

## Antimicrobial Effect of *Lactobacillus kunkeei* Against Pathogenic Bacteria Isolated from Bees' Gut

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### Abstract

The aim of this study was to evaluate antimicrobial activity of *Lactobacillus kunkeei* of bee's gastrointestinal tract (GUT) against pathogenic bacteria isolated from bees' gut. Content of the intestinal tract microbiota was cultured for isolation of different group of bacteria. Gut homogenates were plated on de Man, Rogosa and Sharpe agar plates and incubated for 48-72h at 30 °C anaerobically; on McConkey agar and incubated for 24-48h at 37°C aerobically. Then, the identification of isolates with MALDI-TOF MS Biotyper was done. The bacterial strains of *Lactobacillus kunkeei*, *Bacillus cereus*, *Bacillus megatherium*, *Bacillus simplex*, *Bacillus thuringiensis*, *Delfia acidovorans*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella aerogenes*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Sphingomonas parapaucimobilis* were isolated from gut content of bees. The disc diffusion method was used for the determination of antimicrobial activities of the *Lactobacillus kunkeei* supernatant against other isolated bacterial strains of bees' origin. The best antimicrobial activity of *Lactobacillus kunkeei* supernatant was found against *Klebsiella aerogenes*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens*. Less effective the *L. kunkeei* supernatant was against *Bacillus cereus*, *Bacillus megatherium*, *Bacillus simplex*, *Bacillus thuringiensis*. The moderate antibacterial activity was identified against *Delfia acidovorans*, *Escherichia coli*, *Enterococcus faecalis*, *Sphingomonas parapaucimobilis*.

**Keywords:** *Lactobacillus kunkeei*, antimicrobial activity, bees, pathogenic bacteria

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### 1. Introduction

The honey bee (*Apis mellifera*) is an important pollinator of economically important crops. The loss of honey bees' colonies underlined a need for better understanding of bees and environmental interaction with both environment and bees' microbiota studies to be involved. Pollen and honey storage, pollination environment, beekeeping operation can affect up to 5–10 agricultural

ecosystems within a year. Variable on-site condition impact the particular bee colonies and the most important factors are climate, flora, application of biocides and proximity of water sources. Each of the factors may influence all the bees' colony cycle with many of biocides could later be found in the wax and stored pollen, "beebread" and other products of beekeeping [1,2]. Sub-lethal doses of biocide may affect the microbiological balance of the bees, hive, beekeeping products resulting in long-term

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consequences [3-5]. The comprehensive molecular surveys have shown that the microbiological imbalance may occur at many levels of organization [1,5-12]. The factors influencing the microbial microbiota of the colony, particularly the microbial communities in the gut, crop and pollen, need to be studied.

Honey bee hive can harbor a great variety of microorganisms inside it and the bees themselves are a biota of countless populations of microbes. Microorganisms associated with honey bees can be classified as pathogenic and non-pathogenic [13], and potentially beneficial [14]. While pathogenic microflora gets the most attention due to its importance on revenues, non-pathogenic and beneficial microorganisms may turn out to be as much as important. Several studies are showing that presence of lactic acid bacteria from genera *Lactobacillus*, *Bifidobacterium* and *Leuconostoc* in honey bee intestinal tracts can increase immune response of honey bee larvae [13].

The role of *L. kunkeei* in bees for beehives is not well described. *In vitro* studies have shown that *L. kunkeei* is important for bees' health and can inhibit the growth of bacteria and yeast, including bees pathogens [14-17]. The previous study showed that the morbidity of honeybee larvae caused by specific pathogens was reduced after the diet had been supplemented with LAB, including *L. kunkeei* [14,15]. *L. kunkeei* produces extracellular proteins during tests *in vitro* as a reaction to environmental stress in presence of lipopolysaccharides and other stress-inducing compounds [18]. It was hypothesized that *L. kunkeei* can inhibit the bacteria and fungi which could be pathogenic to bees [14]. The definition of animal probiotics is based on the principle that probiotics are live microorganisms suppressing intestinal infections, increasing production, strengthening animal growth and defending the gastrointestinal environment against morbidity. In the same review, endogenous symbiotic bacteria are described as contributing to host health by their action in digestion, gut wall function and cooperation with the gut immune system [19].

The study aimed to determine antimicrobial activities of honeybee's endogenous *Lactobacillus kunkeei* against pathogenic bacteria isolated from bees.

## 2. Materials and methods

### 2.1. Isolation of *Apis mellifera* gut bacteria

Colonies of *Apis mellifera* from wooden hives in an apiary at the East Slovakia in August 2018 were studied. Foragers (n=10) and returning workers (n=10) of *A. mellifera* from each colony were aseptically taken at the hive entrances and put into a 1.5 ml sterile tubes. For removal of gut, the digestive tracts were aseptically dissected, transferred into a tube with physiological solution and homogenized. Then the material was plated onto de Man, Rogosa and Sharpe agar (MRS, Oxoid), incubated for 48-72 h at 30°C and McConkey agar (MCA), incubated for 24-48 h at 37°C. Bacterial colonies were identified according to size, color and morphology.

### 2.2. Sample preparation and MALDI-TOF MS measurement

Prior to identification, the bacterial colonies were subcultured on Tryptone Soya agar (Oxoid) at 37°C for 18-24 h. One colony of each bacterial isolate was selected for screening. Subsequently, an analysis of the bacteria identification was performed using the MALDI-TOF MS Biotyper.

### 2.3. Bacterial strains for testing

The bacterial strains *Lactobacillus kunkeei*, *Bacillus cereus*, *Bacillus megatherium*, *Bacillus simplex*, *Bacillus thuringiensis*, *Delfia acidovorans*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella aerogenes*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Sphingomonas parapaucimobilis* isolated from gut microflora of bees.

### 2.4. Detection of antibacterial activity of lactobacilli

Culture of *Lactobacillus kunkeei* after 24 h of incubation in MRS broth was 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) was 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 MHA for incubation 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.

### 2.5. Statistical analyses

For tested bacteria, the mean and standard deviation of inhibition zones were calculated.

### 3. Results and discussion

The bacterial strains of *Lactobacillus kunkeei*, *Bacillus cereus*, *Bacillus megatherium*, *Bacillus simplex*, *Bacillus thuringiensis*, *Delfia*

*acidovorans*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella aerogenes*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Sphingomonas parapaucimobilis* were isolated from gut content of bees. The mass spectrum of *L. kunkeei* isolated from gut of bees with MALDI-TOF MS Biotyper is shown in Figure 1. *L. kunkeei* is a symbiotic in honey and usually is the dominating LAB member. The microorganism is colonizing the honeybee crop by interaction with the epithelial layer. *L. kunkeei* is considered as a fructophilic LAB [20, 21]. It was found in wine, flowers and honey [22].

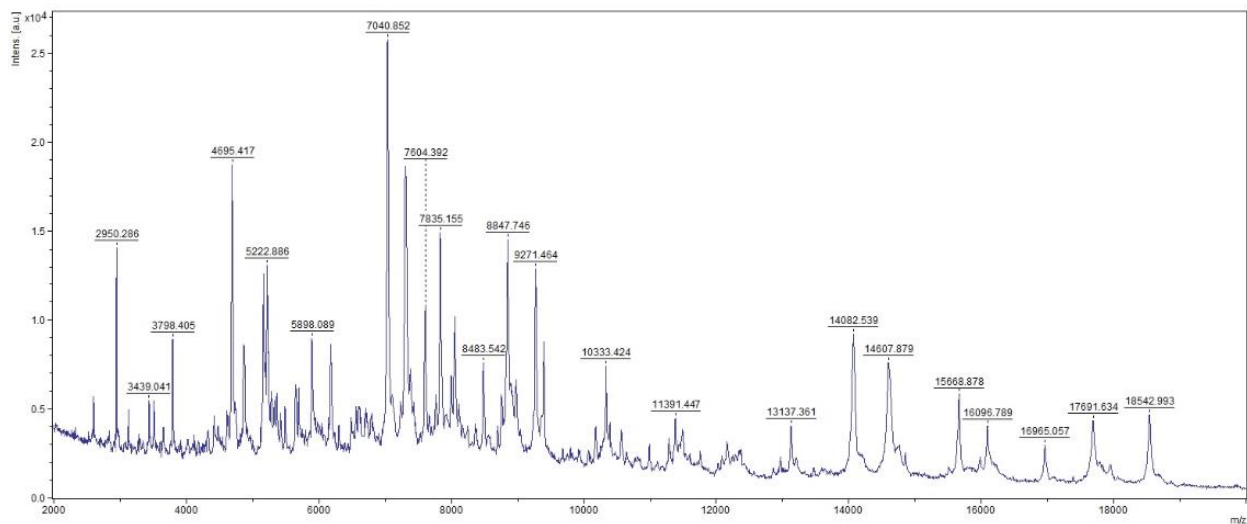


Figure 1. Mass spectrum of *Lactobacillus kunkeei* identified with MALDI-TOF Biotyper

Table 1. Antimicrobial activity of *L. kunkeei* supernatant (in mm)

| Bacterial strain                     | LKS        | AMK | GEN |
|--------------------------------------|------------|-----|-----|
| <i>Bacillus cereus</i>               | 5.33±0.58  | 28  | 29  |
| <i>Bacillus megatherium</i>          | 7.00±1.00  | 28  | 32  |
| <i>Bacillus simplex</i>              | 5.67±0.58  | 28  | 29  |
| <i>Bacillus thuringiensis</i>        | 4.67±0.58  | 33  | 32  |
| <i>Delfia acidovorans</i>            | 11.00±1.00 | 25  | 26  |
| <i>Escherichia coli</i>              | 10.00±1.00 | 28  | 31  |
| <i>Enterococcus faecalis</i>         | 11.00±1.00 | 28  | 31  |
| <i>Klebsiella aerogenes</i>          | 13.67±1.53 | 28  | 30  |
| <i>Morganella morganii</i>           | 14.67±0.58 | 30  | 32  |
| <i>Pseudomonas aeruginosa</i>        | 17.00±1.00 | 30  | 32  |
| <i>Serratia marcescens</i>           | 16.67±1.15 | 27  | 28  |
| <i>Sphingomonas parapaucimobilis</i> | 10.67±1.15 | 25  | 26  |

LKS - *L. kunkeei* supernatant, AMK - amikacin, GEN - gentamicin

The best antimicrobial activity of *Lactobacillus kunkeei* supernatant against *Klebsiella aerogenes*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens*. Less degree of antimicrobial activity was found against *Bacillus cereus*, *Bacillus megatherium*, *Bacillus simplex*, *Bacillus Thuringiensis*. The moderate antibacterial activity against *Delfia acidovorans*, *Escherichia coli*, *Enterococcus faecalis*, *Sphingomonas parapaucimobilis* was found in *L. kunkeei* supernatant.

Infectious diseases caused by pathogenic bacteria are requiring treatment with antimicrobials. However, the increasing antimicrobial resistance to antimicrobial drug require the new approach to address the issue. Assessment of the bioactive compounds could be an alternative to antimicrobial applications that could potentially alter the pathogenic bacteria by inhibiting or killing including those that are resistant to antibiotics. Bioactive compounds with bacteriostatic/bactericidal activity are known to be present as natural ingredients in honey, so honey by itself possess a potential an antibacterial [23]. The LAB is a group of bacteria that are known to produce lactic acid during fermentative metabolism. It is proved that certain species of LAB can produce bioactive compounds, which include organic acids, antimicrobial peptides, free fatty acids, ethanol, benzoic acid, enzymes and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Bioactive compounds via the different antimicrobial action produced a growth inhibition of different microbial pathogens both animal and human origin [24,25]. Sandi and Salasia [23] showed that the inhibitory potential of LAB can differ substantially studying 13 LAB strains. *L. kunkeei* Fhon2, *L. kunkeei* Lahm and *L. kunkeei* Yubipro are considered to be one of the most perspective LAB in order to inhibit the growth of bacterial pathogen by their ability to produce the largest inhibition zones than other *Lactobacillus* species. The inhibition of different pathogens including *Serratia marcescens*, *Klebsiella aerogenes*, *S. aureus*, *P. aeruginosa* and *E. coli* was proved previously [26].

#### 4. Conclusions

Preparations of the *Lactobacillus kunkeei*, which possess probiotic properties, could be used in the beekeeping sector to support the immunity of bee. Resistance to the antimicrobials could be

minimized as the application of those substances in bee's hives could be decreased. It is hoped that future studies on the inhibitory activity of *L. kunkeei* against *Bacillus cereus*, *Bacillus megatherium*, *Bacillus simplex*, *Bacillus thuringiensis*, *Delfia acidovorans*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella aerogenes*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Sphingomonas parapaucimobilis* may prove to be encouraging. As probiotics from fermented foods, the LAB of animal origin might have a potential to be used in a variety of applications, including animal health.

#### Acknowledgements

This study was written during realization of the project REVITAL No. 26210120038 supported by the Operational Programme Research and Development funded from the European Regional Development Fund.

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