Enterobacteriaceae in Gut of Honey Bee (*Apis mellifera*) and the Antibiotic Resistance of the Isolates

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Abstract
Bacterial species of *Enterobacteriaceae* and the antimicrobial resistance of the isolates were detected in *Apis mellifera* L. bees gut. Gut content was cultivated on Meat peptone and McConkey agars at 30 and 37 °C, then, the isolates were identified with MALDI TOF MS Biotyper. Isolated strains were tested for antibiotic resistance to penicillins, cephalosporins, carbapenems, fluoroquinolones and aminoglycosides. Altogether, 12 species representing *Enterobacteriaceae* family were isolated. *Firmicutes* and *Candida were* represented by *Bacillus megaterium* and *Issatchenkia orientalis* . Isolated *Enterobacteriaceae* species were *Enterobacter cloacae, Hafnia alvei, Klebsiella oxytoca, Morganella morganii, Serratia marcescens, Ser. liquefaciens, Raoultella ornithinolytica, R. planticola, R. terrigena, Pantoea ananatis, P. agglomerans, Rahnella aquatilis*. *Enterobacter cloacae, Hafnia alvei, Klebsiella oxytoca, Morganella morganii, Serratia marcescens, Ser. liquefaciens* isolates exhibited the antimicrobial resistance more frequently than *Raoultella ornithinolytica, R. planticola, R. terrigena, Pantoea ananatis, P. agglomerans, Rahnella aquatilis*. Microflora of gut of bees could serve as a source of resistant microorganisms.

Keywords: *Apis mellifera*, intestinal microflora, MALDI TOF MS Biotyper, antibiotic resistance.

1. Introduction

Insects are the most diverse animal group on earth. During evolution the insects had been adapted to get nutrient from a variety of materials ranging from wood or phloem sap to blood. However, the diet alone could not provide all essential structural material and should be complemented by the microflora of insect. Microflora of insect is known to play a major role in the adaptation and evolution [1]. The honeybees are of great importance worldwide because the participation in pollination of crops, wild and fruit plants and trees. This makes the honeybees unreplaceable in ecosystems and sustainable agriculture production. Microflora of honeybee are important for survival of individual bee and for whole the bees community. The gut is colonized with the heterogenic microflora, which take part in nutrition, protection of bees against the bee pathogens and parasites, enhance bees immunity resulting in providing of bees health [2]. Meanwhile, the bees gut microflora can develop the antimicrobial resistance as a result of application of antibiotics in beekeeping. The Tian et al. study reveals the profound effects of tetracycline use in beekeeping and the proliferation and dissemination of resistance genes

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originated from bees gut microflora in the greater environment [3,4].

Therefore, the studies on identification of the intestinal microflora of honeybees alongside with the pattern of antimicrobial resistance of the isolates are needed. The aim of the present study was to characterize the microorganisms inhabiting the honeybees gut with special emphasis on *Enterobacteriaceae* and the detection of antimicrobial resistance of isolated bacterial species.

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=25). The specimens were obtained from the hive. The workers were decapitated and the midgut and rectum were removed. The content of gut was weighed to obtain a 0.1 g of sample material.

Microbiological analyses

Gram-positive, Gram-negative bacteria and yeasts in bees gut were detected. Content of gut was streaked onto MacConkey agar (MCA, Merck, Germany) which was incubated for 24-48 h at 37 °C aerobically. For cultivation of Gram-positive and Gram-negative microflora, the inoculated Tryptone soya agar (TSA, Oxoid) was incubated for 48-72 h at 30 °C. For detection of yeasts, 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 then in 900 µL of absolute ethanol (Bruker Daltonik, Bremen, Germany). Suspension was mixed and centrifuged at 13000 rpm for 2 min. After the supernatant was discarded, the pellet was mixed with 10 µL of formic acid (70%, v/v) (Sigma-Aldrich, USA) and an equal volume of acetonitrile (Sigma-Aldrich, USA) was added. The mixture was repeatedly centrifuged and a quantity of 1 µL of the supernatant was spotted onto a polished steel target plate and air dried at room temperature. Each sample was 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). 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). The MALDI Biotyper output was a log score value in the range 0-3.0 meaning the probability of correct identification of the isolate. The value of identification of each isolate was computed by comparison of the peak list for an unknown isolate with the reference spectrum in the database. The identification criteria were: a score of 2.300 to 3.000 indicated highly probable species level identification; 2.000 to 2.299 - secure genus with probable species identification; 1.700 to 1.999 - probable identification of the genus; <1.700 was considered unreliable identification.

Antibiotic susceptibility testing

At least 50% of isolated bacterial strains of each bacterial species were used for antimicrobial susceptibility testing. A amount of 100µl of bacterial suspension in physiological solutionwith 0.5 McF° density was spread with sterile L-rods on the surface of Mueller Hinton agar (Oxoid, UK). Disc diffusion methods was applied and the following antibiotics discs (Oxoid, UK) were used: ampicilin AMP (10µg), cefotaxime CTX (10µg), doripenem DRP (10µg), imipenem IMP (10µg), ciprofloxacin CPR (10µg), levofloxacin LVX (10µg), amikacin AMK (10µg), gentamicin GEN (10µg). Inoculated agars were incubated at 35±2°C for 16-20 h according to the EUCAST [5]. Interpretation of inhibition zones were done in line with EUCAST [6].

3. Results and discussion

Enterobacteriaceae, *Bacillaceae* and *Saccharomycetaceae* were identified in bees gut. The *Enterobacteriaceae* were the most abundant and were represented with 12 bacterial species The bacterial species identified with MALDI TOF MS Biotyper in bees gut are shown in Table 1.

Table 1. Isolated bacterial strains of bees gut

Family	Bacterial species
Bacillaceae	*Bacillus megatherium*
Enterobacteriaceae	*Enterobacter cloacae*
	Hafnia alvei
	Klebsiella oxytoca
	Morganella morganii
	Pantoea ananatis
	Pantoea agglomerans
	Rahnella aquatilis
	Raoultella ornithinolytica
	Raoultella planticola
	Raoultella terrigena
	Serratia marcescens
	Serratia liquefaciens
Saccharomycetaceae	*Issatchenkia orientalis*

Table 2. Number of isolated and tested bacterial cultures used for antibiotic susceptibility testing

Bacterial species	No. of isolates	No. of tested isolates
Enterobacter cloacae	20	10
Hafnia alvei	15	10
Klebsiella oxytoca	21	10
Morganella morganii	18	9
Pantoea ananatis	25	10
Pantoea agglomerans	18	10
Rahnella aquatilis	19	10
Raoultella ornithinolytica	23	12
Raoultella planticola	22	11
Raoultella terrigena	20	10
Serratia marcescens	30	15
Serratia liquefaciens	31	15
	262	**132**

Altogether, 262 isolates from gut of 25 bees were originated (Table 2). The most frequently the presence of *Serratia liquefaciens*, *Ser. marcescens* and *Pantoea ananatis* were detected in investigated samples.

All the *Enterobacteriaceae* species recovered exhibited the antimicrobial resistance to the antibiotics. *Enterobacteriacea* showed the more frequently resistance to ampicillin (33%), amikacin (32%) and levofloxacin (30%). The resistance to doripenem (17%), ciprofloxacin (26%) and gentamicin (27%) was observed less frequently (Table 3).

Table 3. Antibiotic susceptibility testing of *Enterobacteriacea* from bees gut

Antibiotic tested Resistance/sensitive	AMP R/S	CTX R/S	DRP R/S	IMP R/S	CPR R/S	LVX R/S	AMK R/S	GEN R/S
Enterobacter cloacae	2/8	1/9	2/8	3/7	2/8	5/5	4/6	2/8
Hafnia alvei	5/5	2/8	2/8	4/6	1/9	3/7	5/5	1/9
Klebsiella oxytoca	3/7	2/8	1/9	5/5	4/6	3/7	2/8	2/8
Morganella morganii	5/4	2/7	3/6	2/7	4/5	2/7	3/6	2/7
Pantoea ananatis	1/9	2/8	3/7	1/9	2/8	4/6	2/8	3/7
Pantoea agglomerans	2/8	1/9	2/8	3/7	2/8	3/7	4/6	2/8
Rahnella aquatilis	2/8	2/8	1/9	2/8	1/9	3/7	4/6	2/8
Raoultella ornithinolytica	4/8	3/9	1/11	2/10	4/8	2/10	3/9	4/8
Raoultella planticola	1/10	2/9	3/7	4/7	1/10	2/9	3/8	4/7
Raoultella terrigena	6/4	2/8	1/9	4/6	3/7	5/5	2/8	1/9
Serratia marcescens	7/8	5/10	1/14	2/13	5/10	6/9	5/10	6/9
Serratia liquefaciens	6/9	4/14	3/12	4/11	5/10	3/12	5/10	6/9
	44/88	25/107	23/108	36/96	34/98	41/91	43/89	35/97

Legend to antimicrobial drug abbreviation: ampicilin AMP (10µg), cefotaxime CTX (10µg), doripenem DRP (10µg), imipenem IMP (10µg), ciprofloxacin CPR (10µg), levofloxacin LVX (10µg), amikacin AMK (10µg), gentamicin GEN (10µg)

The microflora in the gut of honey bee was found to be represented mostly with Gram-negative microorganisms in the present study. The presence of Gram-positive microflora were negligible and was associated with contamination from environment [4].

High prevalence of *Enterobacteriaceae* resistant strains to antibiotics identified in the present study. This finding could be explained with bees extensive contact with surrounding environment, when they can acquire antibiotics or antibiotic-resistant microorganisms from other animal species. In Europe, the honeybee-producing farms avoid antibiotic use for their bees because. The explanation for this is relatively low efficiency, development of antimicrobial resistance, development of weak, disease-prone hives. Thus, the elimination of infected hives rather than antibiotic treatment usually is applicable [7,8]. Several studies have shown that the use of antibiotics causes alterations in the microbiomes of humans and livestock. Therefore, the effects of antibiotic treatment on the honeybee gut microbiome are of major interest alongside with the importance of the gut microbiome in animal, including bees, health [9-12] and in context with the unexplained decline of honeybee colonies [13]. This making the studies of composition and the pattern of antimicrobial resistance important for understanding the honey bees nutritional and health-related issues [14].

4. Conclusions

Bacteria from the family *Enterobacteriacea* was predominant in gut microflora of honey bees. Resistant *Enterobacteriaceae* isolates are an issue of concerns with resistance to all the antimicrobials was implicated in the present study. Our study confirms that the gut of bees can be a reservoir of pathogenic and resistant to antibiotics bacterial strains. Thus, is essential to continue studies on the composition and the antimicrobial resistance of the gut microflora isolates.

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