Identification of *Apis mellifera* Gut Microbiota with MALDI-TOF MS Biotyper

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Abstract
The honey bee, *Apis mellifera*, is critically important for the pollination of many economically important crops. Continued colony losses have called for a deeper understanding of both symbiotic and pathogenic microbial interactions, particularly as they relate to food storage and the pollination environment. Therefore, the aim of this study was to explore and characterize the bacteria colonizing the alimentary tract of the native honey bees using MALDI TOF MS Biotyper. Content of the intestinal tract was cultured for isolation of Gram-negative, Gram-positive microorganisms and yeasts. Then, the identification of isolates with MALDI-TOF MS Biotyper was done. Results showed that the most abundant genera in bees' samples were *Lactobacillus*, *Pseudomonas* and *Serratia*. Altogether, 12 genera with 21 bacterial species and one yeast genus with two species were isolated. Bacteria were represented with *Acidovorax facilis, Lactobacillus gasseri, L. amylovorus, L. kunkeei, L. fructivorans, Pseudomonas oryzihabitans, Ps. brenneri, Ps. indica, Micrococcus luteus, Serratia fonticola, Ser. marcescens, Ser. ureilytica, Hafnia alvei, Candida magnolia, Bacillus oleronius, B. horneckiae, Issatchenkia orientalis, Pantoea agglomerans, Enterobacter cloacae, Staphylococcus epidermidis, Staph. pasteuri, Shewanella profunda*. The results of the study shows that the microflora of the bees gut is heterogenic and depend of locality and resources of environment for bees.

Keywords: *Apis melifera*, gut microbiota, identification, MALDI TOF MS Biotyper.

1. Introduction

The gut microbiota can have profound effects on hosts, but the study of these relationships is challenging. The specialized gut microbial community of honey bees is similar to the mammalian microbiota with predominance of host-adapted facultative anaerobic and microaerophilic bacteria. However, the microbial community of the bee gut is accepted to be simpler than the microbiota in mammals. Bees gut microflora was revealed being dominated by only nine bees-specific bacterial species clusters that are transmitted between individuals. Recent developments in science, which include the discovery of variation of bacterial strains, evidence of protective and nutritional functions of gut microflora and reports of eco-physiological or disease-associated shifts in the composition of microbial community, have drawn attention to the role of the microbiota in bee health. The studies of bees gut microflora may have a potential as a model for studying the ecology and evolution of gut symbionts [1].

Social insects as bees provide unique possibility for studying of microbial symbiosis. The high

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density of insects within the individual colony, sharing of food and other resources and the coexistence of colony members from different generations are of great importance [2]. Bacterial communities of insects may vary in different parts of gut, resulting in differences in bacterial counts, composition, location and functions of represented microorganisms[3]. Martinson et al., 2012 reported that bacterial counts in host gut may achieve 10^9 cfu with 8 phylotypes making up to 95% of the total bacteria specific to social bees [4]. The stability of microbial community depends on the nutrition and social lifestyle of the insects [5], environment [7] and ontogenetic stage [9,10]. The dynamic in the bacterial community system has been shown to follow seasonal trends [11,12].
Therefore, the aim of this study was to explore and characterize the bacteria colonizing the gut of the native honey bees using MALDI TOF MS Biotyper.

2. Materials and methods

Samples

Adult worker honey bees (*Apis mellifera*) were used for experiments. The worker honey bees were originated from the apiary from Eastern Slovakia (n=50). All the specimens were obtained from the hive. The workers were decapitated and the midgut and rectum were removed and weighed to obtain a 0.1 g of gut content material.

Microbiological analyses

Gram-positive, including lactic acid bacteria, and Gram-negative bacteria and yeasts counts in bees gut were detected. A sample of gut content was streaked onto MacConkey agar (MCA, Merck, Germany) and inoculated agar was incubated for 24-48 h at 37 °C aerobically. For detection of lactic acid bacteria, the sample was plated onto de Man, Rogosa and Sharpe agar (MRS, Oxoid) and inoculated agar was incubated for 48-72h at 30 °C anaerobically. For detection of Gram-positive and Gram-positive microflora, the sample material also was transferred onto tryptone soya agar (TSA, Oxoid) with subsequent incubation for 48-72 h at 30 °C aerobically. For detection of yeasts, the sample was plated onto malt extract agar (MEA, Merck) and inoculated agar was incubated for 5 days at 25 °C aerobically.

Identification of bacteria with MALDI-TOF MS Biotyper

A sample for MALDI-TOF MS analysis was prepared following the ethanol/formic acid extraction procedure recommended by the manufacturer (Bruker Daltonik, Bremen, Germany). Bacterial colonies were suspended in 300 μL of water (Sigma-Aldrich, St. Louis, USA), and 900 μL of absolute ethanol (Bruker Daltonik, Bremen, Germany) was added and mixed with the cell suspension. After centrifugation at 13000 rpm for 2 min, the supernatant was discarded. The pellet was mixed with 10 μL 70% formic acid (v/v) (Sigma-Aldrich, USA) and an equal volume of acetonitrile (Sigma-Aldrich, USA) was added. The mixture was repeatedly and 1 μL of the supernatant was spotted onto a polished steel target plate, overlaid with 1 μL of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid, HCCA, Bruker Daltonik, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich, USA) and air dried at room temperature. Mass spectra were automatically generated using the microflex LT MALDI-TOF mass spectrometer (Bruker Daltonik, Germany) operated in the linear positive mode within a mass range of 2000-20000 Da. The instrument was calibrated using a Bruker bacterial test standard. Recorded mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker Daltonik, Germany) using standard settings. The MALDI Biotyper output is a log score value in the range of 0-3.0 representing the probability of correct identification of the isolate computed by the comparison of the peak list for an unknown isolate with the reference spectrum in the database. The identification criteria used were as follows: a score of 2.300 to 3.000 indicated highly probable species level identification, a score of 2.000 to 2.299 indicated the secure genus identification with probable species identification, a score 1.700 to 1.999 indicated the probable identification to the genus level, and a score of <1.700 was considered unreliable.

3. Results and discussion

In our study, the presence of 22 different species of bacteria and yeast was recognized in bees gut (Table 1). The most isolated species from the gut

of bees was *Enterobacter cloacae* (100% of samples) followed by *Bacillus oleronius* (98%) and *Serratia fonticola* (96%). The less frequently isolated species were *Issatchenkia orientalis* (10%), *Candida magnolia* (12%), *Pseudomonas brenneri* (15%) and *Shewanella profunda* (15%).

Bacterial species isolated from gut of bees were represented with nine families and 13 genera. The most abundant family in bees gut was *Enterobacteriaceae*. The most abundant microbial genus was *Lactobacillus* where four different *Lactobacillus* species were found.

Table 1 Species isolated from bees GIT samples and their percentage

Species	Number of positive samples	Percentage %
Acidovorax facilis	36	72
Bacillus horneckiae	30	60
Bacillus oleronius	48	96
Candida magnolia	6	12
Enterobacter cloacae	50	100
Hafnia alvei	25	50
Issatchenkia orientalis	5	10
Lactobacillus amylovorus	28	56
Lactobacillus fructivorans	25	50
Lactobacillus gasseri	30	60
Lactobacillus kunkeei	21	42
Micrococcus luteus	20	40
Pantoea agglomerans	35	70
Pseudomonas brenneri	15	30
Pseudomonas indica	29	58
Pseudomonas oryzihabitans	35	70
Serratia fonticola	48	96
Serratia marcescens	42	84
Serratia ureilytica	45	90
Shewanella profunda	15	30
Staphylococcus epidermidis	27	54
Staphylococcus pasteuri	35	70

The honey bee is a key pollinator species worldwide. Bee colonies are exposed to different factors of agricultural ecosystems throughout the year with environmental variables that may affect the microbial balance of individuals and the hive. Therefore the studies of bees microflora are important to understand the origin of microorganisms. Among the studies, the ideas on core microbiota in guts of younger in-hive bees, forager bees leaded transmission of the microflora to the hive and importance of the foregut (crop) for environment and preservation of hive food stores are an area of the research [16].Our results on composition of the digestive tract of Slovakian adult honey-bees differ from the previous reports [13,14]. Previously, the gut was found to be highly populated by anaerobic, rather than aerobic bacteria withcoliforms, *Enterococcus faecium, E. faecalis*, staphylococci, *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* spp. and microscopic fungi and yeast were represented [13,14]. It is iinterestingly that the significant differences were found between the microflora of the gastrointestinal tract of summer and winter bees. In pollen, the presence of of mesophilic anaerobic and aerobic microorganisms, coliforms and microscopic fungi were revealed. Among these, the most representative genera were *Alternaria, Cladosporium* and *Penicillium*. Also in honey, the *Penicillium, Cladosporium* and *Alternaria* were found to be the most abundant microscopic fungi.

Table 2. Isolated bacteria from gut of bees

Family	Genera	Species
Comamonadaceae	*Acidovorax*	*Acidovorax facilis*
Bacillaceae	*Bacillus*	*Bacillus horneckiae*
Bacillaceae	*Bacillus*	*Bacillus oleronius*
Saccharomycetaceae	*Candida*	*Candida magnolia*
Enterobacteriaceae	*Enterobacter*	*Enterobacter cloacae*
Enterobacteriaceae	*Hafnia*	*Hafnia alvei*
Saccharomycetaceae	*Issatchenkia*	*Issatchenkia orientalis*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus amylovorus*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus fructivorans*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus gasseri*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus kunkeei*
Micrococcaceae	*Micrococcus*	*Micrococcus luteus*
Enterobacteriaceae	*Pantoea*	*Pantoea agglomerans*
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas brenneri*
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas indica*
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas oryzihabitans*
Enterobacteriaceae	*Serratia*	*Serratia fonticola*
Enterobacteriaceae	*Serratia*	*Serratia marcescens*
Enterobacteriaceae	*Serratia*	*Serratia ureilytica*
Shewanellaceae	*Shewanella*	*Shewanella profunda*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus epidermidis*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus pasteuri*

The microscopic fungi in bees, pollen and honey can contribute to impairment of the gut microflora and the shifts in ratio of symbiotic microorganisms and their composition lead to favorable conditions for the proliferation of yeasts and moulds. Nevertheless, the intestinal microflora of queens and workers are likely to contain (though in low numbers) microorganisms of the genus *Penicillum*, *Aspergillus*, *Alternaria*, *Hansenula* and *Rhisopus* and *Cladosporium* in workers bees [15]. Our findings indicate that the microflora was balanced with only *Candida* was represented among fungi and yeasts.

4. Conclusions

Altogether, nine families, 12 genera with 21 bacterial species and one yeast genus with two species were isolated. Bacteria were represented with Gram-positive and Gram-negative bacteria. Gut of bees is a heterogenic system and the composition and abundance of microflora depend on the locality and environmental resources for bees life.

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