Intestinal Bacterial Community of the Apis Melifera Carpatica Honey Bee Workers, Depending on Season and Area

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Abstract
It is important to know that the intestinal flora of most organisms play a decisive role in nutrient assimilation or diseases defending action and honeybees are not an exception. The importance and composition of the intestinal micro-flora define the health and growth of honeybees. For this study were been collected as far as 10 honeybee workers from each area, from three different areas: Brasov County, near Perşani Mountains, Timiş County, from plane area and Arad County, from hillside area, from beekeepers, both in June and December. First was been detected the total number of bacteria contained in honeybees intestine samples through dilution method. The largest number of countered bacteria colonies was obtained from Timiş County (2946 colonies/millilitre) in December. The smallest number of countered bacteria colonies was obtained in Braşov County (527 colonies/millilitre) in June. After the growing colonies of bacteria from studied samples on special mediums of culture have been detected many species, Gram-positive including *Bacillus spp., Streptococcus spp.,* and Gram-negative including *Escherichia spp., Proteus, Citrobacter, Pseudomonas.* Also have been detected *Lactobacillus* strains.

Key words: *bacteria colony, Gram-positive, Gram-negative, intestinal micro-flora, medium of culture.*

1. Introduction

Bees are incredible insects, extremely important for nature and man. Today it is considered that there are 40,000 different species of bees of which only 20,000 were found [1]. The basic purpose of bees is to collect pollen as a source of protein and energy for their brood. As part of their debt forage pollen, bees and pollinating at least 80% of the crops we rely on to live. Without bees, many of these crops would not produce fruit, which would mean that there would be no food for the people. It is therefore crucial for the bees not only survive, but thrive, because their life depends on efficient collection activities, which also produces food with medicinal valences such as honey, propolis, and ultimate super food, royal jelly. The main role of bees is of course to pollinate. Currently there are at least 235,000 flowering plants on earth, and bees are responsible for cross-pollination of at least 80% of these plants. Bees are attacked and not about invasive species by itself, of course, unless human beings falling in this category destructive. As it seems, modern farming techniques involving spraying crops with tons of toxic chemicals over extremely kills and soils, and in turn kills bees feed on plants growing in these soils. It is a cycle of death, extremely destructive, that a day would make it virtually impossible to grow enough crops to sustain life. *Apis mellifera* honey bee, as a pollinator is a key species for agricultural production and significantly contributes to the human food supply. Recent

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losses of *Apis mellifera* bees and the possible association of these declines with different infectious agents required a better understanding of these bees microbiota [2]. In many animals, the gut microbial community, in particular, confers functions related to nutrition and susceptibility to disease and thus might also play an important role in the health and resilience of honey bees. Honey bees have pool resources, divided labour, and communicate in highly structured social colonies. Sterile female worker bees predominate within colonies in which they initially clean cells, rear brood, and store food, then leave the hive, and forage for pollen and nectar. Diet and nutrition also shift as workers age but throughout the life cycle, pollen represents the only dietary source of fat and amino acids. Honey bees gut microbiota play an important role in defending processes against pathogens, but also can be involved in nutritional processes such as breakdown and utilization of pollen grains or degradation of toxic compounds encountered in the environment. Several studies have shown that the honey bee gut harbours a simple microbiota that is nonetheless quite distinct from other insects [3,4]. The microbiota means the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share a space. Joshua Lederberg coined the term, emphasizing the importance of microorganisms inhabiting the human body in health and disease. Many scientific articles distinguish microbiome and microbiota to describe either the collective genomes of the microorganisms that reside in an environmental niche or the microorganisms themselves, respectively. However, by the original definitions these terms are largely synonymous. [5,6]. The microbiota contained in honeybees digestive tract is complex but far from being fully understood and even known, having a close relation with health and production of these extraordinary insects. This paper aims to emphasize data about intestinal micro-flora (microbiota) in *Apis melifera carpatica* honey bee workers from three different regions of our country, depending on season and area.

2. Materials and methods

This work was been performed in June-December 2014, when were collected as far as 10 honeybee workers from each area, from three different areas: Brasov County, near Perşani mountains, Timiş County, from plane area and Arad County, from hillside area, from beekeepers, both in June and December. First was been detected the total number of bacteria contained in honeybees intestine samples through dilution method. For this purpose in June were been collected 30 honey bees' workers, each 10 in each location: Braşov County, Timiş County and Arad County. Honey bees workers were transported alive, in sterile tubes, after they were collected near the hives. In the Microbiology laboratory the whole intestinal tracts, from oesophagus to rectum was introduced in distilled water, in sterile tubes, from each colony and location, avoiding through aseptically handled any contamination. After 24 hours of incubation at 37˚C was been calculated the colony-forming units (CFU), also named aerobic plate count, on a nutrient agar poured in thin layer on 60 Petri dishes, two for a sample, finally making an average of the two plates. For this counting were used serial dilutions of incubated samples. It was agreed to use dilution 10^{-3} in the view of aerobic plate counting, starting from the appearance of colonies developed in the initial samples, which had highly developed depot and turbidity, demonstrating an appreciable bacterial colonies development. This 10^{-3} dilution allows the obtaining of isolated colonies which can be counted. All the Petri dishes were been flooding inoculated with sample of honey bee workers digestive tract in 10^{-3} dilution. The Petri dishes were been incubated at 37˚C for 24 hours. Counting of the colony-forming units (CFU) was performed using the Nitech LKB 2002 apparatus. The same method was applied when were collected honey bee workers digestive tract in December. In both seasons (June, December) and three locations collected samples of honey bee workers, Gram-stained smears were made from each sample and were microscopically examined. The collected digestive tract samples from all regions and seasons were also cultured in special culture media to identify bacterial species they contained: blood agar plates (BAPs) contain mammalian blood (usually sheep or horse), typically at a concentration of 5–10%. BAPs are enriched, differential media used to isolate fastidious organisms and detect haemolytic activity. β-haemolytic activity will show lysis and complete digestion of red blood cell contents surrounding colony. MacConkey agar,

Streptococcus selection agar, eosin methylene blue (EMB) also named Levine, agar SS and MRS agar.

3. Results and discussion

In Table 1 are presented the results of colony-forming units (CFU) or aerobic plate counting from the first location, in Braşov County, near Perşani Mountains in both seasons, June and December. In June, in Braşov County was registered the smallest number of countered colonies (527 colonies/millilitre), also in December from this region was registered the lowest value of countered colonies (694 colonies/millilitre). In table 2, the results of CFU counting in Timiş County emphasized an increased number of colonies countered in June (754.2 colonies/millilitre) but in December was registered the highest value of CFU (2946 colonies/millilitre). In Arad County there is a small difference between CFU from Timiş County in June (748.3 colonies/millilitre) and in December The countered CFU had a decreased value in comparison with Timiş County, but increased to the value registered in Braşov County (1513.7 colonies/millilitre). Analyzing the obtained results it is found that in summer season the intestinal microbiota of honey bee workers is more reduced than results obtained in winter season. A difference was observed in the area, the mountain being those in which we obtained the lowest values of intestinal microbiota in honey bee workers. After the growing colonies of bacteria from studied samples on special mediums of culture have been detected many bacteria species. On blood agar plates (BAPs) were developed opalescent greenish colonies apparent with reflect light of *Bacillus spp.* with double zone of beta haemolysis. MacConkey agar (MAC) is a specialized bacterial growth medium selective for Gram negative bacteria and can differentiate bacteria able to ferment lactose. In this medium were differentiated *Escherichia coli* and *Citrobacter*, species able to ferment lactose, named Lac (+) or sugar lactose (+), developing pink coloured colonies. Differentiation of the two species was then made based on morphological characters, Gram stained smears. Also, on this medium was observed the developing of *Pseudomonas aeruginosa*, a Lac (–) or sugar lactose(-) bacteria which use peptone from medium and formed white colonies. From these colonies were made agar plating being observed after two days of incubation the pyocyanin, blue-green pigment elaborated by *Pseudomonas aeruginosa*. In agar SS medium, a differential medium for the isolation of *Salmonella* and *Shigella* species there was no bacterial colony, which demonstrates that these two species was not identified in the intestine bees. In *Streptococcus* selection agar (or selective Strep agar), selective medium use in primary isolation of *Streptococcus* species were observed developed colonies, demonstrating the presence of this bacteria in studied samples. The eosin methylene blue agar (EMB, named also Levine) was used to confirm the presence of *Escherichia coli* which developed distinctive metallic green colonies on this medium of growth.

Table 1. The results of CFU counting in Braşov County

Samples (10^{-3} dillution)	Number of CFU (colonies/milliliter) June	Number of CFU (colonies/milliliter) December
1	103	395
2	150	602
3	470	408
4	448	487
5	889	885
6	561	973
7	630	551
8	777	799
9	574	809
10	668	1031
Mean	527	694

Finally, was used MRS agar (de Man, Rogosa and Sharpe) a growth medium for Lactobacilli, based on the idea that lactobacilli found in the research material. After MRS agar was inoculated with

studied samples from intestinal tract of honey bee workers followed the incubation up to 3 days at 35˚C, under microaerophilic conditions, using Anaerocult ®C by Merck in a crystallizer. Bacterial colonies developed on MRS agar demonstrate the existence of lactobacilli in the material examined.

Results obtained demonstrate that exist a variability of microbial communities in intestinal tract of studied honey bee workers, depending on season and area. A considerable variability of intestinal bacterial communities in bees from the same colony was found by Mohr and Tebbe [7]. Gillian and Morton [8] and Gillian and al. [9] explained that this variability may be induced by several factors such as season and the type of pollen ingested. All results of these studies showed differences in intestinal bacterial communities between honey bees from spring and summer but only small differences between colonies and locations. Also these researchers observed a high similarity between free-flying bees and laboratory-reared bees that were fed only with pollen from maize. This fact suggests that there a small influence of the pollen source and indicates that experimental systems can be representative for the field situation. It is clear that a conclusive assessment of the severity of honey bee life effects detected in many studies depend on a more profound knowledge of essential bacteria in the honey bee gut and on more detailed understanding of their specific physiological functions [10]. Evans and Armstrong [11] in their study suggested that the gut microbial population in honey bee is not constant even within the same species. Honey bees visit flowers of many types, which vary geographically and seasonally. Also, honey bees of different species tend to visit flowers of particular species, so that variation in the food source is likely to be related to the lack of characteristic gut bacterial profile in Apis species.

Table 2. The results of CFU counting in Timiş County

Samples (10^{-3} dillution)	Number of CFU (colonies/milliliter) June	Number of CFU (colonies/milliliter) December
1	297	897
2	484	2771
3	651	3090
4	397	2991
5	855	1789
6	1439	3447
7	798	4494
8	876	4323
9	763	1889
10	982	3769
Mean	754.2	2946

Table 3. The results of CFU counting in Arad County

Samples (10^{-3} dillution)	Number of CFU (colonies/milliliter) June	Number of CFU (colonies/milliliter) December
1	165	697
2	498	984
3	751	2651
4	533	993
5	879	1886
6	993	783
7	1331	2333
8	833	1576
9	599	1021
10	901	2213
Mean	748.3	1513.7

Many studies were made to assess the effects of actual environmental conditions, like transgenic plants on Apis melifera, or pollution and insecticides, in the aim of emphasizing the potential of destructive factors on development of honey bee colonies, especially the effect of insecticidal proteins on the development of the hypo-pharyngeal gland of adult worker bees [12]. The microbial community of digestive tract may be an important factor for the health of honey bees at the individual and colony level. Other studies on honey bee microbial communities have focused on disease-causing microorganisms, while much less emphasis has been given to non-pathogenic microorganisms and their potential benefit for individual bees or whole colonies. A great importance is given to the composition of the intestinal microbial community for the health and growth of honey bees [13], and indirectly on the quality of their mainly products, honey.

4. Conclusions

Trying to identify differences related to digestive tract microbiota of honey bee workers between different regions, with different landforms and seasons, with bacterial species composing microbial flora, this study performed on three regions, one on mountain region (Braşov County), one on hilly region (Arad County) and one on plains (Timiş County). The results emphasized that the largest number of countered bacteria colonies was obtained from Timiş County (2946 colonies/millilitre) in December and the smallest number of countered bacteria colonies was obtained in Braşov County (527 colonies/millilitre) in June. The smallest number of honey bee workers gut colonies was obtained in both seasons in the mountain region, followed by hilly region and the increased number also in both seasons was registered on the plains. Maybe different plants consisting flora from each region influence these results, or maybe the degree of pollution. In winter season, in all three cases was observed an increased number of bacterial colonies, fact observed by other authors, because the bees are in the hive and are fed by beekeeper, perhaps there is a higher concentration of germs. Regarding on identified bacterial species in gut microbiota was highlighted: *Bacillus spp.*, *Streptococcus spp.*, *Escherichia spp.*, *Proteus*, *Citrobacter*, *Pseudomonas and Lactobacillus* demonstrating the existence of considerable variability of intestinal bacterial communities.

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