Scientific Works. Series C. Veterinary Medicine. Vol. LXIII (2)
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

DETERMINATION OF ANTIBIOTIC RESIDUES IN HONEY USING DIFFUSIMETRIC METHODS

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Abstract

One of the major risks resulting from the consumption of honey is the presence of drug residues, especially antibiotics, because they are widely used for treating various diseases. The study aimed to evaluate antibiotic residues in honey, in terms of quality and quantity, by diffusimetric methods. Thus, the studied antibiotics were oxytetracycline and streptomycin, and their action was studied using three bacterial strains, namely Bacillus subtilis, Staphylococcus aureus ATCC 6538 and Staphylococcus aureus ATCC 25923. S. aureus ATCC 25923 and S. aureus ATCC 6538 strains proved to be very sensitive to oxytetracycline and streptomycin. Bacillus subtilis showed no zone of inhibition for the 4 concentrations of oxytetracycline and for all 7 concentrations of streptomycin, indicating the high degree of resistance of the bacteria to these antibiotics. The analysis of honey samples contaminated with oxytetracycline and streptomycin showed inhibition zones with radius segments that were not strictly directly proportional to the antibiotic's concentration. In this respect, the tests carried out revealed the presence of inhibition zones even around the negative control. Both as such and diluted, honey caused the inhibition of bacterial growth, inhibition zones being directly proportional to the percentage of honey. In view of the fact that the honey itself possesses antibacterial properties, testing of honey samples in order to identify antibiotic residues cannot be achieved by microbiological methods, since there is a risk of obtaining false-positive reactions.

Key words*: antimicrobials, residues, honey, oxytetracycline, streptomycin.*

INTRODUCTION

For humans, nutrition is a factor with permanent action, which determines the conduct of metabolic processes, as food is the source and moderator of exchange processes. Also, the character of nutrition affects the system's functions, particularly enzymatic and hormonal factors, in order to maintain body homeostasis (Shils and Shike, 2006).

Food safety has become increasingly more difficult to obtain, both because of the wide range of foods, and also because of the air pollution that does not cease to intensify through the toxic gases, industry and agriculture (Nestle, 2013; Sun et al., 2017).

One of the major risks resulting from the consumption of honey is the presence of drug residues, especially antibiotics, because they are widely used for treating various diseases.

Because antibiotic residues present in food affect human consumers causing various problems such as antimicrobial resistance, immunoreactivity phenomena, imbalances at intestinal level, and toxicity, antibiotics were banned from administration to animals in order to stimulate productions, and, if used to combat certain diseases, a withdrawal period was imposed to allow the elimination of residues from the body (Beilke and Fritz, 2016; Solomon et al., 2006).

Maintaining under control of this chemical risk and keeping the hygienic quality of food were carried out by establishing maximum residue limits meant to keep the residues of antibiotics at a level that can not affect the human body and thus, methods by which they are determined both quantitatively and qualitatively were established. In general, the methods

used for this purpose are complex and require appropriate equipments.
The aim of this paper was to conduct a microbiological study for the qualitative and quantitative determination of antibiotics residues in known concentrations and to assess the effectiveness of these methods for determining antibiotics residues in honey.

MATERIALS AND METHODS

The study aimed to evaluate antibiotic residues in honey, in terms of quality and quantity, by diffusimetric methods. The aim was to determine the effectiveness of the method for two antibiotics present in different known concentrations in honey samples contaminated in the lab. Thus, the studied antibiotics were oxytetracycline and streptomycin, and their action was studied using three bacterial strains, namely *Bacillus subtilis*, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 25923. Therefore, it was targeted a possible relationship of proportionality between the concentration of the antibiotic and the area of the inhibition zone obtained.
To accurately observe the correlation between the antibiotic concentration and the inhibition zone, honey samples were contaminated with the two antibiotics in known concentrations (Table 1). It should be noted that, under the Regulation (EU) No. 37/2010 of the European Commission on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, at present, the presence of antibiotic residues is no longer permitted in honey (virtually no maximum residue limits exist), but supervision in this area should continue as streptomycin and oxytetracycline were the most commonly used antibiotics for treating bacterial diseases in bees (Bargańska et al., 2011; Galarini et al., 2015; Kaufmann et al., 2003; Korkmaz et al., 2017).
Thus, the honey was contaminated with oxytetracycline in concentrations between 6400 µg/kg – 100 mg/kg and streptomycin in concentrations between 6400 µg/kg – 100 µg/kg (Table 1).
Inoculation of Petri plates was done as follows: a sterile cotton swab was immersed in the bacterial suspension, removing the excess liquid by pressing it to the inner wall of the tube, after which it was rubbed uniformly over the entire surface of the plate by making parallel moves and by turning the plate by 60 degrees.
With a pipette tip, wells were made in the inoculated culture medium (agar), the plates being ready for pipetting honey samples. Next, the contaminated honey samples were pipetted in the wells, and also the negative control (there was used a 1:1 mixture of honey and saline solution).
All Petri plates were incubated for 24 hours at a temperature of 37°C without special atmospheric conditions.

RESULTS AND DISCUSSIONS

After the incubation of the Petri plates for 24 hours, the cultures of *Bacillus subtilis*, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 25923 developed uniformly over the entire surface of the agar, with the exception of the zones where the wells were made and contaminated honey samples were pipetted (in the case of some antibiotic concentrations). The zones of inhibition were measured with a ruler, noting the radius segment of each area.
Staphylococcus aureus ATCC 25923 strain proved to be very sensitive to oxytetracycline and streptomycin, which caused the formation of inhibition zones approximately directly proportional to the antibiotic's concentration for all concentrations, including the negative control (Figures 1-4, Tables 2-3).

Table 1. Maximum admitted residue limits and concentrations of antibiotics accomplished experimentally in honey samples

Sample	Antibiotic	Maximum residue limits (µg/kg)	Used concentrations (µg/kg)
Honey	Oxytetracycline	- (0)	100; 200; 400; 800; 1600; 3200; 6400
	Streptomycin	- (0)	100; 200; 400; 800; 1600; 3200; 6400

Figure 1. Inhibitions zones for oxytetracycline (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 25923*)

Figure 2. Inhibitions zones for oxytetracycline (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 25923*)

Figure 3. Inhibitions zones for streptomycin (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 25923*)

Figure 4. Inhibitions zones for streptomycin (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 25923*)

Also, the strain of *Staphylococcus aureus* ATCC 6538 turned out to be very sensitive to the two antibiotics, as evidenced by the inhibition zones present for all of the concentrations used, including the negative control (Figures 5-8, Tables 2-3).

Figure 5. Inhibitions zones for oxytetracycline (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 6538*)

Figure 6. Inhibitions zones for oxytetracycline (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 6538*)

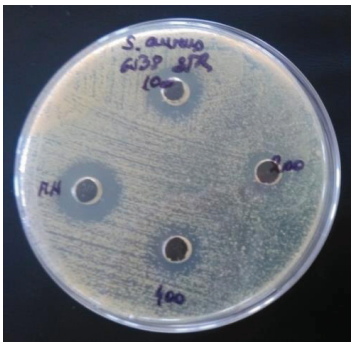

Figure 7. Inhibitions zones for streptomycin (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 6538)*

Figure 8. Inhibitions zones for streptomycin (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 6538*)

The plates inoculated with *Bacillus subtilis* showed no zones of inhibition for oxytetracycline in concentration of 100 µg/kg, 200 µg/kg, 400 µg/kg, 800 µg/kg and for the negative control, but only for 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations. Moreover, the culture of *Bacillus subtilis* showed no zone of inhibition for the 7 concentrations of streptomycin, indicating the high degree of resistance of the bacteria to this antibiotic (Figures 9-12; Tables 2-3). Thus, it was concluded that the strain is not suitable for the determination of streptomycin residues by diffusimetric methods, since it does not develop inhibition zones for the concentrations of interest in the food industry.

Figure 9. Inhibitions zones for oxytetracycline (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*B. subtillis*)

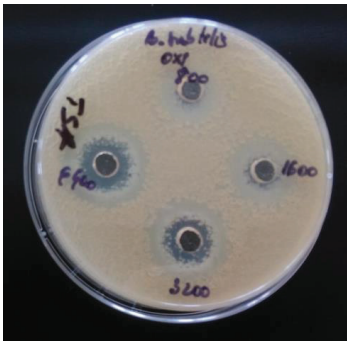

Figure 10. Inhibitions zones for oxytetracycline (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*B. subtillis*)

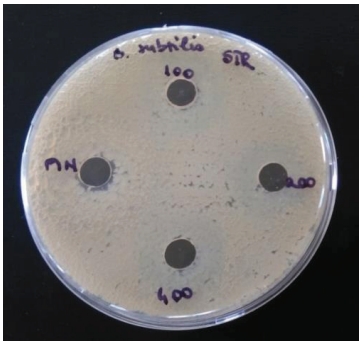

Figure 11. Inhibitions zones for streptomycin (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*B. subtilis*)

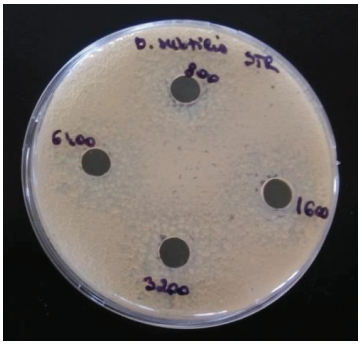

Figure 12. Inhibitions zones for streptomycin (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*B. subtilis*)

Table 2. Radius segments of the inhibition zones caused by oxytetracycline

Concentration (µg/kg)	The radius segments of the inhibition zone (mm)		
	S. aureus ATCC 25923	*S. aureus ATCC 6538*	*B. subtilis*
Negative control	5	4	0
100	3	4	0
200	4	5	0
400	5	5	0
800	7	6	0
1600	5	6	1
3200	8	7	3
6400	4	7	4

Table 3. Radius segments of the inhibition zones caused by streptomycin

Concentration (µg/kg)	The radius segments of the inhibition zone (mm)		
	S. aureus ATCC 25923	*S. aureus ATCC 6538*	*B. subtilis*
Negative control	5	6	0
100	4	4	0
200	3	3	0
400	5	3	0
800	6	4	0
1600	6	4	0
3200	6	2	0
6400	6	5	0

The conflicting results obtained from the analyzes of honey samples contaminated with antibiotics, respectively obtaining inhibition zones with radius segments that were not directly proportional to the concentration of antibiotic (in some cases) and even obtaining inhibition zones around the negative control, led to the suspicion that honey itself could have an antibacterial effect against studied bacterial strains.

To highlight the potential bacteriostatic action of honey, it was tested on the *Staphylococcus aureus* ATCC 6538 strain, both as such and in the form of binary dilutions (2^{-1}, 2^{-2}, 2^{-3}).

The radius segments of the inhibition zones caused by honey are shown in Table 4.

Table 4. Radius segments of inhibition areas caused by honey

Dilution	Radius segment of inhibition area (mm)
2^{0} (M)	17
2^{-1}	13
2^{-2}	4
2^{-3}	2

Thus, it was found that honey, even at 2^{-3} dilution, possesses antibacterial properties (Figure 13). In this situation, the results obtained after testing samples of honey contaminated with different antibiotic concentrations were not considered correct, being highly influenced by the antibacterial properties of honey.

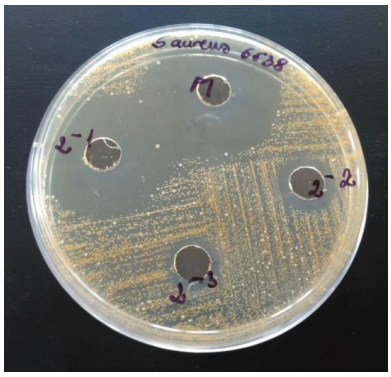

Figure 13. Areas of inhibitionfor honey – dilutions 2^0 (M), 2^{-1}, 2^{-2}, and 2^{-3} (*S. aureus ATCC 6538)*

CONCLUSIONS

The analysis of honey samples contaminated with oxytetracycline and streptomycin showed inhibition zones with radius segments that were not strictly directly proportional to the antibiotic's concentration.

In this respect, the tests carried out revealed the presence of inhibition zones even around the negative control.

Both as such and diluted, honey caused the inhibition of bacterial growth, inhibition zones being directly proportional to the percentage of honey.

In view of the fact that the honey itself possesses antibacterial properties, testing of honey samples in order to identify antibiotic residues cannot be achieved by microbiological methods, since there is a risk of obtaining false-positive reactions.

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