

## IDENTIFICATION AND ANTIBIOTIC RESISTANCE PROFILE OF *ENTEROBACTERIACEAE* SPECIES AND *LACTOBACILLUS* SPP. ISOLATED FROM HONEY BEES (*APIS MELLIFERA*) DIGESTIVE TRACT

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### ABSTRACT

Honey bees play important role in agricultural environment as main pollinators. Its important for many agricultural and wild plants. Also honey bee are producers of honey, which is consumed directly and it should be not a heat treatment. Many bacteria can be survive in honey for long time. Some of these bacteria are human and animal facultative pathogens, including *Enterobacteriaceae* genera. If these bacteria contain antibiotic resistant genes than it can leads to troubles in healing of some of bacterial infections. *Lactobacillus* spp. can be a reservoir of resistant genes for pathogenic bacterial strains. In this study we isolated *Enterobacteriaceae* strains from digestive tracts of honey bees. These strains was tested to the eight selected antibiotics by disc diffusion method and strains were indentified by MALDI TOF MS Biotyper. From this study we determined resistance to piperacillin in the highest level. Equally, we determined that *Citrobacter gillenii* was resistant to three antibiotics (piperacillin, chloramphenicol and levofloxacin) from eight. Resistance to other antibiotics were determined in low levels and other indentified bacteria were resistant to one antibiotic, if any. Also we detected resistance in *Lactobacillus* spp. and determined MICs distribution for some selected antibiotics. For absence of similar studies we could not to discuss our results and we think that further experiments and studies are needed.

**Keywords:** antibiotic resistance, identification, enterobacteriaceae, lactobacilli, honey bee digestive tract

### INTRODUCTION

Honey bees, *Apis mellifera*, play an important agricultural role worldwide (Morse and Calderone, 2000) and are important pollinators in many natural ecosystems. Also honey bees are a main producers of honey (Rinderer et al., 1985), which is consumed directly and it should be not a heat treatment. Many research study considered that many types of microorganisms can survive in honey (Kačániová et al., 2009; Snowdon and Cliver, 1996; Olaitan et al., 2007; Ruiz-Argueso and Rodríguez-Navarro, 1975), mainly yeast and no vegetative bacterial species. However, microorganisms as *Enterobacteriaceae* genera occur in honey as primary (pollen, digestive tracts of honey, dust, air and nectar) or secondary (after-harvest) contamination. Vegetative bacteria can survive in honey, at cool temperature, for several years (Snowdon and Cliver, 1996).

The symbiotic microflora of the digestive tract of adult honeybees (*Apis mellifera*) consists of Gram-negative, Gram-positive and Gram-variable bacteria, moulds, and under some conditions also yeasts (Gilliam 1987).

Several bacterial species included in *Enterobacteriaceae* genera are commonly facultative pathogens of human, animals and plants which causes several types of bacterial infections (Starr and Chatterjee, 1972; Sanders and Sanders, 1997; Drudy et al., 2006; Pitout et al., 2005).

Antibiotic resistant bacteria and drug resistance genes have become an important environmental contamination issue which is receiving an increased attention (Kummerer, 2004; Pruden et al., 2006; Sapkota et al., 2007). The antibiotic resistance genes can be transferred between bacteria in the environment through plasmids, integrons and transposons (Pang et al., 1994; Schwarz and Chaslus-Dancla, 2001; Nordmann and Poirel, 2005; Pruden et al., 2006). Keyser et al. (2008) noted that in recent year, accumulating problems with resistant bacteria, leading to predictions that we are back the period before the discovery of antibiotics. Infections caused by resistant strains of microorganisms causing costly treatment of animals and humans. Such infections prolong the pathological

condition and if not treated with the right antibiotics may be increased mortality (Witte, 2006).

The aim of this study was identification of microorganisms isolated from digestive tracts of honey bees and determination of antibiotic resistant *Enterobacteriaceae* species and *Lactobacillus* spp. from these samples.

### MATERIAL AND METHODS

#### Samples collection

A total of 30 digestive samples of honey bees were collected in 2011 from apiary of Slovak University of Agriculture in Nitra. Honey bees were frozen at -16 °C for 2 minutes for killing. Digestive tracts were removed from bees bodies carefully and content of digestive tracts were suspended in 1 ml of sterile physiological solution. These bacterial suspensions were used immediately.

#### Cultivation and purification of microorganisms

Bacterial suspensions (100 µL) were spread on the surface of MacConkey agar (Biolife, Italy) for *Enterobacteriaceae* genera and MRS agar (Biolife, Italy) for *Lactobacillus* species. *Enterobacteriaceae* species were cultivated in aerobically condition at 35±2 °C for 24 hours (*Enterobacteriaceae*) and 48-72 hours (*Lactobacillus* spp.) in anaerobically condition in anaerobic jars. After incubation we used four-way streak plate method for obtaining the pure culture from each bacterial colonies cultivated in the same conditions. For this method Chromogenic coliform agar (Biolife, Italy) for *Enterobacteriaceae* genera and MRS agar for *Lactobacillus* species was used. Purifying of colonies was repeated as far as we obtained pure culture observed and determined by microbiological laboratory techniques and Gram staining.

**Identification of microorganisms**

For basic identification we used Gram staining (*Enterobacteriaceae* genera, *Lactobacillus* spp.) and Chromogenic coliform agar (Biolife, Italy) for *Enterobacteriaceae* genera only. For biochemical characterization of *Enterobacteriaceae* species Enterotest 24 (Erba Lachema, Czech Republic) was used. Followed computer identification program TNW Lite 7.0 (Erba Lachema, Czech Republic) was used as well. For exact identification of *Enterobacteriaceae* strains and *Lactobacillus* species were used MALDI-TOF MS Biotyper (Brucker Daltonics GmbH, Germany) and method for prepare of samples to identification was done by Kmet' and Drugdová (2012).

**Antibiotic susceptibility testing**

The pure inoculums of *Enterobacteriaceae* strains and *Lactobacillus* species were prepared by suspending of colonies into the physiological solution from agar plates and every suspensions were adjusted to equal a 0.5 (*Enterobacteriaceae*) and 1 (*Lactobacillus* spp.) McFarland standard. The sensitivity of all *Enterobacteriaceae* strains were tested against: piperacillin (PIP 30) 30 µg/disc, ceftriaxone (CRO 30) 30 µg/disc, doripenem (DOR 10) 10 µg/disc, levofloxacin (LVX 5) 5 µg/disc, amikacin (AMI 30) 30 µg/disc, gentamicin (GEN 30) 30 µg/disc, tygecycline (TGC 15) 15 µg/disc and chloramphenicol (CHL 30) 30 µg/disc (discs from OXOID, England). For antibiotic susceptibility testing of *Enterobacteriaceae* genera disc diffusion method was used according by EUCAST (2013a) (Antimicrobial susceptibility testing: EUCAST disk diffusion method, Version 3.0, April 2013). Incubation of *Enterobacteriaceae* strains were done at 35±2 °C on Mueller-Hinton agar (Biolife, Italy). Interpretation of inhibition zones around the disc was according by EUCAST (2013b) (European Committee on Antimicrobial Susceptibility Testing, Breakpoint tables for interpretation of MICs and zone diameter, Version 3.1, valid from 2013-02-11). The inhibition zones were controlled with the references sensitive *Escherichia coli* CCM 3988. For susceptibility testing of *Lactobacillus* species antibiotic M.I.C.E. strips (E-test) (OXOID, England) as erythromycin (ERY 256-0,015 µg/mL), ampicillin (AMP 256-0,015 µg/mL), gentamicin (GEN 256-0,015 µg/mL), meropenem (MEM 256-0,015 µg/mL) and

vancomycin (VAN 256-0,015 µg/mL) and strips MIC diffusion method were used. Incubation of *Lactobacillus* species were done at 35±2 °C on combination of agars (9:1) Mueller-Hinton and MRS agar in anaerobically conditions.

**RESULTS AND DISCUSSION**

A total 30 samples digestive tracts of honey bees (*Apis mellifera*) were investigated. *Enterobacteriaceae* species were cultivated from 22 digestive tracts samples only. We identified *Enterobacteriaceae* species and determined their resistance to some selected antibiotics.

In this experiment we isolated and identified 9 *Enterobacteriaceae* species. Followed bacteria were identified *Escherichia coli*, *Serratia liquefaciens*, *Serratia marcescens*, *Citrobacter gillenii*, *Pantoea agglomerans*, *Hafnia alvei*, *Ewingella americana*, *Moelleralla wisconsensis* and *Yersinia enterocolitica*. Equally these bacteria against 8 selected antibiotics were tested. Resistance to piperacillin, gentamicin, levofloxacin and chloramphenicol were detected. In this study, resistance to ceftriaxone, doripenem, amikacin and tigecycline was not detected. The highest level of resistance was determined in the case of piperacillin, where we determined piperacillin resistance in 14 *Enterobacteriaceae* strains from 22 tested. Low level of resistance were detected for gentamicin where we determined two *Enterobacteriaceae* strains. Resistance for levofloxacin and chloramphenicol we determined one *Enterobacteriaceae* strain only. More detailed spectrum of antibiotic resistance is designed in Table 1.

Besides, authors Ebrahimi and Lotfalian (2005) tested 33 isolates of *E. coli* isolated from digestive tracts of honey bees for several antibiotics and they determined resistance to gentamicin and chloramphenicol in low level as is described in our research. These researcher determined resistance to more antibiotics. They detected the highest level of antibiotic resistance for erythromycin and kanamycin.

We think that further collections and testing are needed, because studies about antimicrobial resistance in *Enterobacteriaceae* strain isolated from digestive tracts of honey bees are a few, if any.

**Table 1** Antibiotic resistance in *Enterobacteriaceae* genera isolated from honey bees (*Apis mellifera*) digestive tracts

Response	Tested antibiotics and counts of isolates							
	PIP	CRO	DOR	LVX	AMI	GEN	TGC	CHL
Resistance	14	0	0	1	0	2	0	1
Intermediate	2	3	3	2	1	2	0	0
Susceptible	6	19	19	19	21	18	22	21

**Legend:** PIP-piperacillin, CRO-ceftriaxone, DOR-doripenem, LVX-levofloxacin, AMI-amikacin, GEN-gentamicin, TGC-tygecycline, CHL-chloramphenicol

From the digestive tracts of honey bees we isolated and identified 9 *Enterobacteriaceae* strains mentioned above. For each isolated and identified strain we tested eight mentioned antibiotics. From this experiment we determined that *Escherichia coli*, *Serratia marcescens*, *Citrobacter gillenii*, *Pantoea agglomerans* and *Moelleralla wisconsensis* were resistant to piperacillin. Also we determined resistance to gentamicin in *Hafnia alvei* and *Ewingella americana*.

Resistance to chloramphenicol and levofloxacin were detected in *Citrobacter gillenii* only. Also multiresistance to three antibiotics (piperacillin, chloramphenicol, levofloxacin) was detected in *Citrobacter gillenii* only. In *Enterobacteriaceae* strains as *Serratia liquefaciens* and *Yersinia enterocolitica* we did not determined resistance to antibiotics which we used in this experiment (Table 2).

**Table 2** Identified *Enterobacteriaceae* strains isolated from digestive tracts of honey bees (*Apis mellifera*) and their resistance

Microorganisms	Resistance	Susceptibility
<i>Escherichia coli</i>	PIP	CRO, DOR, LVX, AMI, GEN, TGC, CHL
<i>Serratia liquefaciens</i>	S	PIP, CRO, DOR, LVX, AMI, GEN, TGC, CHL
<i>Serratia marcescens</i>	PIP	CRO, DOR, LVX, AMI, GEN, TGC, CHL
<i>Citrobacter gillenii</i>	PIP, CHL, LVX	CRO, DOR, AMI, GEN, TGC
<i>Pantoea agglomerans</i>	PIP	CRO, DOR, LVX, AMI, GEN, TGC, CHL
<i>Hafnia alvei</i>	GEN	PIP, CRO, DOR, LVX, AMI, TGC, CHL
<i>Ewingella americana</i>	GEN	PIP, CRO, DOR, LVX, AMI, TGC, CHL
<i>Moelleralla wisconsensis</i>	PIP	CRO, DOR, LVX, AMI, GEN, TGC, CHL
<i>Yersinia enterocolitica</i>	S	PIP, CRO, DOR, LVX, AMI, GEN, TGC, CHL

**Legend:** PIP-piperacillin, CRO-ceftriaxone, DOR-doripenem, LVX-levofloxacin, AMI-amikacin, GEN-gentamicin, TGC-tygecycline, CHL-chloramphenicol, S-susceptible

Identification of *Enterobacteriaceae* genera isolated from digestive tracts of honey bees researched Gilliam and Valentine (1974) too. They identified bacteria as *Enterobacter cloacae*, *Escherichia coli*, *Enterobacter aerogenes*, *Shigella* spp., and *Klebsiella pneumoniae*. These researchers not determined antibiotic profile of these microorganisms. In the same year researchers Gilliam and Morton (1974) identified more *Enterobacteriaceae* strains including *K. pneumoniae*, *Shigella* spp., *E. cloacae*, *E. coli*, *E. hafniae*, *Arizona* spp., *Enterobacter aerogenes*, *Serratia liquefaciens* and *Citrobacter* spp. isolated from digestive tracts of honey bees. Also Jeyaprakash et al. (2003) studied strains

*Enterobacteriaceae* isolated from digestive tracts of honey bees and they identified species as *E. coli* and *S. marcescens*.

In Table 3, the results are described and differed on the basis of the groups of antibiotics by EUCAST (2013b). Also meropenem was excluded from other antibiotics because different MICs dilution was used. Penicillins are presented by ampicillin, macrolides by erythromycin which inhibit protein or mRNA synthesis, aminoglycosides by gentamicin which inhibit the same synthesis as erythromycin, vancomycin presented groups of glycopeptides which belongs to the groups non-β-lactams cell wall acting antimicrobial agents, and meropenem which belongs to the carbapenems groups. The results are presented as

*Lactobacillus* spp. together. For macrolide antibiotic erythromycin, *Lactobacillus* spp. had MICs between 0.03 and 32 µg/mL and results showed 6 strains of *Lactobacillus* spp. where MICs was higher as 2 µg/mL and 5 strains with MICs higher as 8 µg/mL. Resistance to erythromycin is described as MICs value 1 mg.L<sup>-1</sup> established by EFSA (2012). The highest counts of lactobacilli isolates were in MICs value 0.12 µg/mL. The results for penicillins antibiotic ampicillin ranged from 0.03 to 16 µg/mL. The highest number of *Lactobacillus* species (12) were susceptible in 0.5 µg/mL. Results showed 3 isolates of *Lactobacillus* spp. where MICs value with 16 µg/mL and 2 isolates where MICs value with 1 µg/mL. This results showed 5 resistant isolates of lactobacilli to ampicillin. For gentamicin from the aminoglycosides group we determined range of MICs value from 0.25 to ≥256 µg/mL. The highest counts of isolates (9) were detected in the MICs value 16 µg/mL. In this research, resistance to gentamicin was detected in 5, 14 or 20 cases of lactobacilli isolates in depended in strain. However we detected 2 lactobacilli strains were with MICs 64 µg/mL and one strain with MICs value ≥256 µg/mL. In the case of vancomycin from glycopeptides group we determined the highest counts of isolates in MICs value 0.25 µg/mL. However MICs ranged from 0.06 to ≥256 µg/mL and 9 lactobacilli strains were with MICs ≥256 µg/mL which was classified as resistant. Also we observed antibiotic susceptibility for lactobacilli isolated from digestive tracts of honey bees (*Apis mellifera*) for carbapenems antibiotic meropenem. From this study we determined MICs range from 0.008 to 0.5 µg/mL and it resulted that meropenem was the most effective antibiotics against to lactobacilli isolates. More detailed results are described in the Table 3 and 4. Many researchers studied antibiotic resistance of lactobacilli and lactic acid bacteria isolated from dairy products, human samples etc., and they studied antibiotics resistance by variable methods (MIC - D'Aimmo et al., 2007; disc diffusion method - Zhou et al., 2005; Swenson et al., 1990; and Estet - Danielsen and Wind, 2003; Katla

et al., 2001). We could not find study about antibiotic resistance of lactobacilli isolated from honey bees (*Apis mellifera*) digestive tracts. Therefore we compared our results with results of antibiotic resistance of lactobacilli isolated from another sources.

Authors Danielsen and Wind (2003) in themselves study presented MICs distribution for *Lactobacillus* group to erythromycin from 0.03 to 1 µg/mL expect one strain with > 256 µg/mL. In the case of ampicillin these researchers determined similar results as is described in our study, where authors determined distribution of MICs ranged from 0.06 to 2 µg/mL and the highest number of isolates with MICs 0.5 µg/mL. Also similar results they determined in the case of gentamicin where the highest number of isolates was in MICs 16-32 µg/mL. Their isolates of *Lactobacillus* species were obtained from Chr. Hansen Culture Collection. Katla et al., (2001) observed antimicrobial susceptibility of starter cultures used in Norwegian dairy products and they determined lowest MICs range, which was from 0.125 to 1 µg/mL for erythromycin. These authors used interpretation of resistance from breakpoints tables for *Streptococcus* spp. by NCCLS (1999), which was established to 1 µg/mL. Also EFSA (2012) established resistance breakpoint for erythromycin with MICs 1 µg/mL. Authors Zhou et al., (2005) researched new probiotic strains of *Lactobacillus* species by disc diffusion method and they did not determined resistance to erythromycin and ampicillin. They detected resistance to gentamicin and vancomycin. Distribution of MICs for meropenem is not available for lactobacilli strains and therefore we could not compared it. The several researches like Lira et al. (2004), Picozzi et al. (2004), Caro et al. (2007) and Čížek et al. (2007), who researched antibiotic resistance isolated from different samples have argued, that results of antibiotic resistance vary from study to study.

**Table 3** Distribution of MICs of selected antibiotics for *Lactobacillus* spp. isolated from digestive tracts of honey bees (*Apis mellifera*)

ATB groups	ATB	Number of isolates for which the MIC (µg/mL) was as follows														
		≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	≥256
Macrolides	ERY		1	1	12	7	3		1		2	1	2			
Penicillins	AMP		6		3	4	12	2				3				
Aminoglycosides	GEN		1			1		2	5	1	6	9	2	2		1
Glycopeptides	VAN			1	5	10		1	1	1						9

  

ATB group	ATB	Number of isolates for which the MIC (µg/mL) was as follows														
		≤0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	≥32
Carbapenems	MEM			8	2	4	6	2	4	2						

Legend: ERY – erythromycin, AMP – ampicillin, GEN – gentamicin, VAN – vancomycin, MEM – meropenem, ATB – antibiotic(s)

In Table 4, geometrical average (MIC<sub>xG</sub>), MIC<sub>50</sub> and MIC<sub>90</sub> from MICs values which were detected in this study are described. From MICs results we calculated followed values: the highest MIC<sub>xG</sub> was determined for gentamicin with MICs

value 6,95 µg/mL and MIC<sub>50</sub> = 8 µg/mL and MIC<sub>90</sub> = 32 µg/mL. The lowest MIC<sub>xG</sub> was determined for meropenem with MICs value 0,04 µg/mL and MIC<sub>50</sub> = 0,06 µg/mL and MIC<sub>90</sub> = 0,25 µg/mL.

**Table 4** Antibiotic resistance of *Lactobacillus* spp. isolated from digestive tracts of honey bees (*Apis mellifera*)

Antibiotics	Statistical parameters for MICs value						
	Breakpoint resistance (mg/L) <sup>a</sup>	for	No. of resistant isolates	MIC <sub>xG</sub> *	MIC <sub>50</sub>	MIC <sub>90</sub>	Range
Erythromycin	≥ 1		6	0.38	0.12	0.25	0.03 – 32
Ampicillin	≥ 4 ≥ 2 ≥ 1		3-5 <sup>b</sup>	0.33	0.5	1	0.03 – 16
Gentamicin	≥ 8 ≥ 16 ≥ 32		5-14-20 <sup>b</sup>	6.95	8	32	0.03 - > 256
Vancomycin	≥ 2		11	2.21	0.25	256	0.06 - > 256
Meropenem	n <sup>c</sup>		0	0,04	0,06	0,25	0,008 – 0,5

Legend: \*Geometrical average, n<sup>c</sup> – not established, <sup>a</sup>Breakpoint resistance table for lactobacilli strains established by EFSA (2012) for different strains, <sup>b</sup>Resistance depends on the strain or group of lactobacilli

## CONCLUSION

From this research we identified some *in vitro* cultivatable *Enterobacteriaceae* species as *E. coli*, *S. liquefaciens*, *S. marcescens*, *C. gillenii*, *P. agglomerans*, *H. alvei*, *E. americana*, *M. wisconsinensis* and *Y. enterocolitica* by MALDI TOF MS Biotyper which were isolated from digestive tracts of honey bees. Equally we detected antibiotic resistance to some selected antibiotics as piperacillin, chloramphenicol, levofloxacin and gentamicin. The highest resistance level we determined for piperacillin. Also we determined that *Citrobacter gillenii* was resistant to three antibiotics from all tested. Also we determined the highest resistance in *Lactobacillus* spp. to erythromycin and detected 3 lactobacilli species to ampicillin. In conclusion, we would like to mention that this field of study need more experiment for exact decisions.

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