Original Article

INTESTINAL MICROBIOTA OF HONEY BEES (*APIS MELLIFERA*) TREATED WITH AMITRAZ

Anna Żebracka[1]
Anna Chmielowiec-Korzeniowska[2]
Bożena Nowakowicz-Dębek[2]
Łukasz Wlazło[2]
Piotr Dziechciarz[3]
Grzegorz Borsuk[1]*

[1]Department of Animal Hygiene and Environmental Hazards, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences, Lublin, Poland, Poland
[2]University of Life Sciences, Lublin, Poland
[3]Subdepartment of Apidologie, Institute of Biological Basis of Animal Production; Faculty of Animal Sciences and Bioeconomy, University of Life Sciences, Lublin, Poland

*corresponding author: grzegorz.borsuk@up.lublin.pl
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Abstract

The aim of the study was to analyse the intestinal microbiota of honey bees (*Apis mellifera*) treated with amitraz. In the present study, the microbiological profile of the honey bee intestines showed minor changes in the microbiota following the application of amitraz. A comparison of the numbers of bacteria and fungi revealed a positive downward trend in the number of fungi. The number of decreased bacteria, included *E. coli* and especially *Clostridium* spp., which were not isolated from the intestines of bees treated with amitraz. The number of lactic acid bacteria before and after treatment was at a similar level of 8.3 log cfu/g of intestinal contents.

Keywords: amitraz, *Apis mellifera*, microbiota, varroa treatment

INTRODUCTION

Beekeeping plays an important role in the development of agriculture in terms of the valuable bee products produced by beekeepers as well as its influence on the quantity and quality of crops. Unfortunately, bees are increasingly dying off mainly in the United States and Europe. Bober et al. (2016) report that the bee colony mortality rate reached 32.4% in European countries in the years 2012–2014 , while in Poland the rate was 16%, resulting in average production losses of 4.5%. The situation has not improved in the years since then (Semkiw, 2020).

The causes of the decline in the number of pollinators are complex and often highly controversial (Di Noi et al., 2021). However, the indisputable overall weakness and increased mortality of honey bee colonies is due to the synergistic effect of multiple stressors – biological, climatic, nutritional and chemical. Cuesta-Maté et al. (2021) suggest four main areas of human impact responsible for the decline in bee populations: food stress caused by habitat loss and degradation, reduced genetic diversity in honey bees, antibiotics used in beekeeping mainly to control Nosema disease and pesticide use. All of these factors alter the defence mechanisms of the bee immune system, including physical barriers and the general cellular and humoral response, which causes bees to lose their innate ability to resist changes associated with civilization (Iorizzo et al., 2022). These factors, especially pesticides

and medications used in beekeeping, have also been established to affect honey bees' intestinal microbiota, which is of fundamental importance for their growth and development and supports their resistance and vigour (Iorizzo et al., 2022). The role of the intestinal microbiota in the health of bees is crucial, as it influences metabolism, development, immune function and thus protection against pathogens (Raymann & Moran, 2018). Dosch et al. (2021) experimentally both confirmed the positive role of the intestinal microbiota in the condition of honey bees infected with various viruses and showed that environmental stressors altering the composition of the intestinal microbiota of bees, including chemotherapeutics and pesticides, can make them more susceptible to infectious agents.

Laboratory studies confirm that such herbicides and insecticides as glyphosate or highly toxic neonicotinoids, including imidacloprid and thiamethoxam, disturb the population size of dominant members of the bacterial community and make honey bees more susceptible to pathogens (Motta et al., 2018; Blot et al., 2019; Rouzé et al., 2019). The magnitude of disturbances in the microbiota is determined by the concentrations of pesticides, the duration of exposure, the time of year and co-existing stressors (Hotchkiss et al., 2022).

Most studies on the honey bee microbiome concern changes caused by exposure to pesticides (Hotchkiss et al., 2022). However, there is a lack of information on how acaricides, including amitraz which is used to control *Varroa destructor*, affects the intestinal microbiota of honey bees, even though amitraz has known side effects in honey bee larvae, as confirmed in research by Dai (2018) and Gregorc & Bowen (2000).

Varroa destructor mites pose a serious threat to honey bees (Bahreini & Currie, 2015). The mites feed on the fat body and haemolymph of honey bees in all development stages, leading to malnutrition (Iorizzo et al., 2020). The steady increase in the numbers of diseased individuals causes disturbances in the functioning of the colony and reduces the bee population in the nest, until they are completely eliminated (Rosenkranz et al., 2010; Ramsey et al., 2019). Due to the major threat posed by varroosis, the World Organisation for Animal Health (OIE) has placed it on List B of infectious diseases, and in Poland, the reporting of this disease is mandatory. Because *Varroa destructor* causes devastating damage in honey bee colonies worldwide, controlling it is crucial. Research by van Dooremalen et al. (2018) showed that colonies severely attacked by *V. destructor* were 13% smaller than colonies in which mites were controlled, and their risk of death in winter was fifty-nine times greater.

In addition, significant changes in the biology of *V. destructor* in the last 30 years include increasingly frequent feeding on the underside of the bee abdomen, which is not visible to beekeepers inspecting the colonies, and a significant reduction of the parasite's phoretic phase. These factors increase attacks by the mites and reduce the time needed to destroy the colony from 2-3 years to even 2-3 months. Young worker bees, mainly nurses and wax bees, are the most susceptible to mite infection during the season, but with the decline in the number of young workers and brood at the end of the season, secondary infection affects foragers as well (Nowotnik, 2019).

In Poland, many medications of varied compositions are authorized for controlling mites and varroosis (Strachecka et al., 2013). The most effective agents, acaricides, control the mites through direct contact during the development of several parasite generations. These include third-generation acaricides which are based on synthetic pyrethroids and agents whose active substance is amitraz. Although this substance has been used to control *V. destructor* in Poland since 1984, no populations of highly resistant parasites have been observed (Gąbka et al., 2019), but isolated cases of resistance to amitraz have been noted in the United States, Argentina and the Czech Republic (Rinkevich, 2020).

The aim of the study was to analyse the intestinal microbiota of honey bees (*Apis mellifera*) treated with amitraz.

MATERIAL AND METHODS

The study was conducted on six honey bee colonies of similar strengths and structures inhabiting Dadant polystyrene hives. In all colonies, the queens were sisters. Each colony occupied eight combs of the nest, while the free space outside the nest was occupied by two frames filled with polystyrene foam. The microbial composition of the bees' intestines was determined. In addition, the hygiene conditions of the hive environment were assessed through analysis of the microbiological purity of the air and surfaces. Samples were taken twice in the second half of June, the first before the harvest of linden honey and the second seven days afterwards. This means that before amitraz application (2 strips 1000 mg amitraz per colony; 1st sampling) and seven days afterwards (2nd sampling), so that the first samples were taken in colonies in which there had been no treatment to control *V. destructor*.

Analysis of microbiological composition of intestines

Homogenate of intestines collected from ten bees was combined to form one sample and then tested for the total content of bacteria, fungi, lactic acid bacteria, *Escherichia coli* and bacteria of the genera *Bifidobacterium* and *Clostridium*. The material was placed in sterile flasks, and 5 ml volume of Ringer's solution was added. The solution was vortexed for 5 min and left for 15 min to sediment. Then a series of decimal dilutions of the samples was prepared in 0.6% saline and plated on appropriate media (BTL Polska Sp. z o.o.):

- enriched agar for total bacterial count - incubation for 24-48 h at 37°C,
- Sabouraud agar for total fungal count - incubation for 5-7 days at 25°C,
- mFC for *E. coli* - incubation for 18-24 h at 44°C,
- MRS for total count of lactic acid bacteria of the genus *Lactobacillus* - incubation in microaerophilic conditions for 3-5 h at 30°C,
- BSM for bacteria of the genus *Bifidobacterium* - incubation in anaerobic conditions for 24-48 h at 30°C,
- TSC for total *Clostridium* bacteria - incubation in anaerobic conditions for 24-48 h at 37°C.

Following incubation, the colonies were counted and their concentration was expressed as colony-forming units per g of intestines [cfu/g].

Assessment of microbiological purity of hive

Assessment of the microbiological purity of the air in the honey bee colony involved determination of the levels of bacterial and fungal contamination. Air samples were collected by aspiration using a GilAir 5 sampling pump (Sensdidyne, Inc., Clearwater, USA). The total bacterial and fungal counts were determined by dilution plating on appropriate media:

- tryptone soy agar (TSA) for total bacterial count - incubation for seven days at 37°C (1 day), 22°C (3 days) and 4°C (3 days),
- Sabouraud agar with chloramphenicol for total fungal count - incubation at 30°C (4 days) and 25°C (3 days).

Following incubation, the colonies were counted and their concentration was expressed as colony-forming units per m^3 of air [cfu/m^3].

Microbiological assessment of the inner surface of the top bar, brood cappings on the colony's penultimate honeycomb, and the hive walls involved analysis of the total content of bacteria, including *E. coli*, as well as moulds and yeasts. Samples were collected from a 100 cm^2 surface using sterile sampling templates and sterile swabs (Copan, Italy). The specimens were placed in transport test tubes in 10 ml of Ringer's solution (Biolog, Hayward, USA) with Tween 80 (Biolog, Hayward, USA) and transported to the laboratory, where they were shaken for 2 min. The resulting suspension was plated on appropriate media (BTL, Lodz, Poland):

- enriched agar for total bacterial count - incubation for 24-48 h at 37°C,
- Sabouraud agar total fungal count - incubation for 5-7 days at 25°C,
- mFC for *E. coli* - incubation for 18-24 h at 44°C.

After the colonies were counted, the number of microbes per 100 cm^2 of test surface [cfu/100 cm^2] was determined.

Statistical analysis

The obtained research results were analysed statistically. The normality of the distribution was assessed by the Shapiro–Wilk test. If the distribution was normal, one-way analysis of variance (ANOVA) was performed. Statistical analysis of the results was performed by one -way analysis of variance (ANOVA). Differences were considered significant at $p \leq 0.05$. All statistical data were calculated using STATISTICA 13.1 software (StatSoft, Krakow, Poland).

RESULTS

In the present study, the microbiological profile of the honey bee intestines showed minor changes in the microbiota following the application of amitraz (Tab. 1). Comparison of the numbers of bacteria and fungi revealed a positive downward trend in the number of fungi. The number of bacteria decreased as well, including *E. coli* and especially *Clostridium* spp., which were not isolated from the intestines of bees treated with amitraz. The number of lactic acid bacteria before and after treatment was at a similar level of 8.3 log cfu/g of intestinal contents (Fig. 1). Among probiotic bacteria, greater differences were observed for *Bifidobacterium* spp., but they were not statistically significant ($p > 0.05$).

Analysis of air samples collected at seven-day intervals showed a statistically significant ($p \leq 0.05$) increase in both bacteria and fungi (Tab. 2 and Fig. 2). However, in both samplings, the level of bacterial and fungal contamination of the air was low, about 2 and 2.5 log cfu/m^3 (Fig. 2).

No statistically significant differences ($p > 0.05$) were observed in the level of contamination of the hive's surfaces (Tab. 3), which may be due to the thin layer of propolis protecting them against pathogenic bacteria and fungi growth.

DISCUSSION

The intestinal bacteria of the honey bee come from their surrounding environment and food, so the microbiota of mature worker bees may differ somewhat depending on the food source, age of the bee, time of year and geographic location, although some microbe species are common everywhere. The cluster of *Lactobacillus* strains designated phylotype Firm-5 is usually the most abundant, followed by *Lactobacillus* Firm-4, *Bifidobacterium* spp., *Gilliamella apicola* and *Snodgrassella alvi* (Zheng et al., 2018). The small, specialized community of the intestinal microbiota of honey bees suggests a highly stable interdependence, which is crucial to preserving their health (Babendreier et al. 2006; Cox-Foster et al. 2007). Endophytes support production of essential amino acids, sugar metabolism, and conversion of pollen by breaking down the cellulose wall and releasing proteins, amino acids and lipids. They also provide an important defence against pathogens, by synthesizing anti-microbial peptides and organic acids, activating humoral responses, and forming a biofilm impeding colonization by pathogens (Vasquez et al., 2012). Audisio (2012) and Alberoni et al. (2018) have demonstrated that

Table 1.

Total microbial counts in intestinal homogenates [cfu/g]

Group of microbes	1st sampling		2nd sampling		p-value
	M	SD	M	SD	
Bacteria	1.0×10^8	1.3×10^7	1.1×10^8	6.2×10^7	0.837
E. coli	2.2×10^8	3.3×10^7	6.3×10^7	5.3×10^6	0.314
Clostridium spp.	7.9×10^3	1.1×10^2	0.0	0.0	0.143
Lactic acid bacteria	2.2×10^8	4.9×10^7	1.9×10^8	1.0×10^7	0.887
Bifidobacterium spp.	1.7×10^7	1.3×10^7	4.4×10^6	4.2×10^5	0.069
Fungi	2.9×10^7	5.6×10^7	2.0×10^3	1.8×10^2	0.268

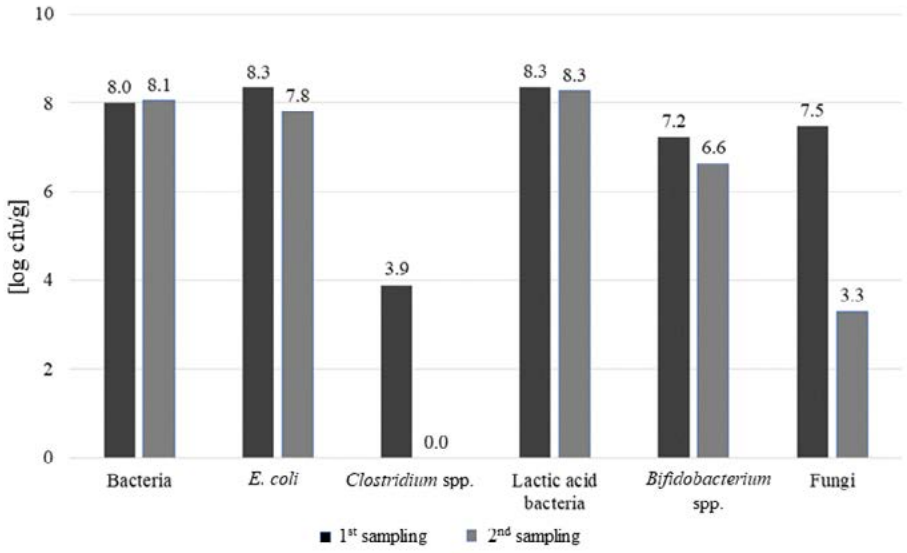


Fig. 1. Log-transformed microbial counts in samples of bee intestines.

Table 2.

Total microbial count in air samples [cfu/m^3]

Group of microbes	1st sampling		2nd sampling		p-value
	M	SD	M	SD	
Bacteria	7.0×10^1	3.3×10^1	2.8×10^2	4.7×10^1	0.0001*
Fungi	7.4×10^1	4.3×10^1	2.9×10^2	1.1×10^2	0.003*

* values differ statistically

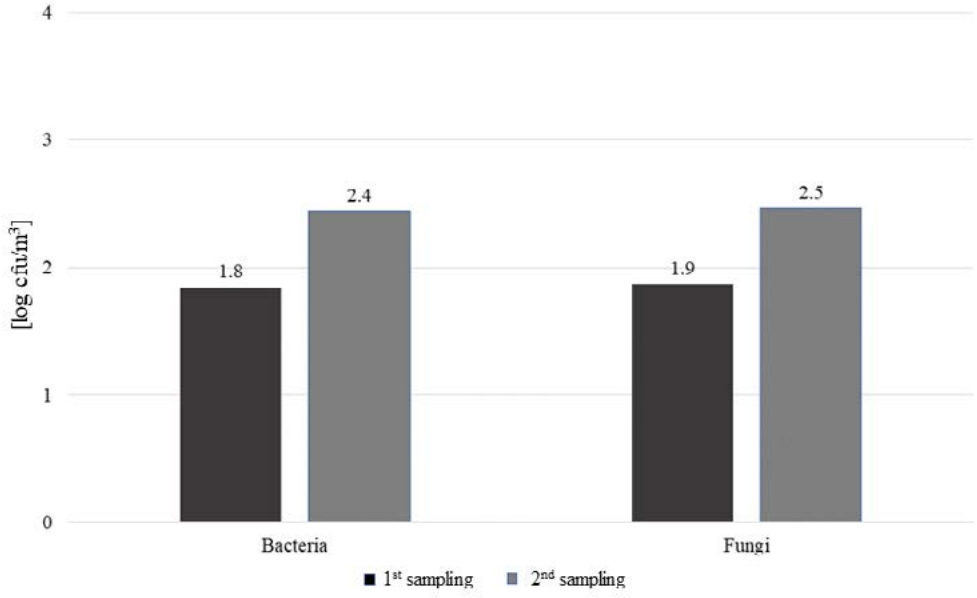


Fig. 2. Log-transformed microbial counts in air samples.

Table 3.

Total microbial count in samples from swabs [cfu/100 cm^2]

Group of microbes	1st sampling		2nd sampling		p-value
	M	SD	M	SD	
			Top bar		
Bacteria	1.2×10^3	1.9×10^2	2.3×10^1	1.7×10^1	0.212
E. coli	9.8×10^1	2.1×10^2	0.0	0.0	0.313
Total fungi	1.0×10^3	1.1×10^2	3.3	4.7	0.064
Yeasts	1.6	3.7	0.0	0.0	0.341
			Honeycomb		
Bacteria	6.7	7. 5	6.7	1.1×10^1	1.000
E. coli	0.0	0.0	0.0	0.0	-
Total fungi	1.7	3.7	1.1×10^2	2.4×10^1	0.340
Yeasts	0.0	0.0	6.8×10^1	1.5×10^1	0.341
			Hive wall		
Bacteria	1.2×10^1	2.2×10^1	5.0	5.0	0.522
E. coli	0.0	0.0	0.0	0.0	-
Total fungi	5.0	1.1×10^1	0.0	0.0	0.341
Yeasts	0.0	0.0	0.0	0.0	-

microbial symbionts associated with the honey bee, particularly probiotic fructophilic lactic acid bacteria (FLAB), have an immunomodulatory effect, helping to fight infection from bacteria and parasites. Therefore, disturbance of the intestinal microbiota of honey bees is harmful to their health, increasing their susceptibility to disease.

The predominant microaerophilic conditions, a temperature of 35°C and the presence of sugars from nectar in the digestive tract of the honey bee are ideal conditions for the development of lactic acid bacteria (Iorizzo et al., 2020). According to Dong et al. (2020), these bacteria colonize the intestines of worker bees up to three days after emergence. The importance of their role in the host organism is evidenced by their involvement in such functions, as inhibiting expansion of pathogens in the intestines during competition for nutrients, fighting pathogens with the products of their metabolism, producing bacteriocins, and significantly influencing immune modulation in the host. In addition, bacteria of the genus *Bifidobacterium* produce short-chain fatty acids (SCFA), which supply the host with rich sources of energy. FLAB also prevent dysbiosis by impeding colonization from conditionally pathogenic bacteria (Nowak et al., 2021).

Hotchkiss et al. (2022) emphasize that changes occur in the abundance of taxa of bees intestinal microbiota during exposure to insecticides, most frequently involving a decline in the populations of *Bifidobacteria* and *Lactobacillus*. A decline in the numbers of *Bifidobacterium* spp. is also observed in insects infected with *Nosema ceranae* (Zhang et al., 2019; Naree et al., 2022). Cuesta-Maté et al. (2021) report that oxalic acid used in *Varroa destructor* infected apiaries also exhibits bactericidal activity against *Lactobacillus* strains isolated from the intestines of the insects.

A balanced microbiota in bees not only supports their defensive strength but are also a significant indicator of the health of bee colonies. Hygiene in the apiary and the hive itself plays an important role, as *V. destructor* is a significant vector in viral infections,

and by damaging the insect's integument, it creates a gateway for bacterial pathogens as well, including conditional pathogens (Buczek, 2008). Poor microbiological quality of the hive creates the risk of co-existing diseases in insects weakened by varroosis. Unfortunately, the available literature devotes little attention to microbial contamination of the hive itself, while Santorelli et al. (2021) argue that every hive has its own microbiota. By visiting flowers multiple times to gather the nectar and pollen needed to produce honey and bee bread, bees transport microorganisms including pathogens between plants and from plants to the hive, transmitting them together with the food to the worker bees. The production of honey and bee bread causes a vast number of microbes to accumulate in the hive, including bacteria from plants and from various body parts of the honey bees, which was confirmed in the present study. Analysis of air samples collected at seven-day intervals showed an increase in both bacteria and fungi, possibly due to horizontal transfer of these microbes from the external environment by forager bees (Keller et al. 2021).

The thin layer of propolis with which bees cover and seal the surface of the hive has antibacterial, antifungal and antiviral properties, and it simultaneously sterilizes the interior of the hive (Anderson et al., 2011). Homeostasis of the nest environment is also influenced through the maintenance of a constant temperature in the hive (33-36°C) and ventilation of the nest, which determines the direction of microbiological changes occurring in bee products, spoilage prevention and removal of harmful substances from the hive together with moist air (Peters et al., 2019).

This study is an introduction to more extensive research. However, the initial observations suggest that the use of amitraz to control *Varroa destructor* may disturb the balance of the microbiome of the honey bee and impair its protection against other pathogens. Therefore, research should be continued to investigate other acaricides used in beekeeping.

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