SUMMARY OF THE PhD THESIS

In vitro impact of apitherapy on the phenomen of antibiotic resistance in *Staphylococcus* strains from extensively raised pigs

PhD student **Cristina Ioana Lazăr**

Scientific advisor **Prof.univ. dr. Marina Spînu**

The UN's objective is to achieve optimal health for people, animals and the environment, and antibiotic resistance is recognized as an "One Health" challenge, due to the emergence and rapid spread of antibiotic-resistant bacteria among people, animals and the environment on a global scale (ROUSHAM, 2018). As well as being a 'One Health' problem, AMR is also a 'One world' problem. The globalization of the food system with the ever-expanding movement of animals and agricultural products, combined with the exponential increase in the number of trips made by humans facilitates the rapid spread and mixing of genes resistant to antimicrobial substances (ROBINSONA, 2016).

Antimicrobial agents are widely used in both veterinary and human medicine. The intensive use of antibiotics in animals can promote the persistence of resistance genes to bacteria with zoonotic potential or able to transfer these resistance genes to pathogens adapted to humans, or directly in their intestinal microbiome, through food or the environment (ARGUDIN, 2017) .

Although staphylococci of animal origin are commensals of the skin and mucous membranes, they do not live in genetic isolation, being in close contact with a variety of other pathogens. In this way, in the case of an increased bacterial density, the genetic material can be transferred not only between staphylococci intra-species or interspecies, but also between staphylococci and other Gram-positive bacteria, a phenomenon called "gender exchange". There are studies demonstrating that staphylococci can act as donors or recipients of resistance genes. Moreover, they can be transferred to other animals or humans, through direct contact, or through contact with animal excretions, through sneezing, coughing or licking (SCHWARZ, 2018).

Therefore, research in the field and identification of a new generation of antibiotics has become a priority. However, testing a new antibiotic before it can be marketed takes a long time. Moreover, limiting their use only to patients who do not respond to existing products on the market leads to a decrease in profitability for large pharmaceutical companies. So the development of new antibiotics is no longer considered an interesting activity (COMBARROS-FUERTES, 2020).

In this context, honey and honey products, an important part from the nutritional, and the therapeutic properties' point of view, as well as due to their implications on the health and hygiene status of people, are known as viable alternatives to antibiotics (STEFANIS, 2023).

The doctoral thesis entitled "The impact of *in vitro* apitherapy on the phenomenon of antibiotic resistance in *Staphylococcus* strains from extensively raised pigs", is structured in two parts, each of them comprising a series of chapters, designed according to the norms in force.

Part 1 entitled "Current state of knowledge" is divided into three chapters and describes the information from the specialized literature regarding the emergence of antibiotic resistance, the mechanisms of antibiotic resistance present in staphylococci, as well as bee products and their antibacterial effect.

The first chapter provides information on the emergence of antibiotic resistance, from the evolution of resistance genes to the emergence of multidrug resistance (MDR).

The second chapter briefly presents the information from the specialized literature regarding antibiotic resistance in staphylococci, and the mechanisms of transmission of resistance genes to them.

In the third chapter, information is presented on the significant antibacterial properties of bee products, especially honey and propolis, which make them potential candidates for fighting bacterial infections and reducing antibiotic resistance.

The second part presents the personal contribution and is structured in 7 chapters that contain the results of personal research according to the purpose and objectives presented in **chapter 4**.

In Chapter 5, data related to the origin of the samples, the protocol for the isolation and identification of the *Staphylococcus* spp. were presented. In this study, 49 samples from the skin behind the ears and from the anterior nostrils of healthy pigs from different households reared extensively, traditionally, were analyzed and 40 strains of *Staphylococcus* spp. were isolated. All the samples that entered the work were individually identified and, to avoid confusion, they received the coding "PD-followed by the current number, ex: PD1 , PD2, PD3, etc.

For the isolation of the strains, specific culture media represented by Chapman agar, Columbia agar with blood were used, and the presumptive colonies were identified based on morphological characters, after which the bacterial species were identified biochemically with the help of ApiStaph galleries.

Three species of staphylococci were identified, represented by *Staphylococcus xylosus* isolated in 47.5% of the strains tested, followed by *Staphylococcus lentus* isolated in 30%, and *Staphylococcus sciuri*, isolated in 22.5% of the strains tested.

Chapter 6 presents the antibiotic susceptibility assessment technique, the antibiotics used in this study, as well as the results of the initial antibiotic susceptibility tests used, all defining the antibiotic resistance profile of the isolated bacteria. In the study, antibiotic susceptibility was tested for all 40 strains of staphylococci isolated from pigs, using the diffusion method (Kirby-Bauer).

Twelve types of antibiotics were applied, which belong to different classes: penicillin-P10, tetracycline-TE30, erythromycin-E30, Clindamycin-CD2, Colistin-CL25,

Trimetroprim/ Sulphamethoxazole-SXT25, Florfenicol-FFC30, Marbofloxacin-MAR5, Vancomycin-VA30, Methicillin-ME30, Imipenem-IPM10, Cefoxitin-FOX10.

Multiple cases of antibiotic resistance were observed, but an increased rate of penicillin resistance should be noted, where 55% of staphylococcal strains isolated were resistant, followed by tetracycline resistance at 30%. 10% of the isolated strains showed resistance to colistin and erythromycin, and 7.5% were resistant to Trimetroprim/ Sulphamethoxazole, while only 2.5% of the strains were classified as resistant to methicillin and marbofloxacin.

In addition, multidrug resistance (MDR) strains have also been identified. Indices of multiple antibiotic resistance (MAR) were calculated with reference to the tested antibiotics. All isolates had a MAR index between 0-0.58, and none had a MAR index=1. The indices of multiple resistance to antibiotics were below 0.2 in 31 of the 40 strains of bacteria studied. However, 10% of the strains were resistant or highly resistant (MAR 0.25, 0.33 and 0.58), posing a risk to animals, caretakers and the environment. Similarly, they could be a source for further contact contamination and the spread of resistance around the low-input farms where the pigs were reared (table 1).

The highest MAR indices were detected in PD40 represented by *Staphylococcus lentus* (0.58), PD30 represented by *Staphylococcus xylosus* (0.33) and PD7 represented by another strain of *Staphylococcus lentus* (0.25).

In **chapter 7**, the antibacterial properties of the tested bee products are evaluated. Rapeseed honey and polyfloral honey were analyzed as such, and propolis was evaluates for its' efficacy in the two different concentrations of 20% and 30%, determining the total polyphenol content and antioxidant capacity.

The total polyphenol content varies significantly between honey and propolis samples, with values between 231.52 mg GAE/100 g and 344.29 mg GAE/100 g. The highest value was recorded for propolis in 20% concentration, and rapeseed honey recorded the lowest value, 231.52 mg GAE/100 g.

The antioxidant capacity also varied, with values between 94.06 RSA (%), in the case of 20% propolis, the lowest value being attributed to polyfloral honey with 18.67 RSA (%).

It has been scientifically proven that the concentration level of phenolic compounds and the antioxidant activity in a sample of honey or propolis are correlated with their antimicrobial potential. Thus, the higher the phenolic content and antioxidant activity are, the higher the antibacterial potential (ISPIRYAN, 2024) is.

Chapter 8 described the testing of the antimicrobial activity of bee products against isolated strains of staphylococci. This chapter includes the steps of describing the experimental protocol, followed by results and discussion.

In order to evaluate the antimicrobial activity of bee products (canola honey, polyfloral honey, 20% propolis and 30% propolis) against strains of *Staphylococcus* spp., both direct exposure to undiluted bee products and exposure to bee products of different concentrations were applied. Subsequently, these strains were also tested with the original antibiotics to observe any changes in their antimicrobial susceptibility.

In the first stage, the diffusion technique was performed in Kirby-Bauer agar gel. The bacterial suspension was prepared with a density of 0.5 McFarland. With the help of a sterile swab soaked in the bacterial solution, the excess liquid being removed, the bacterial culture was seeded on the surface of the plate containing Mueller Hinton agar, after which the plates were left for 10 min for surface drying. After the plates had dried, wells were made in the agar approximately 2 cm apart using a sterile perforator (6 mm diameter).

Using a pipette, the two types of honey: rapeseed honey and polyfloral honey and the two different concentrations of propolis (20 and 30%) were added to each particular well, after which the plates were incubated at 37°C for 24 for hours. The highest diameter for an inhibition zone was of 22 mm in *Staphylococcus xylosus* strain (PD30) and 20 mm in *Staphylococcus xylosus* strain (PD23) exposed to rapeseed honey, suggesting superior antimicrobial efficacy when compared to the other tested products. In the case of polyfloral honey and 20% propolis, the largest inhibition zone was of 17mm, and in case of 30% propolis, the largest zone of inhibition was of 16mm. This indicates, comparatively, the presence in rapeseed honey of some powerful bioactive compounds that can effectively inhibit the growth of staphylococci.

Table 1 Overview of the comparison of the obtained results with the initial ones.

	CL25	**E30**	**SXT25**	**P10**	**FFC30**	**VA30**	**TE30**	**MAR5**	**FOX30**	**CD2**	**ME30**
Rapeseed honey	+12 mm	+10 mm	+10 mm	+10 mm	+12 mm	+14 mm	+12 mm	+10 mm	+15 mm	+10 mm	+10 mm
Polyfloral honey	+12 mm	+11 mm	+13 mm	+10 mm	+14 mm	+14 mm	+12 mm	+9 mm	+10 mm	+11 mm	+12 mm
Propolis 20%	+8 mm	+13 mm	+14 mm	+12 mm	+14 mm	+15 mm	+11 mm	+12 mm	+15 mm	+10 mm	+14 mm
Propolis 30%	+7 mm	+13 mm	+13 mm	+15 mm	+14 mm	+11 mm	+12 mm	+12 mm	+15 mm	+14 mm	+14 mm

In the next stage of the study, the staphylococci strains were exposed to 10% rapeseed and polyflora honey, and to propolis. The following formulas were used to test the activity of honey and propolis on bacterial isolates: for honey: 9.9ml Mueller Hinton bullion+1.1 ml honey+0.01 ml bacterial suspension, and for propolis: 9.9ml Mueller Hinton broth +0 .01 ml propolis 20% or 30% + 0.01 ml bacterial suspension After

homogenization, they were incubated at 37°C for 24 hours, after which they were seeded on nutrient agar and tested for antibiotic resistance, using the Kirby Bauer technique. The results obtained from these tests were recorded and analyzed and compared with the initial results (Table 2). Nine of the 30 strains exposed to polyfloral honey were completely inhibited, 3 strains tested after exposure to rapeseed honey and 20% propolis were also completely inhibited, and 30% propolis completely inhibited only one strain of staphylococcus.

Tabel2. The percentage of strains tested for which the classification changed from resistant to susceptible

%	**CL25**	**E30**	**SXT25**	**P10**	**FFC30**	**VA30**	**TE30**	IPM5	**MAR5**	**FOX30**	**CD2**	**ME30**
Rapeseed honey	0	0	3,84	19,23	0	0	23,07	0	0	3,84	0	3,84
Polyflower honey	0	0	5	40	5	0	25	0	0	5	0	5
Propolis 20%	0	0	3,84	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

The results revealed the impact of apitherapy, in different concentrations, on the susceptibility of *Staphylococcus* spp. strains to antibiotics.

After all the tests, it was observed that the bee products used in the study have the ability to increase the susceptibility to all tested antibiotics. However, none of the strains tested that did not develop an inhibition zone in the initial antibiogram, did not become sensitive or intermediately sensitive following the application of treatment with bee products.

Depending on the tested antibiotic, significant differences were observed, so tetracycline and vancomycin showed the most differences in the diameter of the zone of inhibition of strains treated with bee products, compared to the initial antibiogram.

The general conclusions that emerge from the paper entitled: "The impact of *in vitro* apitherapy on the phenomenon of antibiotic resistance in *Staphylococcus* strains from extensively raised pigs" can be found in **chapter 9**.

The results of this study indicated that the distribution of isolated strains is unexpectedly narrow (three species: *Staphylococcus xylosus* 43.34%; *Staphylococcus sciuri* 33.33%; *Staphylococcus lentus* - 26.66%).

Although the MAR index was relatively low in 90% of the strains (0 in 26.7%, 0.08 in 20% and 0.16 in 43.3% of the strains), 10% of the strains were resistant or highly resistant (MAR 0.25; 0.33 and 0.58) suggesting an increased risk to animals, caretakers and the environment. Similarly, they could be a source for further contamination of contacts and spread of resistance around the farms where the pigs were reared.

The increased resistance observed to penicillins, followed by tetracycline is justified, these antibiotics being the most widely used in the different pathologies in pigs, while for colistin and erythromycin the resistance was lower.

In this study, *in vitro* exposure of bacterial strains to honey or propolis resulted in a significant increase ($p<0.05$-0.001) in the size of the inhibition zone when compared to the initial resistance/susceptibility assessment. The results were influenced by the type of product and the concentration used.

Both honey and propolis have demonstrated significant antibacterial properties. Both substances were able to inhibit the growth of staphylococci, indicating their potential in fighting bacterial infections.

The antibiogram performed before and after treatment with honey and propolis revealed a significant change in the sensitivity of the tested bacteria. Initially resistant strains became sensitive to common antibiotics, suggesting that treatment with honey and propolis may reduce bacterial resistance

Chapter 10 highlighted the originality and innovative contributions of the thesis:

It is a study that investigated staphylococci isolated from healthy pigs, initially evaluating their resistance to antimicrobials, and subsequently treating the strains with bee products (polyfloral honey, rapeseed honey, propolis 20% and propolis 30%), to observe changes in resistance to the initial antibiotics.

This approach is innovative and offers significant contributions in the field of microbiology as well as the therapy of bacterial infections.

The study is part of a specific agricultural framework, where the use of antibiotics is frequent and the surveillance of antimicrobial resistance is crucial.

The study provides important data on resistance profiles of staphylococci from pigs to various antibiotics, the information being essential due to the close owner/keeper contact in the extensive system both for the proper management of infections within farms and for preventing the transmission of resistance to humans.

The application of bee products (polyflora honey, rapeseed honey and propolis) is innovative, considering the known antimicrobial properties of these substances, still insufficiently studied in the context of resistant staphylococci of animal origin.

The study is innovative because of the comparison of the effects of the two types of honey (rapeseed and polyfloral) and propolis (20% and 30%) adding an original element to the study, highlighting the variations in the effectiveness of bee products.