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Honey and propolis reduce the antibacterial resistance of staphylococci

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Summary

Antimicrobial resistance (AMR) is a significant public health concern of the 21st century, contributing to an increasing number of deaths in both human and veterinary medicine. Multi-antibiotic-resistant bacteria, such as *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Streptococcus pneumoniae*, and *Klebsiella pneumoniae*, pose substantial challenges. This research investigated changes in antibiotic susceptibility of *Staphylococcus* spp. Isolates from extensively farmed pigs (n = 421) when exposed to different types of honey (multi-floral, n = 1, and rapeseed, n = 2) or different commercial propolis concentrations (20% and 30% in 70% ethanol). Thirty species of staphylococci were identified using classical bacteriology techniques. Initial antimicrobial resistance/susceptibility was tested against colistin, erythromycin, sulfamethoxazole/trimethoprim, penicillin, florfenicol, vancomycin, tetracycline, imipenem, marbofloxacin, cefoxitin, clindamycin, and methicillin using the Kirby Bauer disk-diffusion method. Following exposure to bee products, susceptibility to antibiotics was reassessed using the same protocol. The MAR index and percentage of strains resistant to each antibiotic were calculated. Results showed a relatively low MAR index in 90% of the strains, with 26.7% showing no resistance, 20% with a MAR index of 0.08, and 43.3% with a MAR index of 0.16. However, 10% of the strains exhibited mild resistance or high resistance (MAR index of 0.25, 0.33, and 0.58), posing risks to animals, caretakers, and the environment. Following exposure to bee products, changes in susceptibility to different antibiotics were observed. The highest number of strains initially classified as resistant and reclassified as sensitive were found for penicillin and tetracycline, followed by sulfamethoxazole, methicillin, and cefoxitin. Changes were more pronounced in strains classified as moderately resistant, with significant differences ($p < 0.05$-$p < 0.001$) observed between antibiotics. The findings suggested that honey and propolis could reduce antimicrobial resistance in staphylococci isolated from healthy pigs, thus serving as natural alternatives to antimicrobial treatment for staphylococcal infections.

Keywords: staphylococci, antibiotic resistance, honey, propolis

Antibiotic resistance has significant implications for humans, animals, and the environment, posing a continuously escalating global concern (5, 22). The inappropriate use of antimicrobials in swine, particularly as growth promoters, has contributed to the rise in antimicrobial resistance (AMR), which is recognized as a major public health risk (18). Indiscriminate antibiotic use against bacterial pathogens in both human and animal populations has resulted in numerous fatalities. Of particular concern is the persistent increase in the number of multi- and extensively drug-resistant strains, which exhibit resistance to a wide range of antibiotics, including those commonly used. The significant concern is that AMR may compromise certain medical procedures, essentially interventions such as surgeries or transplants (11) because the antimicrobial resistance genes can be transmitted reciprocally between bacteria sourcing from animals and humans (15, 24). Generally, bacteria develop resistance through multiple mechanisms including gene mutation, horizontal gene transfer, antimicrobial agent inactivation by enzymes, drug target modification, the alteration of membrane permeability, and efflux pumps (12). The UE current legislation regarding antibiotic resistance has been preoccupied with reducing the use of the latest antimicrobials used in therapy in animals (32). Nevertheless, antibiotics are prescribed by veterinarians to treat mainly bacterial diseases but not only (29). Based on available data, AMR is estimated to produce hundreds of millions of human deaths, severe financial losses, and a significant fall in livestock production by 2050 (14). Therefore, nowadays an increase in antibiotic resistance is considered to be one of the major healthcare sector crises (7).

For millennia, bees provided humanity with their products (honey, royal jelly, bee bread, propolis, and pollen) used for nutritional, prophylactic or therapeutic purposes (6, 11, 16, 19). Propolis is a traditional, potentially medicinal product with several health benefits (9, 11, 30). Nowadays, bee-products' uses have expanded, e.g. that of propolis, which has antibacterial, anti-inflammatory and antioxidant properties (21) due to its high content of phenolic compounds (28).

Analysis of the propolis action mechanisms revealed involvement in the permeability of the cellular membrane of microorganisms by reducing the mitochondrial membrane potential and adenosine triphosphate (ATP) production, as well as in decreasing bacterial mobility (25). Propolis is active both against Gram-positive and Gram-negative bacteria, but also on aerobic and anaerobic bacteria (23). Similarly, propolis actively inhibits growths of MRSA and VRE isolates (10). The composition of honey produced by honeybees (*Apis melifera*) is closely related to the geographical area, flora, climate, and other environmental conditions (25, 28). The use of honey in traditional medicine to treat infections is one of the oldest therapeutic procedures applied during microbial infections; honey inhibiting the growth of over sixty species, including aerobic and anaerobic, Gram-positive, and Gram-negative bacteria (8, 9).

Bee products from a variety of geographical areas possess different levels of antimicrobial activities (25). Honey is composed of a complex mixture of carbohydrates (77-86%), the most abundant being fructose 38.5% and glucose 31.0% (26). The high viscosity and high osmotic pressure are produced by a low percentage of water (20%) (20). Additionally, honey contains vitamins, flavonoids, aminoacids, enzymes, minerals, and phenolic acids (4). It has been reported that other phytochemicals, especially phenolic compounds, are essential for their antibacterial potency. The antibacterial activity of honey is mainly attributed to its osmolarity, H_2O_2 content, low pH, phenolic acid levels, and flavonoids. Phytochemical factors, such as fatty acids, peroxides, ascorbic acid, amylase, terpenes, phenols, benzoic acid, and benzyl alcohols, are factors that make honey active against pathogenic bacteria and produce either bacteriostatic or bactericidal efficacy (4), which biological activity increases its importance for various therapeutic fields (3).

The aim of the present research was to evaluate the efficacy of Romanian products such as polyfloral and rapeseed honeys as well as 20% and 30% commercial propolis for human use in controlling the susceptibility of resistant *Staphylococcus* spp. strains isolated from extensively farmed pigs by changing their classification category from resistant and intermediate to sensitive subsequent to bee product exposure.

Material and methods

Sampling. The antibacterial activity of honey and propolis were tested against a collection of staphylococci from the skin and nostrils of extensively farmed pigs (n = 421). The samples were collected in an interval of four years and were included in the bacterial strain deposit of the Veterinary Direction of Alba County Romania.

Antimicrobial susceptibility tests were performed on 30 isolates to investigate their antibiotic-resistance profile by the agar dilution and disk diffusion methods as described in the Clinical and Laboratory Standards Institute (CLSI). These isolates were tested against twelve different antimicrobial agents (see below) using the Kirby-Bauer disk-diffusion method, before the *in vitro* treatment with honey and propolis.

Preparation of the bacterial inoculum. For the antimicrobial resistance testing, only pure, viable cultures, not older than 18-24 hours, were used. Each organism (bacterial strain deposit of the Veterinary Direction of Alba County Romania) was revitalized by cultivation on nutrient agar at 37°C, for 18-24 h. Three to six identical colonies were selected and transferred to sterile demineralized distilled water, adjusting the suspension to 0.5 degrees on the McFarland scale. The density of suspension was confirmed by the Nephelometer Sensititre.

Antibacterial testing of bee products: honey and propolis. Polyfloral (n = 1) and rapeseed honey (n = 2) and two different concentrations of propolis (20% and 30%, dissolved in 70% ethanol) were tested. The propolis and rapeseed honey used were those available on the Romanian biological product market, extracted in 70% alcohol, while the polyfloral honey was obtained directly from individual beekeepers. To obtain the 10% honey dilution, 9.9 mL cation-adjusted Mueller Hinton broth was mixed with 1.1 mL of each rapeseed and polyfloral honey. Since in preliminary studies it was demonstrated that a 10% concentration propolis is totally destructive against bacteria, the ready-to-use, commercially available 20 and 30% concentrations were chosen to work with this bee-product.

For testing the activity of honey and propolis on bacterial isolates the subsequent formulas were used:

For honey

9.9 mL Mueller Hinton broth + 1.1 mL of honey + 0.01 mL bacterial inoculum

For propolis

9.9 mL Mueller Hinton broth + 0.01 mL propolis 20% or 30% + 0.01 mL bacterial inoculum

The mixtures were incubated for 24 h at 37°C, then spread on nutrient agar, and new inocula were prepared for the Kirby Bauer method repetition, following the steps described below.

Antimicrobial susceptibility testing. The Kirby-Bauer diffusion method was used to investigate the antibiotic resistance/sensitivity profile of all isolated staphylococci.

Twelve antibiotics, namely: colistin (CL), erythromycin (E), sulfamethoxazole/trimethoprim (SXT), penicillin (P), florfenicol (FFC), vancomycin (VA), tetracycline (TE), imipenem (IMP), marbofloxacin (MAR), cefoxitin (FOX), clindamycin (CD), methicillin (ME) were used. The results were interpreted according to the CLSI M100-Ed33, the strains ranking as resistant (R), intermediate (I) and sensitive (S). The MAR index was calculated by the method described by Krumperman (16) for all tested *Staphylococcus* spp. strain. Similarly, the same diffusion method was applied to staphylococci following the contact between the isolated bacterial strains and the tested bee products.

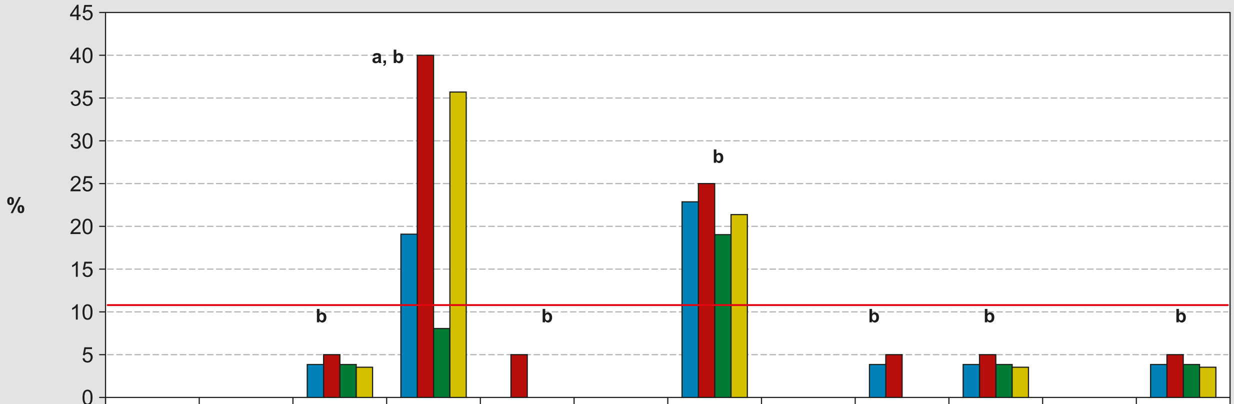
Results and discussion

Honey and bee products in general represent the most frequently used natural products for dietary and therapeutic purposes in different cultures all over the world (1, 2). Antimicrobial susceptibility compared to the *in vitro* effects of polyfloral, rapeseed honeys and propolis on thirty staphylococci strains were tested to evaluate the potential of natural bee products in reducing bacterial antimicrobial resistance. For that the Kirby Bauer diffusion method was applied by use of twelve antibiotics (colistin (CL), erythromycin (E), sulfamethoxazole/trimethoprim (SXT), penicillin (P), florfenicol (FFC), vancomycin (VA), tetracycline (TE), imipenem (IMP), marbofloxacin (MAR), cefoxitin (FOX), clindamycin (CD), methicillin (ME)) against the staphylococcal strains exposed to polyfloral, rapeseed honeys and propolis treatment for 24 hours.

The results of this study indicated that 43.34% of the staphylococci isolated from pigs represented *Staphylococcus xylosus*, 33.33% *Staphylococcus sciuri*, and 26.66% – *Staphylococcus lentus*. A relatively low MAR index was calculated for 90% of the strains, as follows: 0 in 26.7%, 0.08 in 20%, and 0.16 in 43.3%

Tab. 1. The initial sensitivity/resistance of the isolated *Staphylococcus* spp. strains to antibiotics

	CL	E	SXT	P	FFC	VA	TE	IPM	MAR	FOX	CD	ME	MAR index
Staphylococcus sciuri	I	I	S	R	S	R	S	S	S	S	I	S	0.16
Staphylococcus sciuri	I	I	S	R	S	R	S	S	S	S	I	S	0.16
Staphylococcus xylosus	I	I	S	R	S	R	S	S	S	S	S	S	0.16
Staphylococcus sciuri	I	I	S	R	S	R	S	S	S	S	I	S	0.16
Staphylococcus lentus	I	R	S	R	S	R	S	S	S	S	I	S	0.25
Staphylococcus xylosus	I	I	S	R	I	S	R	S	I	S	S	I	0.16
Staphylococcus xylosus	I	I	S	S	S	S	S	S	S	S	I	I	0
Staphylococcus sciuri	I	S	S	S	I	S	S	S	S	S	S	S	0
Staphylococcus lentus	I	I	S	R	S	S	S	S	S	S	S	S	0.08
Staphylococcus lentus	I	I	S	R	S	S	S	S	S	S	I	I	0.08
Staphylococcus sciuri	I	I	S	I	S	S	S	S	S	S	I	S	0
Staphylococcus sciuri	I	I	S	S	I	S	S	S	S	S	S	S	0
Staphylococcus sciuri	I	I	S	R	S	R	S	S	S	S	I	I	0.16
Staphylococcus sciuri	I	I	S	S	S	S	S	S	S	S	S	I	0
Staphylococcus lentus	I	R	I	S	S	S	R	S	S	S	I	I	0.16
Staphylococcus lentus	I	I	I	S	S	S	S	S	S	S	I	S	0
Staphylococcus xylosus	I	I	S	R	S	S	R	S	S	S	I	S	0.16
Staphylococcus xylosus	R	S	S	S	S	S	S	S	S	S	S	S	0.08
Staphylococcus xylosus	I	I	S	S	I	S	S	S	S	S	S	S	0
Staphylococcus xylosus	I	I	S	R	S	S	S	S	S	S	S	I	0.08
Staphylococcus xylosus	I	I	S	S	S	S	S	S	S	S	I	S	0
Staphylococcus xylosus	I	I	S	R	I	S	R	S	S	S	I	I	0.16
Staphylococcus xylosus	I	S	R	R	S	S	R	S	S	S	S	R	0.33
Staphylococcus xylosus	I	I	S	S	I	S	R	S	S	R	I	I	0.16
Staphylococcus sciuri	I	I	S	R	S	S	S	S	S	S	I	I	0.08
Staphylococcus lentus	R	I	S	S	I	R	S	S	S	S	I	I	0.16
Staphylococcus xylosus	R	I	S	R	S	S	S	S	S	S	I	S	0.16
Staphylococcus sciuri	I	I	S	R	S	S	S	S	S	S	I	S	0.08
Staphylococcus lentus	R	R	R	R	R	S	R	S	R	S	S	S	0.58
Staphylococcus lentus	I	R	S	S	R	S	S	S	S	S	S	I	0.16
Percentage of strains resistant by antibiotic	13.3	13.3	0.07	56.7	6.66	23.3	23.3	0	3.33	0	0	3.33	



	CL	E	SXT	P	FFC	VA	TE	IMP	MAR	FOX	CD	ME
Rapeseed honey	0	0	3.84	19.23	0	0	23.07	0	3.84	3.84	0	3.84
Polyfloral honey	0	0	3.84	40	5	0	25	0	5	5	0	5
Propolis 20%	0	0	5	8	0	0	19.23	0	0	3.84	0	3.84
Propolis 30%	0	0	3,57	35,71	0	0	21.42	0	0	3.57	0	3.57

Fig. 1. The percentages of tested strains for which the classification has changed from resistant to sensitive. It is visible that polyfloral honey is the most effective in changing the antibiotic resistance of staphylococci against most antibiotics. The bar indicates the average percentage of strains with changing classification for pooled tested antibiotics
Explanations: CL – colistin; E – erythromycin; SXT – sulfamethoxazole/trimethoprim; P – penicillin; FFC – florfenicol; VA – vancomycin; TE – tetracycline; IMP – imipenem; MAR – marbofloxacin; FOX – cefoxitin; CD – clindamycin; ME – methicillin; a – indicates the statistical significance of the differences $p < 0.05$, 0.01, 0.001 between different honeys for the same antibiotic; b – $p < 0.05$, 0.01, 0.001 between different antibiotic categories and their R or I status reclassification induced by honeys and propolis, as represented by the bars

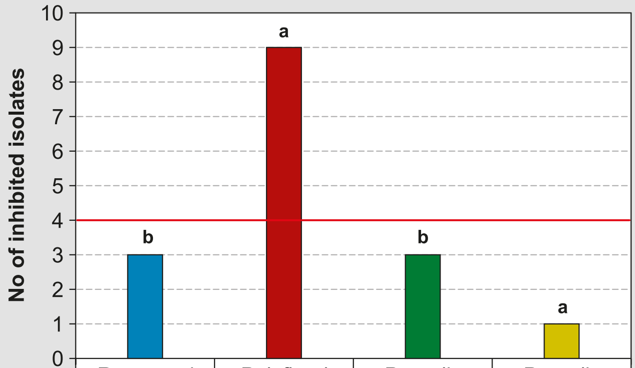
of the strains. Nevertheless, 10% of the strains were resistant or highly resistant (0.25; 0.33, and 0.58), posing a risk to the animals, caretakers, and the environment. Similarly, these could represent a source for further contamination of contacts and spread of the resistance around the farms where the swine were raised (Tab. 1).

The highest resistance was observed against penicillin, followed by vancomycin, and tetracycline, while for colistin and erythromycin, the resistance was lower. Very high sensitivity was observed towards imipenem, cefoxitin, and clindamycin (Tab. 1).

In this study, exposing *in vitro* bacterial strains to honey or propolis led to a significant ($p < 0.05$-0.001) increase in the size of the inhibition zone when compared to the initial evaluation of the resistance/sensitivity. The results varied from one type of honey and propolis to another (Fig. 1-3).

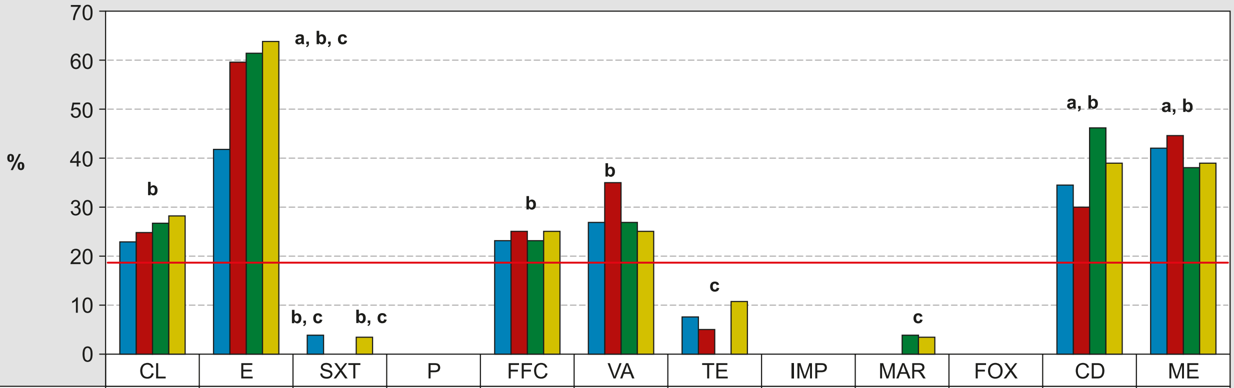
According to the antimicrobial susceptibility results, the highest number of strains for which the classification from R to S changed were found to penicillin and tetracycline, followed by sulfamethoxazole, methicillin, and cefoxitin where just one strain became sensitive after the exposure. 40% of the strains treated with polyfloral honey switched from their initial classification towards sensitive in the case of penicillin, followed by 35.71% when 30% propolis was used. Lower percentages were obtained with rapeseed honey and 20% propolis. The lowest percentage (8%) was obtained for 20% propolis, while rapeseed honey was more efficient. In the case of increased sensitivity to tetracycline, the results were approximately equal for honey and propolis.

The literature showed that honey contains mainly carbohydrates, water, and minor components (proteins, minerals, phytochemicals, and antioxidants), which are



Rapeseed honey	Polyfloral honey	Propolis 20%	Propolis 30%
3	9	3	1

Fig. 2. Number of isolates which were inhibited by the activity of honey and propolis: an enhanced activity of the polyfloral honey could be observed against more numerous staphylococcal strains, as opposed to rapeseed honey or propolis. Interestingly, the higher propolis concentration (30%) inhibited less strains. The bar indicates the average number of all tested strains treated with honeys and propolis
Explanations: a – indicates the statistical significance of the differences $p < 0.01$ between polyfloral honey and the rapeseed honey or propolis used; b – indicates the statistical significance of the differences $p < 0.05$ between polyfloral honey and the rapeseed honey or propolis used



	CL	E	SXT	P	FFC	VA	TE	IMP	MAR	FOX	CD	ME
Rapeseed honey	23.07	42.3	3.84	0	23.07	26.92	7.69	0	0	0	34.61	42.3
Polyfloral honey	25	60	0	0	25	35	5	0	0	0	30	45
Propolis 20%	26.92	61.53	0	0	23.07	26.92	0	0	3.84	0	46.15	38.46
Propolis 30%	28.57	64.28	3.57	0	25	25	10.71	0	3.57	0	39.28	39.28

Fig. 3. The percentages of tested strains for which the classification has changed from intermediate to sensitive. For the strains with an intermediate resistance, an increase in sensitivity is seen towards CL and E, but also CD and ME, with no significant differences by bee product, but by antibiotic. The bar indicates the average percentage of strains with changing classification for pooled tested antibiotics

Explanations: CL – colistin; E – erythromycin; SXT – sulfamethoxazole/trimethoprim; P – penicillin; FFC – florfenicol; VA – vancomycin; TE – tetracycline; IMP – imipenem; MAR – marbofloxacin; FOX – cefoxitin; CD – clindamycin; ME – methicillin; a – $p < 0.05$, b – $p < 0.01$, c – $p < 0.001$ between different antibiotic categories and their R or I status reclassification induced by honeys and propolis, as represented by the bars

responsible for its medicinal and biological activities. According to this, different types of honey possessed different efficacies and mechanisms of action against the same type of bacteria (2).

In addition to the increase in the inhibition zone, honey and propolis exhibited inhibitory activity against several tested strains. The highest number of strains inhibited by the treatment was observed with polyfloral honey (9 strains), followed by rapeseed honey and 20% propolis (3 strain), while only one strain was inhibited by 30% propolis. The antibacterial activity of propolis was conditioned by the variations in extraction procedures, and in the flora, which influenced the collection by the bees and made a difference inits composition (1, 27). In recent years, the evaluation of the antibacterial activity of propolis was based on the quantification of total phenolics and flavonoids. Total phenolics in the samples with the highest and lowest content were directly proportional to the content of flavonoids and antioxidant properties (23). Phenolic compounds are bioactive compounds, defined as organic compounds with an aromatic ring chemically bound to one or additional hydrogenated substituents in the presence of corresponding functional derivatives (33).

The results obtained by using the propolis in conditioning the antimicrobial resistance of staphylococci indicated that most strains changed their category from moderately sensitive to sensitive, with the highest percentage being recorded in the case of erythromycin and 30% propolis, followed by 20% propolis (61.53%), polyfloral honey (60%), and rapeseed honey 42.3% (Fig. 3).

Honey and propolis offer natural alternatives to antimicrobial treatment and can be considered as effective means of influencing antimicrobial resistance in staphylococcal strains. The effectiveness of various types of honey and propolis against different staphylococci depends on the specific type of honey, likely influenced by factors such as geographic location, vegetation type, weather conditions, and preservation methods. To develop tailored strategies aimed at targeting various bacterial genera and influencing their resistance to antimicrobials, further studies involving diverse isolates and types of honey/propolis are necessary. Ultimately, the goal of these studies is to contribute to the reduction of overall antimicrobial usage.

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