

## ANTAGONISTIC EFFECT OF GUT MICROBIOTA OF HONEYBEE (*APIS MELLIFERA*) AGAINST CAUSATIVE AGENT OF AMERICAN FOULBROOD *PAENIBACILLUS LARVAE*

Miroslava Kačaniová<sup>1,2</sup>, Jaroslav Gasper<sup>3</sup>, Margarita Terentjeva<sup>4</sup>

### Address(es):

<sup>1</sup>Slovak University of Agriculture in Nitra, Faculty of Horticulture and Landscape Engineering, Department of Fruit Sciences, Viticulture and Enology, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia, phone number: +421 37 641 4715.

<sup>2</sup>University of Rzeszow, Faculty of Biology and Agriculture, Department of Bioenergy and Food Technology, Zelwerowicza St. 4, PL-35601 Rzeszow, Poland.

<sup>3</sup>Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia.

<sup>4</sup>Latvia University of Life Sciences and Technologies, Institute of Food and Environmental Hygiene, Faculty of Veterinary Medicine, Latvia K. Helmaņa iela 8, LV-3004, Jelgava, Latvia.

\*Corresponding author: [miroslava.kacaniova@gmail.com](mailto:miroslava.kacaniova@gmail.com)

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### ABSTRACT

The aim of our study was to isolate and identify the microorganisms of gut microbiota of honeybees with MALDI-TOF MS Biotyper and to identify their anti-*Paenibacillus larvae* affect. In our study, 200 samples of bee's gut originated from honeybee of Slovak origin were tested. A total of 23 species of 17 bacterial genera (10 Gram-negative and 6 Gram-positive, one yeast) were identified in gut of bees with MALDI-TOF Mass Spectrometry. The bacterial strains *Bacillus cereus*, *B. megatherium*, *B. oleronius*, *B. thuringiensis*, *Delftia acidovorans*, *Escherichia coli*, *Enterococcus cloacae*, *Issatchenkia orientalis*, *Klebsiella oxytoca*, *Lactobacillus acidophilus*, *L. crispatus*, *L. kumei*, *Morganella morgani*, *Pantotea agglomerans*, *P. ananatis*, *Pseudomonas oryzihabitans*, *Rahnella aquatilis*, *R. terrigena*, *Raouella ornithinolytica*, *Serratia liquefaciens*, *S. marcescens*, *Sphingomonas parapaucimobilis*, *Staphylococcus epidermidis* were representative part of gut microflora of bees. Bees isolates of *Paenibacillus larvae* and *Paenibacillus larvae* CCM 4438 were tested for antimicrobial activity. The best antimicrobial activity against *P. larvae* isolated from bee's gut showed *L. kumei*, *L. crispatus* and *L. acidophilus*. The lower antibacterial activity against *P. larvae* was found in *Raouella ornithinolytica*, *Pantotea ananatis* and *Serratia liquefaciens* isolated from bees' gut. The strongest antimicrobial activity of *L. kumei*, *L. acidophilus* and *L. crispatus* and the lowest antimicrobial activity of *Pantotea ananatis* and *Rahnella aquatilis* were found against *P. larvae* CCM 4438.

**Keywords:** GIT microbiology, mass spectrometry, *Apis mellifera*, antimicrobial activity, *Paenibacillus larvae*

### INTRODUCTION

The Western honeybee (*Apis mellifera*) is the most important insect pollinator around the globe. Human food consumption relies on insect pollination and almost 90% of crops depends on honeybee pollination services (Klein *et al.*, 2007). Honeybee is important not only for its services and products consumed by humans, because the honeybee plays unique role in sustaining of natural plant biodiversity in ecosystem (Potts *et al.*, 2010). Honeybees are social insects living in colonies consisted of different generations, taking cooperative brood care and a reproductive division of labor. The colony consists of female worker bees which number could be between 15000-50000 in different seasons with a peak in the summer, male drones usually present in the spring and one reproducing female queen bee (Vojvodic *et al.*, 2013). Honeybees share a diverse microbiome with different bacterial taxa, ranging from Gram-positive bacteria to alpha-, beta-, and gamma-proteobacteria (Gilliam, 1997; Jeyaprakash *et al.*, 2003). Only some of those bacterial species are pathogenic to bees, most of them have never been reported in honeybee disease and their impacts on honeybee are still unknown. Gut bacteria is associated with stimulation of the immunity of honeybee larvae, because in this stage the organism is especially vulnerable to infection, and anti-pathogen immune responses. Feeding larvae with non-pathogenic gut bacteria containing diet stimulated the expression of genes involved in immune response (Evans and Lopez, 2004). Supplement of probiotic bacteria into diet of the honeybee larval food decreased the number of infections with *Paenibacillus larvae* (Forsgren *et al.*, 2010). It has been concluded that the addition of probiotic bacteria will improve the honeybee immune response and protection against pathogens. *Paenibacillus larvae*, a Gram-positive sporulated bacterium, causes the American foulbrood disease is of broad distribution, extremely contagious diseases and could kill the honeybee colonies (Williams, 2000). Infectious dose of a 24-h-old larva ill dose is 10 spores. Since disease is mostly

incurable, and the significant economic loss in apiculture are attributed due to high mortality rates (Sabate *et al.*, 2009).

The aim of this study was to study microbiota isolated from gut of local honeybee (*Apis mellifera*) of East Slovakia with MALDI-TOF MS Biotyper and to evaluate the effect of isolated gut bacteria from the *P. larvae*.

### MATERIAL AND METHODS

#### Samples

Altogether, 100 samples of the Slovak worker honeybees examined in this study. Additionally, a total of 100 gastrointestinal samples from the Slovak beekeeper located in the east, middle and west part of Slovakia were collected (Košice, Poprad, Liptovský Hrádok, Nitra, Brezno). The specimens were obtained from the hive. All samples were placed in sterile sample containers and transported to the laboratory on ice for microbiological investigations. 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 bee gut were detected. A 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 microorganisms, the inoculated Tryptone soya agar and Enterococcus selective agar (TSA, ESA, Oxoid, UK) was incubated for 48-72 h at 30°C. Also blood agar (BA, Oxoid, UK) and de Man, Rogosa and Sharpe agar (MRS, Oxoid, UK) were incubated for 48-72 h at 37°C anaerobically. 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.

**Sample preparation and MALDI-TOF MS measurement**

Prior to identification, the bacterial colonies were sub-cultured on TSA agar (Tryptone Soya Agar, Oxoid, UK) at 37°C for 18-24 h. One colony of eight bacterial cultures was selected. The identification was performed with Maldi TOF MS Biotyper as described by Kačaniová et al. (2017). Totally, a number of 534 isolates were identified with score higher than 2.

**Detection of antibacterial activity of different isolates of bacteria against Paenibacillus larvae**

The bacterial strains of *Bacillus cereus*, *B. megatherium*, *B. oleronius*, *B. thuringiensis*, *Delftia acidovorans*, *Escherichia coli*, *Enterococcus cloacae*, *Issatchenka orientalis*, *Klebsiella oxytoca*, *Lactobacillus acidophilus*, *L. crispatus*, *L. kunkei*, *Morganella morgani*, *Pantotea aglomerans*, *P. ananatis*, *Pseudomonas oryzihabitans*, *Rahnella aquatilis*, *R. terrigena*, *Raoutella ornithinolytica*, *Serratia liquefaciens*, *S. marcescens*, *Sphingomonas parapaucimobilis*, *Staphylococcus epidermidis*, *Paenibacillus larvae* were isolated from gut microflora of bees. *Paenibacillus larvae* CCM 4438 was originated from the Czech Collection of microorganisms (Brno, Czech Republic). Bacterial strains after 24 h of incubation in MRS broth and TSA broth were centrifuged at 5500 g for 10 min at 4°C and 0.1 mL of the supernatant was used for detection of antibacterial activity. The suspension of tested bacteria isolated from gut of bees in saline (0.1 mL of 10<sup>5</sup> cfu.mL<sup>-1</sup>) were spread on Mueller Hinton Agar (MHA, Oxoid). Filter paper discs (6 mm in diameter) were impregnated with 15 µL of supernatant and placed on the inoculated agars. Agars were incubated at 4°C for 2 h and at 37°C for 24 h. All the tests were performed in triplicate. Filter discs impregnated with a 10 µL of distilled water were used as the negative, but antibiotic (amikacin 10 µg and gentamicin 10 µg) as the positive control (Kačaniová et al., 2018).

**RESULTS AND DISCUSSION**

The composition of the gut microbiota of social insects has been shaped changed by coevolution. The social behaviour of honeybees creates favorable conditions for exchange with microorganisms, which are going to be transmitted either between colonies members or different generations (Engel and Moran, 2013a). The gut microbial composition of honeybees is unique with microorganisms present to be niche-adapted bacterial species with a high degree of genetic diversity (Engel and Moran, 2013b).

**Isolated bacterial group**

Total count of microorganisms bacteria in bees gut ranged from 5.25 to 6.58 log cfu.g<sup>-1</sup>. Enterococci count ranged from 4.25 to 4.87 log cfu.g<sup>-1</sup>. Coliforms bacteria counts were from 4.55 to 5.80 log cfu.g<sup>-1</sup>, lactic acid bacteria counts - from 2.22 to 2.51 log cfu.g<sup>-1</sup> and yeast from 1.18 to 1.23 log cfu.g<sup>-1</sup>. Kačaniová et al. (2004) found in the midgut and rectum of the honeybee that counts of aerobic microorganisms were significantly lower than counts of anaerobes (10<sup>5</sup>-10<sup>6</sup> vs. 10<sup>8</sup>-10<sup>9</sup> viable cells per g of intestinal content). Total numbers of anaerobic microorganisms were almost identical with the count of anaerobic Gram-positive acid resistant rods. A higher number of coliform bacteria and *Bacillus* spp. was detected in the rectum (10<sup>5</sup> per g). Anaerobic and aerobic microorganisms, coliforms, enterococci, *Bacillus* spp., *Pseudomonas* spp. and yeasts were found in all bees; lactobacilli, staphylococci and moulds were not found.

**Microorganisms identified with MALDI-TOF MS Biotyper**

A total of 23 species of 17 bacterial genera (10 Gram-negative and 6 Gram positive, 1 yeast) were identified in gut of bees with MALDI-TOF Mass Spectrometry. Gram-negative, Gram-positive and yeast isolates comprised 43.63% (233 isolates), 51.87% (277 isolates) and 4.49% (24 isolates), respectively. Isolated species of microorganisms from bees' gut are shown in Table 1. Percentage of isolated species is at Figure 1. Number of isolates of each species for Gram-positive, Gram-negative bacteria and yeasts are shown in Table 2. The most abundant Gram-negative bacteria were *Delftia acidovorans*, *Serratia marcescens*, *Escherichia coli* and *Serratia liquefaciens*. *Lactobacillus* spp. was the most abundant within 3 different species of Gram-positive bacteria with *Lactobacillus acidophilus*, *L. crispatus* and *L. kunkei* were the most distributed.

**Table 1** Isolated species of microorganisms from gut of honeybees

Family	Genera	Species
Bacillaceae	Bacillus	Bacillus cereus
Bacillaceae	Bacillus	Bacillus megatherium
Bacillaceae	Bacillus	Bacillus oleronius
Bacillaceae	Bacillus	Bacillus thuringiensis
Comamonadaceae	Delftia	Delftia acidovorans
Enterobacteriaceae	Escherichia	Escherichia coli
Enterococaceae	Enterococcus	Enterococcus cloacae
Saccharomycetaceae	Issatchenka	Issatchenka orientalis
Enterobacteriaceae	Klebsiella	Klebsiella oxytoca
Lactobacillaceae	Lactobacillus	Lactobacillus acidophilus
Lactobacillaceae	Lactobacillus	Lactobacillus crispatus
Lactobacillaceae	Lactobacillus	Lactobacillus kunkei
Enterobacteriaceae	Morganella	Morganella morgani
Enterobacteriaceae	Pantotea	Pantotea aglomerans
Enterobacteriaceae	Pantotea	Pantotea ananatis
Pseudomonadaceae	Pseudomonas	Pseudomonas oryzihabitans
Enterobacteriaceae	Rahnella	Rahnella aquatilis
Enterobacteriaceae	Rahnella	Rahnella terrigena
Enterobacteriaceae	Raoutella	Raoutella ornithinolytica
Enterobacteriaceae	Serratia	Serratia liquefaciens
Enterobacteriaceae	Serratia	Serratia marcescens
Sphingomonadaceae	Sphingomonas	Sphingomonas parapaucimobilis
Staphylococcaceae	Staphylococcus	Staphylococcus epidermidis

**Table 2** Number of isolates identified with MALDI-TOF MS Biotyper in bees' gut

Microorganisms	No. of isolates	No. of isolates in %
<i>Delftia acidovorans</i>	42	18.1
<i>Escherichia coli</i>	21	9.1
<i>Klebsiella oxytoca</i>	18	7.73
<i>Morganella morgani</i>	15	6.44
<i>Pantotea aglomerans</i>	10	4.29
<i>Pantotea ananatis</i>	12	5.15
<i>Pseudomonas oryzihabitans</i>	10	4.29
<i>Rahnella aquatilis</i>	15	6.44
<i>Rahnella terrigena</i>	14	6.01
<i>Raoutella ornithinolytica</i>	18	7.73
<i>Serratia liquefaciens</i>	20	8.58
<i>Serratia marcescens</i>	22	9.44
<i>Sphingomonas parapaucimobilis</i>	16	6.87
<b>Gram-negative bacteria</b>	<b>233</b>	
Microorganisms	Total	Percentage of bacterial species
<i>Bacillus cereus</i>	22	7.94
<i>Bacillus megatherium</i>	5	1.81
<i>Bacillus oleronius</i>	8	2.89
<i>Bacillus thuringiensis</i>	12	4.33
<i>Enterococcus cloacae</i>	27	9.75
<i>Lactobacillus acidophilus</i>	46	16.61
<i>Lactobacillus crispatus</i>	52	18.77
<i>Lactobacillus kunkei</i>	69	24.91
<i>Staphylococcus epidermidis</i>	36	12.99
<b>Gram-positive bacteria</b>	<b>277</b>	
<i>Issatchenka orientalis</i>	24	100
<b>Yeasts</b>	<b>24</b>	
<b>Total</b>	<b>534</b>	

The microbiota associated with honeybee is complex, and previously mainly yeasts, Gram-positive bacteria (*Lactobacillus rigidus apis*, *S. constellatus*, *Bacillus* spp., *Streptococcus*, and *Clostridium* spp.), and Gram-negative or Gram-variable bacteria (*Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus*, and *Pseudomonas*) were identified (Mohr and Tebbe, 2006).

The presence of *Enterobacteriaceae*, represented by *Pantoea* spp. and *Enterobacter gergoviae* were identified (Rada et al., 1997; Ebrahim and Lotfalian, 2005; Chahbar and Mahamed, 2014). Distribution of the same

genera of *Enterobacteriaceae* was explained with trophallaxie is made between the worker bees (Hansen et al., 2004). *Enterococcus* group is not abundant in honeybee intestinal samples in comparison to *Lactobacillus* spp. Coinfection of

*Enterococcus faecalis* with *Melissococcus plutonius* was reported as developing (Evans and Schwarz, 2011).



Figure 1 Composition of honeybees gut microbiota

**The anti-Paenibacillus larvae activity**

All tested microorganisms exhibited the antimicrobial activity against *Paenibacillus larvae*. The strongest antimicrobial activity was shown by *Lactobacillus* spp., while the weakest antimicrobial activity was exhibited by *Enterobacteriaceae* (Table 3). The best antimicrobial activity against *P. larvae* were expressed by *L. kunkei*, *L. crispatus* and *L. acidophilus*. *Raoutella ornithinolytica*, *Pantotea ananatis* and *Serratia liquefaciens* were less active against *P. larvae* isolated from bees' gut. The strongest antimicrobial activity of *L. kunkei*, *L. acidophilus* and *L. crispatus* and the weakest antimicrobial activity of *Pantotea ananatis* and *Rahnella aquatilis* were found against *P. larvae* CCM 4438.

*In vitro* inhibition of *P. larvae* has been previously reported (Evans and Armstrong, 2005; Alippi and Reynaldi, 2006; Sabaté et al., 2009).

Endogenous strains of lactic acid bacteria of bees did not inhibit honeybee pathogenic *P. larvae* or *L. sakei* subsp. *sakei* JCM 1157 used as the indicator bacteria. The bacteriocin-producing exogenous bacterial strains - *E. durans* A5-11, *E. faecalis* KT2W2G and *L. lactis* subsp. *lactis* KT2W2L - inhibited *P. larvae* strains (Janashia et al., 2016).

The bacteriocin-producing strains were absolutely inactive against *Lactobacillus* spp. group with different patterns were observed for other bacterial groups tested (Sabate et al., 2009).

Endogenous administration of LAB inhibited *Melissococcus plutonius*, which causes European Foulbrood (Forsgren et al., 2009, Vasquez et al., 2012). According to their works, the effectiveness of endogenous LAB administration against the bacterial pathogens increased when a mixture of LAB was used, but this antimicrobial action has never been attributed to bacteriocin action. Lactic acid bacteria strains of the bee gut, namely *Lactobacillus* AJ5, IG9, A15 and CRL1647 were able to inhibit *S. aureus* ATCC29213, *B. cereus* C1, *E. coli* O157:H7, *L. monocytogenes* and *P. larvae*; but strain A15 failed to inhibit two of eight *P. larvae* strains. *E. faecium* SM21 exhibited activity only against *L. monocytogenes* (Audisio et al., 2011).

**Table 3** Antimicrobial activity of microorganisms isolated from gut of honeybees

Species	<i>Paenibacillus larvae</i>	<i>Paenibacillus larvae</i> CCM 4438
	mean±SD	mean±SD
<i>Bacillus cereus</i>	14.67±0.58	15.33±0.58
<i>Bacillus megatherium</i>	13.67±1.53	12.67±1.15
<i>Bacillus oleronius</i>	13.67±1.53	13.33±0.58
<i>Bacillus thuringiensis</i>	13.67±1.53	13.67±0.58
<i>Delftia acidovorans</i>	16.67±1.53	15.33±0.58
<i>Escherichia coli</i>	16.33±1.53	14.67±2.52
<i>Enterococcus cloacae</i>	15.33±2.52	14.33±1.15
<i>Issatchenkia orientalis</i>	15.67±2.08	13.67±1.53
<i>Klebsiella oxytoca</i>	13.67±1.53	12.67±1.15
<i>Lactobacillus acidophilus</i>	20.33±0.58	19.67±1.53
<i>Lactobacillus crispatus</i>	20.67±1.15	19.33±1.15
<i>Lactobacillus kunkei</i>	24.33±0.58	23.67±1.53
<i>Morganella morgani</i>	11.33±0.58	11.67±0.58
<i>Pantotea aglomerans</i>	9.67±0.58	9.33±1.53
<i>Pantotea ananatis</i>	7.67±0.58	6.67±0.58
<i>Pseudomonas oryzihabitans</i>	11.33±1.15	10.67±0.58
<i>Rahnella aquatilis</i>	8.67±0.58	6.67±1.53
<i>Rahnella terrigena</i>	8.33±1.53	7.33±1.15
<i>Raoutella ornithinolytica</i>	6.33±1.53	7.67±0.58
<i>Serratia liquefaciens</i>	7.67±0.58	8.33±0.58
<i>Serratia marcescens</i>	8.33±0.58	7.33±0.58
<i>Sphingomonas parapaucimobilis</i>	11.33±0.58	10.67±0.58
<i>Staphylococcus epidermidis</i>	12.67±1.15	13.67±1.53

## CONCLUSION

*Bacillus cereus*, *B. megatherium*, *B. oleronius*, *B. thuringiensis*, *Delftia acidovorans*, *Escherichia coli*, *Enterococcus cloacae*, *Issatchenkia orientalis*, *Klebsiella oxytoca*, *Lactobacillus acidophilus*, *L. crispatus*, *L. kunkei*, *Morganella morgani*, *Pantotea aglomerans*, *P. ananatis*, *Pseudomonas oryzihabitans*, *Rahnella aquatilis*, *R. terrigena*, *Raoutella ornithinolytica*, *Serratia liquefaciens*, *S. marcescens*, *Sphingomonas parapaucimobilis*, *Staphylococcus epidermidis* strains associated with adult worker bees were isolated and characterized to extend the knowledge on microorganisms inhabiting the bees gut. The best antimicrobial activity against both *P. larvae* isolated from bees' gut were shown by *L. kunkei*, *L. crispatus* and *L. acidophilus*.

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