Applied and Environmental Microbiology

Biotransformation Strategy To Reduce Allergens in Propolis

Claudio Gardana, Andrea Barbieri, Paolo Simonetti and Simone Guglielmetti

Appl. Environ. Microbiol. **2012, 78(13):4654. DOI: 10.1128/AEM.00811-12.**
Published Ahead of Print 20 April 2012.

AEM
Journals.ASM.org

Biotransformation Strategy To Reduce Allergens in Propolis

Claudio Gardana,[a] Andrea Barbieri,[b] Paolo Simonetti,[a] and Simone Guglielmetti[b]

Division of Nutrition[a] and Division of Food Microbiology and Bioprocesses,[b] Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Milan, Italy

Propolis (bee glue) is a resinous, sticky, dark-colored material produced by honeybees. Propolis today, due to its medicinal properties, is increasingly popular and is extensively used in food, beverages, and cosmetic products. Besides its numerous positive properties, propolis may also have adverse effects, such as, principally, allergic eczematous contact dermatitis in apiarists and in consumers with an allergic predisposition. In this study, we found appropriate conditions for removing caffeate esters, which are the main allergenic components, from raw propolis. The proposed method consists of the resuspension of propolis in a food grade solvent, followed by a biotransformation based on the cinnamoyl esterase activity of *Lactobacillus helveticus*. We showed that the reduction of caffeate esters by *L. helveticus* did not affect the content of flavonoids, which are the main bioactive molecules of propolis. Furthermore, we verified that the biotransformation of propolis did not cause a loss of antimicrobial activity. Finally, we demonstrated that the ability of *L. helveticus* to hydrolyze caffeate esters in propolis is strain specific. In conclusion, the proposed strategy is simple, employs food grade materials, and is effective in selectively removing allergenic molecules without affecting the bioactive fraction of propolis. This is the first study demonstrating that the allergenic caffeate esters of propolis can be eliminated by means of a bacterial biotransformation procedure.

Propolis (bee glue) is a resinous, sticky, dark-colored material produced by honeybees (*Apis mellifera*), which collect exudates from trees, mix them with wax, and use the resulting material to seal and protect honeycombs. The chemical composition of propolis varies and depends mainly on the plants accessible to bees and on the season. Propolis originating from plants of the genus *Populus* (for instance, poplar), which are typical of the temperate zones of Europe, China, and North America, comprises resins (20 to 25%), waxes (30 to 40%), volatile oils (5 to 10%), and many phenolic compounds (10 to 30%), which include, in particular, flavonoids. In addition, the phenolic fraction of propolis contains aromatic acids, such as cinnamic, caffeic, ferulic, and *p*-coumaric acids, and their esters (3).

The attribution of a potential medicinal value to propolis dates back to ancient Egyptians, Greeks, and Romans, who used propolis to heal wounds, sores, and ulcers (11). Registered as an official drug in the London pharmacopeias of the 17th century, propolis today, due to its medicinal properties, is increasingly popular and is extensively used in food, beverages, and cosmetic products. Indeed, the ethanolic extract of propolis reportedly has a wide variety of biological actions, including antimicrobial (18), antiherpes and antifungal (20), anti-inflammatory (22), antioxidant (17), immune-stimulating (19), cariostatic (8), anticancer (16), and anti-*Helicobacter pylori* (1) activities. The pharmacological properties of propolis have been correlated with the presence of specific molecules. For instance, its antibacterial and antiviral activities have been linked with diterpenic acids and dihydrobenzofuranic lignans, and its anti-inflammatory and antioxidant activities seem to be due to flavonoids, whereas hepatoprotective and anticancer activities have been proposed for several propolis constituents, including caffeoylquinic acid derivatives and phenylethyl caffeate (4).

Besides the numerous above-mentioned positive effects, propolis, though seemingly relatively safe (calculated safe dose, ~1.4 mg per kg of body weight per day in humans [5]), may have adverse effects, such as xerostomia, gastric pain, and allergic eczematous contact dermatitis, in apiarists (10) and in consumers with an allergic predisposition (23). Less than 10% of the population is sensitive to propolis (sensitization rate, 1.2 to 6%); nevertheless, a significant increasing trend in sensitization has been observed in adolescents in recent decades (from 2 to 13.7% in the period 1995 to 2002) (15), possibly as a consequence of a considerably augmented use of propolis in numerous commercial products. For this reason, a strategy to eliminate allergens from propolis would be of wide interest. Nonetheless, to the best of our knowledge, propolis with a high content of flavonoids and a reduced content of allergens is not industrially available at present.

In this context, we describe a strategy using food grade materials to selectively and effectively reduce the allergenic molecules in propolis. The allergens in propolis are mainly caffeic acid (CA) derivatives (14), and in this study, we show how to treat propolis in order to eliminate these molecules by the cinnamoyl esterase activity of a dairy bacterium, namely, *Lactobacillus helveticus*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. helveticus* strains were grown overnight anaerobically at 42°C in MRS broth (Difco, Detroit, MI).

Propolis biotransformation. Raw propolis samples (collected from the Puglia region of Italy) were chilled at −20°C (since it becomes hard and very brittle at lower temperatures), finely ground in a mill, and passed through a 500-μm (35-mesh) sieve. At the same time, the bacterial-cell concentration of an overnight culture of *L. helveticus* was determined microscopically with a Neubauer improved counting chamber (Marienfeld GmbH, Lauda-Königshofen, Germany). Then, 1 g of propolis was placed in each 50-ml tube in which we had previously pelleted different amounts of *L. helveticus* cells (10^8 to 10^{14} bacterial cells). Finally, we added 2 ml of phosphate-buffered saline (PBS) (pH 7.3) containing ethanol

Received 12 March 2012 **Accepted** 2 April 2012
Published ahead of print 20 April 2012
Address correspondence to Simone Guglielmetti, simone.guglielmetti@unimi.it.

doi:10.1128/AEM.00811-12

(from 5 to 70%) or polyethylene glycol (PEG) 400 (from 1 to 80%). After 24 h of incubation at 37°C under constant agitation by a magnetic stirrer, samples were collected and analyzed by liquid chromatography/mass spectrometry. Bacterial-cell concentrations were also determined microscopically with the counting chamber after incubation.

Analytical methods for the study of the phenolic fraction of propolis. After biotransformation, phenolic fractions were extracted from propolis samples and analyzed as described previously (9). In brief, we added 70 ml of ethyl acetate to the sample (bacteria-propolis mixture) and incubated it at room temperature under agitation for 10 min. The mixture was centrifuged at 1,500 × *g* for 5 min, and the supernatant was transferred into a flask. The solid residue was extracted again with ethyl acetate as described above. The extracts were dried under nitrogen, and the pellets were dissolved in 100 ml methanol. After a final centrifugation at 4,000 × *g* for 1 min, the supernatants were diluted and analyzed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). UHPLC-MS/MS analyses were performed with an Acquity UHPLC system (Waters, Milford, MA) coupled with a Quattromicro triple-quadrupole mass spectrometer (Waters). A 1.7-μm C_{18} BEH Shield column (150 by 2.1 mm; Waters) was used for the separation at a flow rate of 0.45 ml min^{-1}. The separation was carried out at 40°C with linear gradient elution (eluent A, 0.05% HCOOH; eluent B, 0.05% HCOOH in acetonitrile). The gradient was as follows: 20 to 30% B in 4 min, 30 to 40% B in 5 min, 40 to 60% B in 3 min, 60 to 90% B in 1 min, and then 90% B for 2 min. The capillary voltage was set to 2.7 kV, and the cone voltage and the energy applied during fragmentation (Elab) were specific for each compound, as previously reported (9). All mass data were obtained in the negative ion mode. The source temperature was 130°C, the desolvation temperature was 380°C, and argon was used at 0.21 Pa to improve fragmentation in the collision cell. Masslinx 4.0 acquired data with the Quan-Optimize option for the fragmentation study. The fragmentation transitions for the multiple-reaction monitoring (MRM) were as previously reported (9). CA, 3-methyl-2-butenyl-CA (3M2B), internal standard (dihydrocaffeic acid [DHCA]), and caffeic acid phenyl ethyl ester (CAPE) were purchased from Sigma-Aldrich (St. Louis, MO). Methyl caffeate was from Chromadex (Milan, Italy), ethyl caffeate from Waterstone Technology (Carmel, IN), and ethyl ferulate from Santa Cruz Technology (Santa Cruz, CA). Methanol, acetonitrile, ethyl acetate, and formic acid were supplied by Merck (Darmstadt, Germany). 2-Methyl-3-butenyl-caffeate (2M3B), benzyl-caffeate (CABE), and 3-methyl-3-butenyl-caffeate (3M3B) were synthesized as described previously (9). An unpaired Student's *t* test was run for statistically significant differences.

Antimicrobial assay. Antimicrobial activity was determined by the agar dilution method on two different samples: (i) conventional propolis extract (60% ethanol, 18.1 ± 1.0 mg ml^{-1} flavonoids, 7.1 ± 0.4 mg ml^{-1} phenolic acids [including 4.3 ± 0.2 mg ml^{-1} allergenic caffeate esters]) and (ii) MIMLh5-treated propolis extract (60% ethanol, 18.1 ± 1.0 mg ml^{-1} flavonoids, 5.2 ± 0.3 mg ml^{-1} phenolic acids [including 1.5 ± 0.2 mg ml^{-1} allergenic caffeate esters]).

Furthermore, a 60% ethanol solution in water was used as a control. The assay was performed against five indicator microorganisms: *Staphylococcus aureus* (three strains), *Staphylococcus haemolyticus* (four strains), *Streptococcus pneumoniae* (six strains), *Candida albicans* (eight strains), and *Candida tropicalis* (six strains). Bacteria were cultivated in Mueller-Hinton medium (Difco), whereas fungi were grown on Sabouraud's dextrose medium (Difco). In brief, the samples were serially 2-fold diluted in the range of 0.07 to 9.0 mg ml^{-1} flavonoids (equally for the ethanol control) and spiked on agar plates. The agar plates had been previously inoculated with approximately 10^4 CFU of the indicator strain, whose cells were collected at the mid-log phase of growth. The plates were read after 24 h of incubation at 37°C for bacteria and after 4 days at 35°C for fungi. The MICs of samples promoting growth inhibition of 50% of the strains under study were denoted MIC_{50}s.

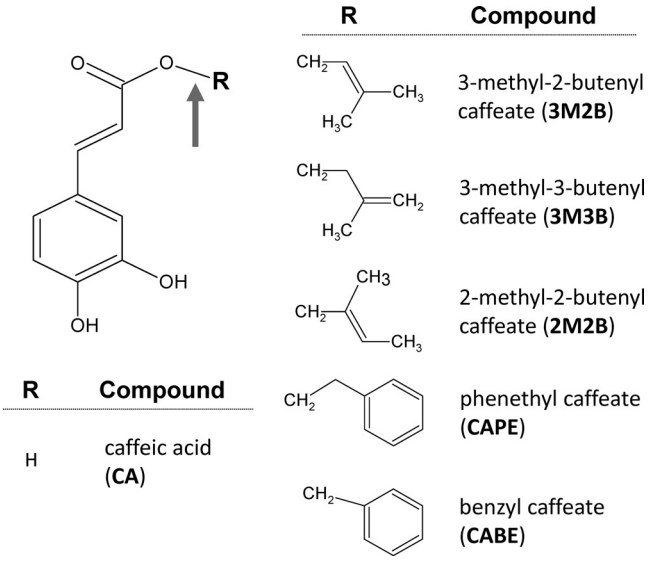

FIG 1 Structural formulas of the molecules constituting the allergenic fraction of propolis. The arrow indicates the chemical bond hydrolyzed by cinnamoyl esterase activity.

RESULTS AND DISCUSSION

A few methods have been proposed to obtain propolis with a reduced proportion of allergenic substances. For instance, a recent patent reported a protocol consisting of chemical-physical purification steps to produce hypoallergenic propolis extracts (K.-H. Sensch, 27 April 2009, international patent application WO2009/133073). However, these methods come with several drawbacks, such as altered taste, reduced polyphenol content, or high energy consumption during production steps. The methods also lack selectivity, and they cannot preserve the biological/pharmacological efficacy of the extract obtained. In this study, we aimed to identify a strategy using food grade materials to selectively and effectively decrease the allergenic molecules in propolis while preserving its bioactive components (mainly flavonoids).

Reduction of allergenic compounds in propolis. The allergenic components identified in propolis are mainly caffeic acid derivatives (14) (Fig. 1), among which 3M2B seems to be the most active (2). Specifically, since the allergenic molecules of propolis characteristically contain a cinnamoyl ester bond (Fig. 1), we studied the possibility of employing a bacterial strain, namely, *L. helveticus* MIMLh5, which we had recently selected over 100 lactic acid bacteria for its high cinnamoyl esterase activity (12).

To remove allergenic molecules and to make them accessible to *L. helveticus*, we first looked for a way to properly mix propolis with bacterial cells. Propolis is a very dense, resinous material that is sticky above room temperature and insoluble in water. Propolis is partially soluble in ethanol, the solvent commonly used in industry to prepare extracts. For this reason, we tested the stability of the cinnamoyl esterase activity of MIMLh5 at different ethanol percentages by using 0.5 mg ml^{-1} chlorogenic acid. Chlorogenic acid was chosen as a reference molecule in these preliminary experiments because it is a stable molecule, easy to detect chromatographically, and already successfully employed to quantitatively study the cinnamoyl esterase activity of lactic acid bacteria (12). We found that above a concentration of 10% (vol/vol) ethanol, the enzymatic ability of strain MIMLh5 to hydrolyze chlorogenic

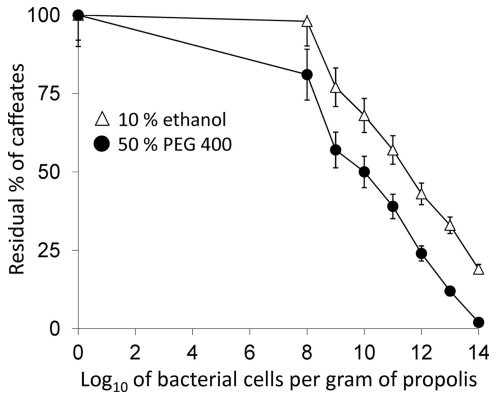

FIG 2 Degradation of caffeic acid esters in raw propolis samples by increasing concentrations of *L. helveticus* MIMLh5 cells. Propolis was dispersed in PBS buffer plus 10% ethanol or 50% PEG 400. The data are reported as means (three experiments) of the relative amount of all caffeates shown in Fig. 1 ± standard deviations.

acid was significantly reduced (data not shown). We then dispersed a raw propolis sample in a solution of PBS and 10% (vol/vol) ethanol (e10PBS) in the presence of different numbers of MIMLh5 cells (10^8 to 10^{14} bacterial cells per g of propolis). After 24 h of incubation at 37°C under constant agitation, we found that 10^{11} bacterial cells reduced allergenic caffeic acid esters in 1 gram of raw propolis sample by 43%. With more bacterial cells, the reduction of allergenic caffeic acid esters increased proportionally, from 57% with 10^{12} cells to 67 and 81% reduction when 10^{13} and 10^{14} cells, respectively, were employed (Fig. 2). Quantitative analysis revealed that all the main caffeate esters in propolis decreased during the biotransformation (Table 1), while the caffeic acid concentration increased, confirming that the reduction of caffeates resulted from the hydrolysis of the cinnamoyl ester bond (Table 1). By means of an HPLC-diode array detector (DAD) method, we also observed that the flavonoid fraction, considered the main bioactive component of propolis, was not affected (Table 1).

Improved propolis solubilization allowed a bigger reduction of caffeate esters. In the next part of our study, we assessed the possibility of employing a different solvent to disperse raw propolis sample in order to improve the biotransformation rate. To this end, we considered PEG 400, for two main reasons: (i) PEG 400 is a food-grade additive, recently approved by the European Food Safety Authority (EFSA) (7), and (ii) PEG 400 reportedly solubilizes hydrophobic molecules, preserving the stability of enzymes (21). In accordance with the latter statement, the stability of the cinnamoyl esterase activity of *L. helveticus* MIMLh5 was unaffected up to 80% (wt/vol) PEG 400 in PBS (data not shown). Next, we performed biotransformation experiments with 50% PEG 400 instead of ethanol, and we observed a significant improvement in the reduction of caffeic acid esters. Precisely, under our experimental conditions (i.e., 1 g of propolis in 2 ml of 50% PEG 400), 10^{11} bacterial cells reduced the allergenic molecules by 61% (Fig. 2 and Table 1). This result corresponds to a 42% increase in caffeate ester reduction compared to the same experiment carried out in 10% ethanol. This amelioration of the biotransformation rate was also maintained in higher numbers of cells; in fact, when 10^{14} bacterial cells were used, about 98% of caffeic acid esters were removed in 1 gram of propolis (Fig. 2).

We also tested a propolis sample of different origin, specifically, from China. The reduction of allergenic caffeates in Chinese propolis occurred only when a greater concentration of PEG 400 was used (80%). Plausibly, this depended on the wax concentration, which was 60% higher in Chinese than in Italian propolis (about 40 and 25 g of wax, respectively, per 100 g of raw propolis [data not shown]). Therefore, optimal experimental conditions should be set for any specific propolis sample, since its composition can affect dispersion efficacy.

Different *L. helveticus* strains display dissimilar abilities to reduce allergens in propolis. We also tested if diverse strains of *L. helveticus* can display different performances during propolis biotransformation. We dispelled 1 g of propolis in 2 ml of e10PBS in the presence of four different *L. helveticus* strains, namely, MIMLh5, SLh02, SLh13, and SLh37. *L. helveticus* SLh02, SLh13, and SLh37 were included in the study because they are commercial strains with known growth performances at an industrial level. In particular, strains SLh13 and SLh37 are commercialized as diary starters and SLh02 as a probiotic. We used 2×10^{10} bacterial cells, because the amount approximately corresponded to 50% activity, and therefore, it was suitable to detect possible differences in the biotransformation rate as a function of the bacterium employed. The results showed marked differences among strains; in particular, two of them, SLh13 and SLh37, displayed faint activity on propolis, whereas SLh02 and MIMLh5 markedly reduced caffeate esters (Fig. 3). In a previous report, the cinnamoyl esterase activities varied markedly among *L. helveticus* strains, and MIMLh5 displayed the highest activity (12). Furthermore, we observed that the microscopically determined bacterial cell concentrations were the same before and after incubation with propolis. Differences in the biotransformation rates among *L. helveticus* strains, therefore, were not a consequence of bacterial growth dissimilarities during the biotransformation (data not shown).

The antimicrobial activity of MIMLh5-treated (low-allergen) propolis was not significantly dissimilar from that of conventional propolis. In order to confirm that propolis conserved its beneficial features after treatment with *L. helveticus* MIMLh5,

TABLE 1 Concentrations of allergenic caffeic acid esters and flavonoids in the Italian propolis employed in our study

Molecule or parameter	Concn[a]		
		Biotransformation with 2×10^9 MIMLh5 cells g^{-1}	
	Control	10% ethanol	50% PEG 400
3M3	2.66 ± 0.11	1.69 ± 0.10	1.53 ± 0.10
3M2	4.33 ± 0.19	2.53 ± 0.14	2.41 ± 0.14
2M2	2.43 ± 0.13	1.52 ± 0.10	1.35 ± 0.09
CABE	6.76 ± 0.41	4.83 ± 0.30	4.36 ± 0.30
CAPE	34.62 ± 1.42	26.01 ± 1.12	21.84 ± 1.10[b]
Total amt	50.80 ± 2.59	36.58 ± 1.91	31.50 ± 1.73[b]
Total flavonoids	208.00 ± 11.90	204.00 ± 12.64	212.00 ± 13.81
Flavonoid esters	46.00 ± 2.44	45.08 ± 2.44	46.80 ± 2.55
Caffeic acid	3.80 ± 0.17	4.40 ± 0.19	4.51 ± 0.22

[a] Data are shown as mg of molecule per g of propolis and describe experiments carried out using 2×10^9 cells of MIMLh5 to treat 1 g of propolis dispersed in 10% ethanol or 50% PEG 400. The data represent the means of at least three independent experiments ± standard deviations. With the exception of total flavonoids and flavonoid esters, all molecule concentrations were significantly modified by the biotransformation ($P < 0.001$; unpaired Student's *t* test).

[b] Statistically significant difference between PEG- and ethanol-treated propolis ($P < 0.05$).

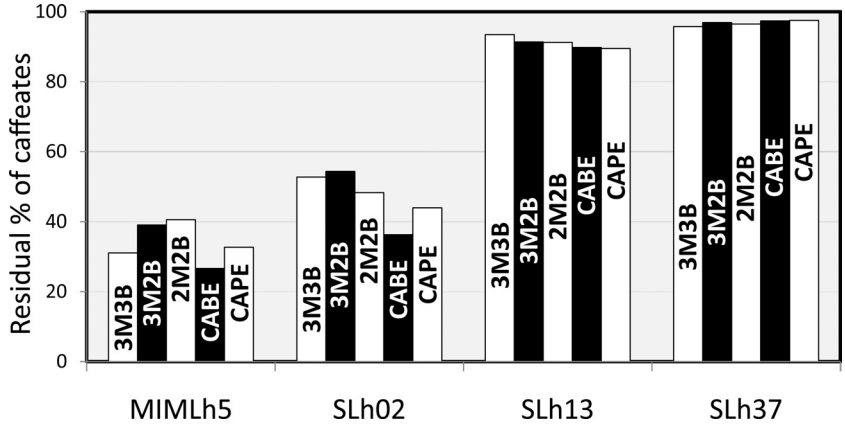

FIG 3 Relative amounts of allergenic esters of caffeic acid in propolis treated with *L. helveticus* strains. The names of the molecules match those in Fig. 1. The amount of each molecule in the control sample (propolis incubated without bacteria) was set to 100.

we assessed its best-known and most widely researched property, i.e., the ability to inhibit pathogenic microorganisms. In this experiment, we compared a MIMLh5-treated (low-allergen) propolis alcoholic extract with an identical preparation that was obtained from non-treated (conventional) propolis. The antimicrobial activity was tested against three bacterial (13 strains) and two fungal (14 strains) species, which were selected because they are ubiquitous microorganisms, often involved in opportunistic infections. The MIC_{50} of the low-allergen propolis did not significantly differ from that of conventional propolis for any of the indicator microorganisms (Table 2). We observed a reduced MIC_{50} (i.e., stronger antimicrobial ability) only for low-allergen propolis against *S. aureus*. Nonetheless, this difference was not statistically significant ($P = 0.08$) (Table 2). The reduction of caffeate esters by the activity of strain *L. helveticus* MIMLh5, therefore, did not result in a loss of antimicrobial activity by propolis. This result is in agreement with the flavonoid content, which did not significantly change during the biotransformation.

Conclusions. We proposed a novel biotransformation strategy to significantly reduce allergenic molecules in propolis without affecting its bioactive fraction. This method is based on a food-grade solvent (ethanol or PEG 400) and the cinnamoyl esterase activity of *L. helveticus*, a dairy bacterium generally recognized as safe, included in the EFSA QPS list of microorganisms (6), and demonstrated to have probiotic properties (13).

A clinical study will be carried out in the near future in order to confirm that the reduction of caffeate esters by means of the proposed biotransformation can actually result in attenuated allergic symptoms in propolis-sensitive people. At the moment, this strategy (recently patented [G. M. Ricchiuto, C. Gardana, and S. Guglielmetti, 22 September 2011, international patent application WO2011/114291]) is under industrial development. The first promising scale-up experiments suggest that hypoallergenic high-quality propolis extracts, prepared according to this strategy, will soon be industrially available.

TABLE 2 Flavonoid concentrations corresponding to MIC_{50}s of the propolis preparations

Microorganism	Concn of flavonoids in preparation to achieve MIC_{50} (mg ml^{-1})[a]		P^b
	Conventional propolis	MIMLh5-treated propolis	
S. aureus	1.50 ± 0.52	1.04 ± 0.28	0.08
S. haemolyticus	1.00 ± 0.35	0.87 ± 0.35	0.28
S. pneumoniae	0.26 ± 0.09	0.26 ± 0.07	1.00
C. albicans	3.00 ± 1.04	2.55 ± 0.94	0.42
C. tropicalis	2.55 ± 0.94	2.7 ± 0.78	0.42

[a] The means (± standard deviations) of three independent experiments are shown. Ethanol solutions, tested as a control, did not affect microbial growth.
[b] The *P* values refer to the statistical difference between the two propolis samples according to an unpaired Student's *t* test.

ACKNOWLEDGMENTS

We are grateful to Specchiasol S.r.l. (Bussolengo, Italy) for providing the raw propolis samples and to Sacco S.r.L. (Cadorago, Italy) for providing strains SLh02, SLh13, and SLh37.

REFERENCES

1. **Abdel-Latif MMM, Windle HJ, Homasany BSE, Sabra K, Kelleher D.** 2005. Caffeic acid phenethyl ester modulates *Helicobacter pylori*-induced nuclear factor-kappa B and activator protein-expression in gastric epithelial cells. Br. J. Pharmacol. **146**:1139–1147.
2. **Accial MC, Ginanneschi M, Braci S, Sertoli A.** 1990. Studies of the sensitizing properties of propolis. Contact Dermatitis **23**:274.
3. **Bankova VS, de Castro SL, Marcucci MC.** 2000. Propolis: recent advances in chemistry and plant origin. Apidologie **31**:3–15.
4. **Banskota AH, Tezuka Y, Kadota S.** 2001. Recent progress in pharmacological research of propolis. Phytother. Res. **15**:561–571.
5. **Burdock GA.** 1998. Review of the biological properties and toxicity of bee propolis (propolis). Food Chem. Toxicol. **36**:347–363.
6. **European Food Safety Authority.** 2004. Scientific colloquium summary report. QPS: qualified presumption of safety of microorganisms in food and feed. European Food Safety Authority, Brussels, Belgium.
7. **European Food Safety Authority.** 2006. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the commission related to an application on the use of polyethylene glycol (PEG) as a film coating agent for use in food supplement products. EFSA J. **414**:1–22.
8. **Feres M, et al.** 2005. In vitro antimicrobial activity of plant extracts and propolis in saliva samples of healthy and periodontally-involved subjects. J. Int. Acad. Periodontol. **7**:90–96.
9. **Gardana C, Simonetti P.** 2011. Evaluation of allergens in propolis by ultra-performance liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. **25**:1675–1682.
10. **Garrido Fernandez S, et al.** 2004. Direct and airborne contact dermatitis from propolis in beekeepers. Contact Dermatitis **50**:320–321.
11. **Ghisalberti EL.** 1979. Propolis: a review. Bee World **60**:59–84.
12. **Guglielmetti S, et al.** 2008. Bacterial cinnamoyl esterase activity screening for the production of a novel functional food product. Appl. Environ. Microbiol. **74**:1284–1288.

13. **Guglielmetti S, et al.** 2010. A dairy bacterium displays in vitro probiotic properties for the pharyngeal mucosa by antagonizing group A streptococci and modulating the immune response. Infect. Immun. **78**:4734–4743.
14. **Hausen BM.** 2005. Evaluation of the main contact allergens in propolis (1995 to 2005). Dermatitis **16**:127–129.
15. **Jappe U, Schnuch A, Uter W.** 2005. Rosacea and contact allergy to cosmetics and topical medicaments—retrospective analysis of multicentre surveillance data 1995–2002. Contact Dermatitis **52**:96–101.
16. **Orsolić N, Saranović AB, Basić I.** 2006. Direct and indirect mechanism (s) of antitumour activity of propolis and its polyphenolic compounds. Planta Med. **72**:20–27.
17. **Pietta PG.** 2000. Flavonoids as antioxidants. J. Nat. Prod. **63**:1035–1042.
18. **Scazzocchio F, D'Auria FD, Alessandrini D, Pantanella F.** 2006. Multifactorial aspects of antimicrobial activity of propolis. Microbiol. Res. **161**:327–333.
19. **Sforcin JM, Orsi RO, Bankova V.** 2005. Effect of propolis, some isolated compounds and its source plant on antibody production. J. Ethnopharmacol. **98**:301–305.
20. **Silici S, Koç NA, Ayangil D, Cankaya S.** 2005. Antifungal activities of propolis collected by different races of honeybees against yeasts isolated from patients with superficial mycoses. J. Pharmacol. Sci. **99**:39–44.
21. **Talukder MMR, et al.** 2003. Improvement in enzyme activity and stability by addition of low molecular weight polyethylene glycol to sodium bis (2-ethyl-L-hexyl) sulfosuccinate/isooctane reverse micellar system. Appl. Biochem. Biotechnol. **110**:101–112.
22. **Tan-No K, et al.** 2006. Anti-inflammatory effect of propolis through inhibition of nitric oxide production on carrageenin-induced mouse paw edema. Biol. Pharm. Bull. **29**:96–99.
23. **Walgrave SE, Warshaw EM, Glesne LA.** 2005. Allergic contact dermatitis from propolis. Dermatitis **16**:209–215.