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# Anti-pathogenic activity on the body surface of adult workers of Apis mellifera

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Summary

**The aim of the study was to determine the anti-pathogenic activity on the body-surface of bee workers after applying selected acaricides, such as Amitraz or oxalic and formic acids. In two consecutive years, four experimental groups (5 colonies per group) were created. The 1st group was the control, the 2nd was treated with Apiwarol, the 3rd with oxalic acid, and the 4th with formic acid. Hive worker bees were collected three times a year from each colony: just before treatment as well as two and four weeks after treatment. The samples were shaken/rinsed for 10 min. at 8000 rpm, filtered and lyophilized. Anti-pathogenic activities were determined by the double application method, with SABG (Aspergillus niger, A. fumigatus), YPD (Candida albicans) and LB (Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Salmonella Typhimurium, Pseudomonas aeruginosa and Escherichia coli). The control group displayed antifungal and antibacterial activities against all of the pathogens analysed. After acaricide treatment, anti-pathogenic activities were reduced or disappeared within the four weeks of the experiment.**

**Keywords: acaricides, honey bee, anti-pathogenic activity, bacteria, fungus**

Behavioral, physiological and individual traits of bees and their parasites have been interactively developed by these organisms to better adapt to each other during their co-evolution. Pathogens have evolved specialized mechanisms for transmission either between individuals within a single generation, or between individuals from one generation and the next, or both, and the differences in the mode of transmission greatly affect the host's fitness (4). In the case of colonies of social insects, a subdivision of horizontal and vertical transmission into both intracolony and intercolony components can be made. Disease virulence and pathogen transmission in colonies of honeybees present a special case because the host's fitness depends on the ability of the colony to produce swarms. To understand apian epidemiology and disease virulence, one must distinguish between pathogen transmission and virulence at both the individual and colony level (4, 6).

Standard beekeeping practices, which include swarm control and apiaries with large numbers of colonies, will inevitably increase the horizontal transmission and decrease the vertical transmission of pathogens. One of these pathogens is *Varroa destructor*. Parasites feeding on the hemolymph of capped brood cause disturbances in the protein metabolism of the host. This destroys the neurohormonal balance and resistance mechanisms of bees (5, 6). Varroatosis is a global apicultural problem and an etiologic factor of CCD (Colony Collapse Disorder), which has been identified in Europe and the USA (2, 9). This disease is combatted with acaricides, which increases drug resistance of the mites and causes fatalities among bees in the apiary. Beekeepers have reported that a frequent use of these acaricides weakens apian resistance and thus favours the spread of other diseases, e.g. viral, bacterial or fungal ones.

The aim of the studies was to investigate the anti-pathogenic activity on the body surface of worker bees after the application of selected acaricides.

## Material and methods

In two consecutive years, four experimental groups (5 colonies per group) were created:

– in the first group, the control, no treatment with chemical compounds was performed,

– each colony in the second group was fumigated with one tablet of Apiwarol AS (12.5 mg Amitraz),

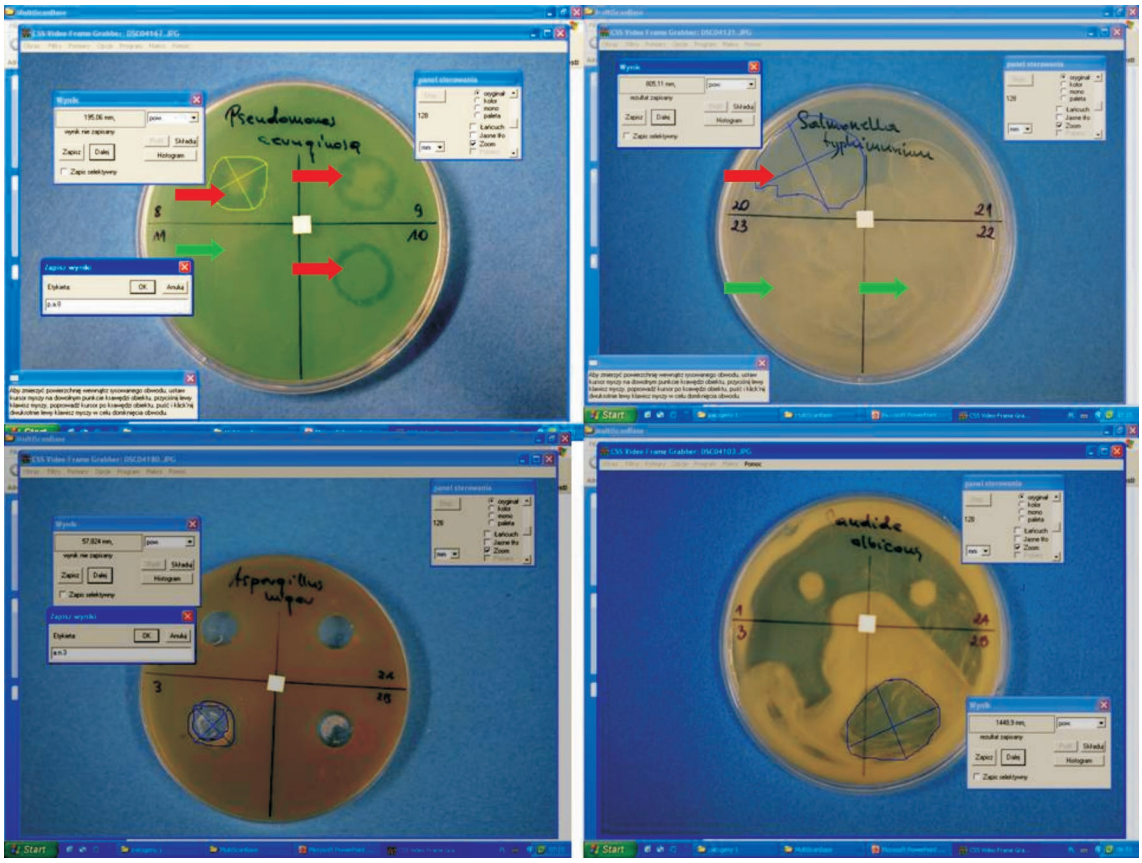

**Fig. 1. Determination of the antifungal and antibacterial activity of the proteins washed out of the body surface of the bees by calculating the area of the contaminated surface (mm²) on which the microorganisms did not develop, using MultiScan Base. Example**
Explanations: ➡ antifungal and antibacterial activity present – lack of development of the microorganisms; ➡ antifungal and antibacterial activity absent – microorganism development

– in the third group, bees were treated with a 3.2% oxalic acid solution in sugar syrup, 50 ml per colony,

– in the fourth group, experimental colonies were treated with formic acid vapours (concentration 60%).

Three samples comprising 10 mature hive worker bees were collected three times a year from each colony: just before treatment, as well as two and four weeks after treatment (5 colonies × 3 samples × 3 samplings × 2 seasons = 90 samples = 900 bees per group). The material was frozen in sterile bags at –8°C and stored for 1-2 months. Then, the samples were successively defrozen, 10 ml of a 1% detergent solution (Triton X-100) was added, and the samples were shaken/rinsed for 10 min. at 8000 rpm. After filtering each of the samples through Miracloth, a solution was obtained. The solution was frozen in a refrigerator at –40°C. The solutions were lyophilized for 18 hours with a Labconco FreeZone 2.5 unit. To this end, the lyophilizates were combined with 200 µl of distilled water. Subsequently, the mixtures (10 µl of each) were sprinkled on the following culture media by the double application method:

– SABG (11) – to determine the activity against *Aspergillus niger* (ATCC 16404) and *A. fumigatus* (ATCC 1022),

– YPD (10) – to determine the activity against *Candida albicans* (ATCC 10231),

– LB (1) – to determine the activity against *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 7468), *Salmonella* Typhimurium (ATCC 13311), *Pseudomonas aeruginosa* (ATCC 17853), and *Escherichia coli* (ATCC 10536).

The dishes with *A. niger* were incubated for 3 days at 37°C, those with *C. albicans* for 24 hours at 28°C, and those with bacteria for 24 hours at 30°C.

In all the microbiological tests, each dish was photographed (SONY α100) to determine the area on which there was no microorganism growth, using the MultiScan Base software (Fig. 1).

Statistical calculations were performed with the SAS software (11). Statistical differences between experimental factors were analysed by ANOVA (t-Student test).

## Results and discussion

In the first group, the control, treatment with any of the chemical substances produced antifungal or antibacterial activities against all of the pathogens analysed (Tab. 1 and 2). In all other groups, before treatment with varroacides, these activities were the same as in the control group during the four weeks of experiment. No activity against *A. niger*, *A. fumigatus* or *S. aureus* was observed in bees treated with Amitraz or oxalic and formic acid. Moreover, after applying oxalic acid, no activity against *S.* Typhimurium or *P. aeruginosa* (Tab. 2) was observed in the second and the fourth week. In workers treated with formic acid, no activity against *S.* Typhimurium appeared. After the treatment with acaricides, anti-pathogenic activities against *C. albicans* and *B. subtilis* were reduced (Tab. 1 and 2) and were lower in the fourth week than in the second week.

The results of an *in vivo* test on microorganisms confirmed a weaker activity of body-surface barriers in *A. mellifera* workers.

During the four weeks of the experiment, antifungal and antibacterial activity was markedly lower after treatment with acaricides than it was in the control group. These results correspond with reports from bee-

**Tab. 1. The antifungal activities in the washed-out body-surface samples of *A. mellifera* workers, calculated as the area (mm²) without pathogen development**

| Groups | Area (mm²) with antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | after two weeks | | | after four weeks | | |
| | *C. albicans* | *A. niger* | *A. fumigatus* | *C. albicans* | *A. niger* | *A. fumigatus* |
| Control | $1105.15^{a} \pm 0.23$ | $60.24 \pm 0.52$ | $62.54 \pm 0.35$ | $1110.00^{a} \pm 0.45$ | $60.55 \pm 0.25$ | $63.12 \pm 0.39$ |
| Apiwarol | $215.75^{b} \pm 0.54$ | 0 | 0 | $87.12^{b} \pm 0.25$ | 0 | 0 |
| Oxalic acid | $320.55^{c} \pm 0.52$ | 0 | 0 | $210.23^{c} \pm 0.37$ | 0 | 0 |
| Formic acid | $257.35^{d} \pm 0.65$ | 0 | 0 | $152.00^{d} \pm 0.48$ | 0 | 0 |

Explanations: various lowercase letters – the differences are statistically significant for comparisons in the columns for $p \leq 0.05$

**Tab. 2. The antibacterial activities in the washed-out body-surface samples of *A. mellifera* workers, calculated as the area (mm²) without pathogen development**

| Groups | Area (mm²) with antibacterial activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | after two weeks | | | | after four weeks | | | |
| | *B. subtilis* | *S. aureus* | *S.* Typhimurium | *P. aeruginosa* | *B. subtilis* | *S. aureus* | *S.* Typhimurium | *P. aeruginosa* |
| Control | $952.43^{a} \pm 0.54$ | $130.55 \pm 0.35$ | $105.95^{a} \pm 0.47$ | $195.05^{a} \pm 0.63$ | $952.55^{a} \pm 0.55$ | $130.21 \pm 0.45$ | $104.98^{a} \pm 0.55$ | $195.32^{a} \pm 0.47$ |
| Apiwarol | $210.42^{b} \pm 0.67$ | 0 | $74.00^{b} \pm 0.55$ | $101.32^{b} \pm 0.70$ | $55.41^{b} \pm 0.74$ | 0 | $21.11^{b} \pm 0.43$ | $43.12^{b} \pm 0.54$ |
| Oxalic acid | $150.55^{c} \pm 0.35$ | 0 | 0 | 0 | $35.05^{c} \pm 0.45$ | 0 | 0 | 0 |
| Formic acid | $300.05^{d} \pm 0.55$ | 0 | 0 | $95.15^{c} \pm 0.40$ | $120.87^{d} \pm 0.66$ | 0 | 0 | 0 |

Explanations: as in Tab. 1

keepers, who have observed the spread of various diseases in their hives after intensive use of the above drugs. This effect stems from the toxicity of the varroacides. Amitraz-containing agents, such as Bayvarol Flivalinate and Apivar Amitraz, considerably reduced the amount of the main biochemical compounds in the hemolymph of adult workers, and to a lesser extent, the amount of proteins in the body tissues of the workers (8). Howis et al. (6) proved that organic acids damage anatomic and physiological structures of the alimentary tract and body cover, and can facilitate pathogen penetration by debilitating the protein barrier within the proteolytic system (12). Additionally, organic acids create a favourable environment for fungal development. Moreover, oxalic acid probably has a negative effect on the Krebs cycle in honeybees, reducing their energy levels and making them more suceptible to pathogenic fungi, bacteria and viruses. This pattern was observed in our experiment. Drugs used against *V. destructor* weaken bee colonies, and thus it becomes necessarily to use acaricides repeatedly and in increasing quantities, which creates a "vicious circle". Therefore, it is important to use these drugs as instructed and to take care of the apiary so that no infection with pathogens occurs, because pharmaceutical treatment considerably reduces bees' immunity (Tab. 1 and 2).

## References


1. *Bertani G.*: Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. J. Bacteriol. 1952, 62, 293-300.
2. *Buczek K.*: Honey bee colony collapse disorder (CCD). Annales Un. Mariae Curie-Skłodowska 2009, 64, 1-7.
3. *Fries I., Camazine S.*: Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. Apidologie 2001, 32, 199-214.
4. *Gliński Z., Jarosz J.*: Alterations in hemolymph proteins of drone honey bee larvae parasitized by Varroa jacobsoni. Apidologie 1984, 15, 329.
5. *Gliński Z., Kostro K., Luft-Deptuła D.*: Choroby pszczół. PWRiL, Warszawa 2006, 34-79.
6. *Howis M., Chorbiński P., Nowakowski P.*: Influence of exposure to formic acid on the physiological status of the apian (Apis mellifera L.) midgut. Nauk. Konf. Pszczelarska, Puławy 2010, p. 21.
7. *Loucif-Ayad W., Aribi N., Smagghe G., Soltani N.*: A scientific note on the impact of acaracides on the nutritional biochemistry of Apis mellifera intermissa (Hymenoptera: Apidae). Apidologie 2010, 41, 135-137.
8. *Luis M. M., Stephen J. M., Espinosa-Montaño L., Ratnieks F.*: Reproduction of Varroa destructor in worker brood of Africanized honey bees (Apis mellifera). Exp. Appl. Acarol. 2002, 27, 79-88.
9. *Murthy M. S., Rao B. S., Reddy N. M., Subrahmanyam P., Madhvanath U.*: Non-equivalence of YEPD and synthetic complete media in yeast reversion studies. Mutat. Res. 1975, 27, 219-223.
10. *Sabouraud R.*: Contribution a l'etude de la trichophytie humaine. Etude clinique, microscopique et bacteriologique sur la pluralite des trichophytons de l'homme. Ann. Dermatol. Syphil. 1892, 3, 1061-1087.
11. SAS Institute (2002-2003) SAS/STAT User's Guide release 9.13, Cary, NC, Statistical Analysis System Institute, license 86636.
12. *Strachecka A., Paleolog J., Borsuk G., Gryzińska M., Olszewski K., Grzywnowicz K., Kasperek K.*: Proteases on the body surface of honeybee Apis mellifera L. in cage and beehive. Annales Un. Mariae Curie-Skłodowska 2011, 29, 20-26.


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