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. 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.

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
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 \textit{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 \textit{P. fluorescens}), bacterial counts were determined on the following media (Oxoid): Listeria Selective Agar Base with Oxford Supplement for \textit{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 \textit{B. cereus} (FDA BAM, 2017), Pseudomonas Agar Base with CFC Supplement for \textit{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 \textit{L. monocytogenes}, \textit{S. aureus} and \textit{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   | Ethanol | MIC   | Ethanol |
| Lm. 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. Lm: 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**|          |          |          |          |          |
| Lm. 7644     | 6.40±0.48| (1.87)   | 3.92±0.14| (4.35)   | 8.04±0.19| (0.23)   | 7.50±0.34| (0.77)   | 8.27±0.07| (0.98)   |
| S.a. 25923   | 5.88±0.50| (1.98)   | 5.31±0.26| (2.65)   | 7.84±0.16| (0.12)   | 7.46±0.07| (0.50)   | 7.96±0.08| (0.15)   |
| S.a. 35556   | 5.74±0.33| (2.63)   | 5.33±0.42| (3.04)   | 8.19±0.05| (0.18)   | 8.09±0.13| (0.28)   | 8.37±0.08| (0.15)   |
| B.c. DSV12   | 3.31±0.35| (2.72)   | 2.94±0.32| (3.09)   | 6.57±0.92| (0.54)   | 6.86±0.24| (0.83)   | 6.03±0.44| (0.15)   |
| P.f. 13525   | 6.78±0.11| (1.09)   | 6.55±0.07| (1.32)   | 7.45±0.41| (0.42)   | 6.95±0.20| (0.92)   | 7.87±0.18| (0.15)   |
| **Pasteurized cow’s milk**|          |          |          |          |          |
| Lm. 7644     | 5.59±0.31| (0.47)   | 4.66±0.22| (1.40)   | 5.79±0.45| (0.27)   | 5.82±0.14| (0.24)   | 6.06±0.31| (0.15)   |
| S.a. 25923   | 6.30±0.16| (1.12)   | 5.09±0.13| (2.33)   | 7.10±0.31| (0.32)   | 6.62±0.27| (0.80)   | 7.42±0.21| (0.15)   |
| S.a. 35556   | 7.04±0.35| (0.67)   | 4.49±0.09| (3.22)   | 7.44±0.43| (0.27)   | 7.13±0.36| (0.58)   | 7.71±0.32| (0.15)   |
| B.c. DSV12   | 6.21±0.27a| (0.24)  | 3.12±0.56a| (2.85)   | 5.75±0.28a| (0.22)   | 6.25±0.21a| (0.28)   | 5.97±0.47a| (0.15)  |
| 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| (0.15)   |

EEP: 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); Lm: Listeria monocytogenes; S.a.: Staphylococcus aureus; B.c.: Bacillus cereus; P.f.: Pseudomonas fluorescens. Results are the 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. **Different letters in the same row denote significant differences (P<0.05).**
respectively, 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 (0.09) |
| t7             | 6.53±0.66 (0.67) | 5.38±0.36 (1.82) | 6.06±0.40 (1.91) | 5.39±0.92 (3.39) | 7.20±0.89 (1.91) |
| t14            | 7.30±0.74 (0.00) | 5.02±0.24 (2.28) | 7.32±0.02 (0.02) | 5.79±0.12 (1.51) | 7.30±0.74 (1.51) |
| t21            | 7.12±0.91 (0.01) | 4.87±0.21 (3.26) | 7.67±0.19 (4.6)  | 6.45±1.06 (6.05) | 8.13±0.10 (6.05) |
| t28            | 7.71±0.08 (0.51) | 4.53±0.48 (5.69) | 7.99±0.17 (0.23) | 6.86±0.96 (1.36) | 8.22±0.18 (1.36) |
| 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 (0.12) |
| t7             | 4.75±0.29 (0.10) | 4.56±0.45 (0.29) | 4.97±0.38 (0.12) | 4.88±0.21 (0.03) | 4.85±0.16 (0.03) |
| t14            | 4.38±0.51 (0.00) | 3.81±1.11 (0.57) | 4.81±0.32 (0.43) | 4.58±0.08 (0.20) | 4.38±0.35 (0.20) |
| 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 (0.21) |
| t28            | 4.29±0.91 (0.18) | 3.34±0.50 (0.77) | 4.63±0.21 (0.52) | 4.12±0.08 (0.01) | 4.11±0.38 (0.01) |
| 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 (0.14) |
| t7             | 1.57±0.96 (1.66) | 1.33±0.58 (1.90) | 3.37±0.58 (0.14) | 2.71±0.98 (0.32) | 3.23±0.46 (0.32) |
| t14            | 1.35±0.49 (0.95) | 1.33±0.49 (0.95) | 2.44±1.05 (0.14) | 2.00±0.43 (0.30) | 2.30±0.85 (0.30) |
| t21            | 1.00±0.00 (0.85) | 1.00±0.00 (0.85) | 1.35±0.49 (0.85) | 1.00±0.00 (0.85) | 1.85±0.21 (0.85) |
| 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 (0.00) |
| **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 (0.01) |
| t7             | 7.00±0.11 (0.10) | 5.44±0.28 (1.66) | 6.90±0.24 (2.1)  | 6.86±0.31 (2.42) | 7.10±0.08 (2.42) |
| t14            | 7.01±0.15 (0.15) | 5.70±0.72 (1.46) | 7.06±0.14 (0.10) | 6.97±1.18 (0.19) | 7.16±0.14 (0.19) |
| 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 (0.04) |
| t7             | 5.10±0.19 (0.08) | 4.95±0.30 (0.23) | 5.25±0.31 (0.87) | 5.11±0.10 (0.07) | 5.18±0.03 (0.07) |
| 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 (0.15) |
| B.c. DSV12     |        |        |        |        |        |
| t0             | 3.86±0.25 (0.02) | 3.91±0.24 (0.07) | 3.83±0.32 (0.01) | 3.85±0.36 (0.19) | 3.84±0.26 (0.19) |
| t7             | 3.04±0.88 (0.74) | 2.27±0.38 (0.74) | 3.07±0.75 (0.86) | 3.12±0.81 (0.11) | 3.01±0.38 (0.11) |
| t14            | 1.00±0.00 (0.35) | 1.00±0.00 (0.35) | 1.35±0.49 (0.35) | 1.00±0.00 (0.35) | 1.35±0.49 (0.35) |

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. 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., Querucus 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)-5.55 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 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 t17 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 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.

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.

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