *P. fluorescens*, E5 effect was lower of, at least, 1.16 log cfu/mL in comparison with P5.

Effect of EEP and ethanol on bacterial growth in whey cheese

Results are shown in Table 3. In cow's product, in P5 *L. monocytogenes* counts were lower than in control at each time during storage, with differences progressively higher and statistically significant from t14. In comparison with E5, differences in counts, negligible at t7, increased in time, showing an inhibiting effect of propolis rather than of ethanol. As for *S. aureus*, the 2 strains were enumerated together, but the 2 types of colonies were different enough to confirm that the strains were balanced in their growth. EEP didn't obtain significant antibacterial effects; this was probably influenced by the fact that, unsurprisingly, staphylococci didn't grow at 8.5°C and even decreased over time, including in control. Anyway, P5 was the most active, with differences of 0.86 (t21) and 0.76 log cfu/g (t28) between P5 and E5. Overall, *B. cereus* showed lower counts than the other bacteria in milk and whey cheese controls. In cow's whey cheese, as for *S. aureus*, counts decreased already in control, with too low values to correctly evaluate the effect of EEP and ethanol. Anyway, at t7 P5 gave rise to 1.90 and 1.38 log cfu/g differences versus control and versus E5, respectively. In goat's product, as for *L. monocytogenes*, significant differences versus control were present at t7 and t14. Significant differences between P5 and E5 were obtained at t7, but not at t14, due to high standard deviations; differences were in both cases higher than 1 log cfu/g.

Discussion

The chemical composition of propolis strictly depends from the plant sources and is a result of geographical location, climate conditions and environmental factors (Bankova *et al.*, 2016). The pollen types found in our propolis were related to the type of vegetation of the geographical environment, with woodland and rural areas. Particularly, chestnut groves were situated within the production area and were responsible for the high content in *Castanea* pollen. Dry residue of EEP was analogous to that of Gutiérrez-Cortés and Suarez-Mahecha (2014), and in the range recorded by Cveck *et al.* (2007) and Barbeira *et al.* (2013). Flavonoids content was in the range determined by Marghitas *et al.* (2010) in ethanolic extracts of Romanian propolis. The same authors used EEP to determine the antimicrobial activity with a disk diffusion test on *L. monocytogenes*, *S. aureus*, *B. cereus, E. coli* and *P. aeruginosa* and obtained inhibition values comparable to ours. Considering EEP MIC values, various authors found results similar to ours: Miorin *et al.* (2003) obtained values of 0.36-3.65 mg/mL for *S. aureus* strains, slightly higher than those of Freitas Santana *et al.* (2012). More recently, Ristivojević *et al.* (2016) found MIC of 0.1-1.9 mg/mL for *L. monocytogenes* and 0.4-13.7 mg/mL for *S. aureus*. Finally, Mascheroni *et al.* (2014) using chitosan-propolis beads found MIC values of 0.8-1 mg/mL for *S. aureus*, *L. innocua* and *B. cereus*. Our study focused on milk and whey cheese, and, at the best of our knowledge, trials in milk at *optimum* bacterial temperature and in whey cheese during refrigerated storage were not performed before. Results showed some differences in bacterial behavior in the different growth substrates, but, noteworthily, in all tested matrices, EEP revealed a not negligible antimicrobial effect, higher than that determined by ethanol, particularly against *L. monocytogenes*.

Conclusions

The noteworthy antimicrobial activity of propolis, particularly on *L. monocytogenes*, together with its beneficial properties, could be advantageously exploited, especially in ready-to-eat dairy products to be stored in refrigerated conditions. The development of non-alcoholic formulations (Jansen-Alves *et al.,* 2018) could make it suitable for all consumers.

References

Baldini M, Fabietti F, Giammarioli S, Onori R, Orefice L, Stacchini A, 1996. Metodi di analisi utilizzati per il controllo chimico degli alimenti. Rapporti ISTISAN 96/34, Istituto Superiore di Sanità, Roma, Italy. ISSN 1123-3117.

Bankova V, Popova M, Trusheva B, 2016. New emerging fields of application of propolis. Maced J Chem Chem Eng 35:1-11.

Banskota AH, Tezuka Y, Kadota S, 2001. Recent progress in pharmacological research of propolis. Phytoter Res 15:561-71.

Barbeira PJS, Paganotti RSN, Ássimos AA, 2013. Development of a multivariate calibration model for the determination of dry extract content in Brazilian commercial bee propolis extracts through UV-Vis spectroscopy. Spectrochim Acta A Mol Biomol Spectrosc 114:441-8.

Chiesa F, Lomonaco F, Nucera D, Garoglio D, Dalmasso A, Civera T, 2014. Distribution of *Pseudomonas* species in a dairy plant affected by occasional blue discoloration. Ital J Food Saf 9:1722.

Clinical and Laboratory Standards Institute (CLSI), 2013. Performance standards for antimicrobial susceptibility testing;

twenty-third informational supplement. CLSI document M100-S23. Wayne, PA, USA.

Cveck J, Medić-Sarić M, Jasprica I, Zubčić S, Vitali D, Mornar A, Vedrina-Dragojević, Tomić S, 2007. Optimisation of an extraction procedure and chemical characterisation of Croatian propolis tinctures. Phytochem Anal 18:451-9.

De Vecchi E, Drago L, 2007. Propolis antimicrobial activity: what's new? Infez Med 15:7-15.

FDA BAM (U. S. Food and Drug Administration Bacteriological Analytical Manual). *Bacillus cereus*. Available from: https://www.fda.gov/food/laboratory-methods-food/bam-bacillus-cereus. Accessed July 28th 2019.

Freitas Santana H, Teixeira Barbosa AA, Ferreira SO, Cuquetto Mantovani H, 2012. Bactericidal activity of ethanolic extracts of propolis against *Staphylococcus aureus* isolated from mastitic cows. World J Microbiol Biotechnol 28:485-91.

Gutiérrez-Cortés C, Suarez Mahecha H, 2014. Antimicrobial activity of propolis and its effect on the physicochemical and sensorial characteristics in sausages. Vitae Columbia 21:90-6.

Heo EJ, Song BR, Park HJ, Kim YJ, Moon JS, Wee SH, Kim JS, Yoon Y, 2014. Rapid detection of *Listeria monocytogenes* by Real time PCR in processed meat and dairy products. J Food Prot 77:453-8.

Huang S, Zhang C-P, Wang K, Li GQ, Hu F-L, 2014. Recent advances in the chemical composition of propolis. Molecules 19:19610-32.

Jansen-Alves C, Fernandes KF, Crizel-Cardoso MM, Krumreich FD, Borges CD, Zambiazi RC, 2018. Microencapsulation of propolis in protein matrix using spray drying for application in food systems. Food Bioprocess Tech 11:1422-36.

Louveaux J, Maurizio A, Vorwohl G, 1978. Methods of melissopalynology. Bee World 59:139-57.

Marghitas LA, Mihai CM, Chirila F, Dezmirean DS, Fit N, 2010. The study of the antimicrobial activity of Transylvanian (Romanian) propolis. Not Bot Horti Agrobo 38:40-4.

Mascheroni E, Figoli A, Musatti A, Limbo S, Drioli E, Suevo R, Talarico S, Rollini M, 2014. An alternative encapsulation approach for production of active chitosan-propolis beads. Int J Food Sci Tech 49:1401-7.

Metwalli SAH, 2011. Extended shelf-life of Kareish cheese by natural preservatives. Egypt. J Agric Res 89:639-49.

Miorin PL, Levy Junior NC, Custodio AR, Bretz WA, Marcucci MC, 2003. Antibacterial activity of honey and propolis from *Apis mellifera* and *Tetragonisca angustula* against *Staphylococcus aureus*. J Appl Microbiol 95:913-20.

Pobiega K, Kraśniewska, K, Gniewosz M, 2019. Application of propolis in antimicrobial and antioxidative protection of food quality – A review. Trends Food Sci Technol 83:53-62.

Popova M, Bankova V, Butovska D, Petkov V, Nikolova-Damianova B, Sabatini AG, Marcazzan GL, Bogdanov S, 2004. Validated methods for the quantification of biologically active constituents of poplar-type propolis. Phytochem Anal 15:235-40.

Ricciardelli D'Albore G, 1979. L'origine géographique de la propolis. Apidologie 10:241-67.

Ristivojević P, Dimkić I, Trifković J, Berić T, Vovk I, Milojković-Opsenica D, Stanković S, 2016. Antimicrobial activity of Serbian propolis evaluated by means of MIC, HPTLC, bioautography and chemometrics. PLoS One 11:e0157097.

Sforcin JM, Bankova V, 2011. Propolis: is there a potential for the development of new drugs? J Ethnopharmacol 133:253-60.

Thamnopoulos I-A, Michailidis GF, Fletouris DJ, Badeka A, Kontominas MG, Angelidis AS, 2018. Inhibitory activity of propolis against *Listeria monocytogenes* in milk stored under refrigeration. Food Microbiol 73:168-76.

UNI, 2004. UNI EN ISO 6888-1: 2004. [Microbiologia di alimenti e mangimi per animali - Metodo orizzontale per la conta di stafilococchi coagulasi- positivi (*Staphylococcus aureus* e altre specie) - Tecnica che utilizza il terreno agar Baird-Parker]. [Regulation in Italian]. Italian Unification Institute ed., Milan, Italy.

Wiegand I, Hilpert K, Hancock RE, 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163-75.

Zabaiou N, Fouache A, Trousson A, Baron S, Zellagui A, Lahouel M, Lobaccaro J-MA, 2017. Biological properties of propolis extracts; something new from an ancient product. Chem Phys Lipids 207: 214-22.