Antimicrobial Effect of Bee Collected Pollen Extract to *Enterobacteriaceae* Genera after Application of Bee Collected Pollen in their Feeding

Lukáš Hleba[1], Jaroslav Pochop[1], Soňa Felšöciová[1], Jana Petrová[1], Juraj Čuboň[2], Adriana Pavelková[2], Miroslava Kačániová[1],

[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia
[2] Faculty of Biotechnology and Food Sciences, Department of Animal Products Evaluation and Processing, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia

Abstract
In this study we researched antimicrobial activity of bee pollen extracts to *Enterobacteriaceae* genera isolated from chicken intestinal tract after application of bee collected pollen in their feeding. We used well plate agar diffusion method for antimicrobial testing of bee pollen extract and disc diffusion method for antibiotic susceptibility testing of bacteria by EUCAST. Identification of bacteria was done by test kit Enterotest 24. We identified tree bacterial strains: *E. coli*, *P. mirabilis* and *K. oxytoca*. We determined that *K. oxytoca* was resistant to ampicillin only and others identified strain were sensitive to used antibiotics. Also we determined antimicrobial effect of bee pollen extract to all tested strains of *Enterobacteriaceae* genera which were isolated from intestinal tract of chicken after application of bee collected pollen extract in their feeding. From obtained results we could be conclude that bacteria isolated from chicken after application of bee pollen extract had more resistance to bee collected pollen extract in *in vitro* experiment as *E. coli* CCM 3988, which did not be in contact with bee pollen extract.

Keywords: antimicrobial effect, bee collected pollen extract, *Enterobacteriaceae* genera

1. Introduction

Nowadays, the products of the honey bee, *Apis mellifera* L., are of great concern in many fields, e.g. nutritional and pharmaceutical industries. One of these products is bee-collected pollen [1]. Honeybees collect bee pollen in order to use it as food for all the developmental stages in the hive. Pollen is a fine, powder-like material produced by flowering plants and gathered by bees. Pollens grains are the male reproductive cells of flowers [2]. Flower pollens, whose composition can vary due to their botanical and geographic origin [3], contain carbohydrates, amino acids, proteins, lipids, vitamins, minerals, phenolic compounds, flavonoids, concentrations of phytosterols and are also rich in phytochemicals [5-7].
Phytochemicals, such as phenolic compounds are considered beneficial for human health since they decrease the risk of degenerative diseases by reducing oxidative stress and inhibiting acromolecular oxidation [8, 9]. They have been shown to possess free radical-scavenging and metal-chelating activity in addition to their reported anticarcinogenic properties [10]. Bee pollen has also been successfully used for the treatment of some cases of benign prostatitis and for oral desensitization of children who have allergies [11, 12]. There are some reports about the antimicrobial [13, 14] and antioxidant [15-17] activities of pollen separated into families.

* **Corresponding** author: Ing. Lukáš Hleba, lukas.hleba@gmail.com

In this study we researched antimicrobial activity of bee collected pollen extracts to *Enterobacteriaceae* genera isolated from chicken intestinal tract after application of bee collected pollen in their feeding.

2. Materials and methods

Collection of samples

Bacterial samples were collected from chicken intestinal tract by sterile cotton swabs tampons (Copan Inovation, Italy) after killing. Samples of pollen were obtained from 20 cultivated honeybee hives placed in 2 localities in the central part of Slovakia (Nitra region). Partial samples were collected several times during the period of April to August in the year 2009. Bee pollen samples containing mainly monofloral pollen loads from sunflower (*Helianthus annuus*), poppy (*Papaver somniferum*) and rape (*Brassica napus)* were stored frozen. *Escherichia coli* CCM 3988 was obtained from Czech Collection of Microorganisms.

Cultivation and identification of microorganisms

Bacterial cells captured on tampons were spread on the surface of MacConkey agar (Biolife, Italy) by sterile cotton swabs tampons. Petri dishes with bacteria were cultivated on 35±2 °C at 24 hours in aerobic conditions. After incubation we picked up some bacterial colonies randomly and we cleaned these colonies by four-ways streak plate method to obtaining of pure bacterial culture. Recultivation was done in the same condition. Initial identification of *Enetrobacteriaceae* strains were done on Chromogenic coliform agar (Oxoid, UK) and Triple sugar iron agar (Biolife, Italy). Biochemical identification of *Enterobacteriaceae* strains was done in ENTERO test 24 (Erba Lachema, Brno). Working procedure for biochemical identification is described into the manufacturer manual. Evaluation of biochemical test was done in identifying computer program TNW Lite 7.0 software (Erba Lachema, Brno).

Antibiotic susceptibility testing

The pure inoculum of *Enterobactericeae* strains were prepared by suspending of colonies into the physiological solution from agar plates and every suspensions were adjusted to equal a 0.5 McFarland standard. The sensitivity of all *Enterobacteriaceae* strains were tested against: ampicillin (AMP 10) 10 μg.disc^{-1}, chloramphenicol (C 30) 30 μg.disc^{-1}, meropenem (MEM 10) 10 μg.disc^{-1}, ceftriaxone (CRO 30) 30 μg.disc^{-1} and ofloxacin (OFX 5) 5 μg.disc^{-1}. For antibiotic susceptibility testing was used disc diffusion method according by EUCAST [18] (The European Committee on Antimicrobial Susceptibility Testing). Incubation of *Enterobacteriaceae* strains were done at 35±2 °C on Mueller-Hinton agar (Biomark, Pune). Interpretation of inhibiotion zones around the disc was according by EUCAST [18] (Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, valid from 2013/2/11). The inhibition zones were controlled with the references sensitivity *Escherichia coli* CCM 3988.

Preparing of bee collected pollen extract

A 50 g bee collected pollen (BCP) was added to 200 ml Et-OH and it was stayed 2 weeks at the room temperature in the dark. Than we filtered bee collected pollen solution by filter paper 185 mm (Whatman, UK). After filtration we evaporated bee collected pollen filtrate by vacuum rotary evaporator RE300DB (Stuart, UK). Yield of crude BCP was dissolved in DMSO and it was prepared to 50 % of BCP solution. We prepared next concentrations (25 %, 12.5 % and 6.25 %) of BCP by half dilution method with DMSO. More information about BCP extract is described into the Table 1.

Table 1 Detail info rations about bee collected pollen and extract

Composition of bee collected pollen	Yield of BCP extract (g)	Used concentration	Origin of bee collected pollen
Helianthus annuus *Papaver somniferum* *Brassica napus*	5.58	50 %, 25 %, 12.5 %, 6.25 %	Nitra, Slovakia

Well plate agar diffusion method

One hundred microliters of the standardized bacterial suspensions (0.5 McF°) were evenly spread on the Muller Hinton Agar, using a glass spreader. Wells were then bored into the agar using a sterile 6 mm diameter cork borer and the wells filled with the solution of the sterile extract with DMSO of Bee Collected pollen extract (50%, 25%, 12.5% and 6.25%). After pre-incubation for 2h to allow for proper diffusion of the extract into the media, plates were incubated at 35 ± 2 °C for 24 hours. Inhibition zones were then measured and recorded as the mean semi-diameter (mm) of inhibition zone. *Escherichia coli* CCM 3988 was used as a positive control. Pure DMSO was used as negative control.

Statistical evaluation

From obtained data were calculated basic variation-statistical values by using statistical program Statgraphic. In this study were calculated values like: average, standard deviation, minimum, maximum, coefficient of variation. Equally we used Tukey HSD test for determine of statistical differences between used concentrations of BCP extract.

3. Results and discussion

Antimicrobial activity of BCP extract

In this study we determined that bee collected pollen had antimicrobial effect to some wild *Enterobacteriaceae* genera isolated from chicken after application of bee collected pollen in their feeding. We researched that every percentage of bee collected pollen extracts had antimicrobial effect in the similar values. For example, semi-diameter of inhibition zone around the 50 % BCP extract was 3.1 ± 1.37 mm. The bigger semi-diameter of inhibition zone was in 12.5 % BCP extract (3.35 ± 0.99 mm) and the smaller semi-diameter zone was in 6.25 % BCP extract (2.95 ± 1.1 mm). Semi-diameter of inhibition zone in 25 % extract was 3.3 ± 1.26 mm (Table 2). However, we did not found statistical differences between researched concentrations (Table 3). Equally, we determined that *E. coli* CCM 3988 which was tested to 50 % BCP extract was very sensitive to antimicrobial effect of BCP extract (11 ± 4.17 mm). More detail information about semi-diameter of inhibition zones are described in Table 2.

Table 2 Summary statistics for inhibition zones of BCP extract to *Enterobacteriaceae* genera

Percentage of extracts of BCP	Count	Average	Standard deviation	Coeff. of variation	Min	Max	Range
50 % extract	20	3.1	1.37	44.28%	2	6	4
25 % extract	20	3.3	1.26	38.20%	2	6	4
12.5 % extract	20	3.35	0.99	29.50%	2	6	4
6.25 % extract	20	2.95	1.10	37.26%	1	5	4
50 % extract to *E. coli* CCM 3988	20	11	4.17	37.89%	6	17	11

After the evaluation of differences between semi-diameter of inhibition zones of antimicrobial effect of different concentration of BCP extracts to *Enterobacteriaceae* genera isolated from chicken after application of BCP extract into their feeding and *E. coli* CCM 3988 we determined that the bigger differences were between 50 % BCP extract to *E. coli* CCM 3988 and others used concentration of BCP extract to *Enterobacteriaceae* genera. Every detailed pieces of information about statistical values are described in Table 3.

Antibiotic resistance profile

Also we researched antibiotic resistance profile for *Enterobacteriaceae* genera isolated from chicken after application of BCP extract in their feeding. We determined that some microorganisms from *Enetrobacteriaceae* genera isolated from chicken were resistant to used antibiotics. Samples number 126-2 was resistant to ampicillin. More results from antibiotic resistance are visible in Table 4.

Table 3 Multiple range text between BCP extracts by Tukey HSD test (95 %)

Percentage of extracts of BCP	Count	Mean	Homogeneous Groups
50 % extract	20	3.1	A
25 % extract	20	3.3	A
12.5 % extract	20	3.35	A
6.25 % extract	20	2.95	A
E. coli	20	11	B
Contrast	**Sig.**	**Difference**	**+/- Limits**
50 % - 25 %		-0.2	1,88709
50 % - 12.5 %		-0.25	1,88709
50 % - 6.25 %		0.15	1,88709
50 % - *E. coli*	*	-7.9	1,88709
25 % - 12.5 %		-0.05	1,88709
25 % - 6.25 %		0.35	1,88709
25 % - *E. coli*	*	-7.7	1,88709
12.5 % - 6.25 %		0.4	1,88709
12.5 % - *E. coli*	*	-7.65	1,88709
6.25 % - *E. coli*	*	-8.05	1,88709

Table 4 Antibiotic resistance profile of *Enterobacteriaceae* genera isolated from chicken after application of BCP extract in their feeding

Samples / ATB	AMP 10		C 30		MEM 10		CRO 30		OFX 5	
120-1	24	S	28	S	34	S	34	S	30	S
77-2	22	S	26	S	32	S	30	S	28	S
103-1	21	S	25	S	24	S	28	S	28	S
126-2	**d**	**R**	27	S	29	S	29	S	24	S
120-2	21	S	26	S	30	S	30	S	31	S

Identification of bacteria

From isolated *Enterobacteriaceae* genera isolated from chicken after application of BCP extract in their feeding we identified these strains by Enterotest 24 and TWn software: *Escherichia coli*, *Proteus mirabilis* and *Klebsiella oxytoca*. Equally, from previous table (Table 4) is shown that sample 126-2 (*Klebsiella oxytoca*) was resistant to ampicillin. Others identified bacteria were not resistant to used antibiotics.

Table 5 Identified bacteria isolated from chicken after application of BCP extract in their feeding

Samples	Identification by TWN software	
120-1	*E. coli*	99.94%
77-2	*P. mirabilis*	99.95%
103-1	*E. coli*	99.91%
126-2	*K. oxytoca*	99.74%
120-2	*E. coli*	99.94%

4. Discussion

Antimicrobial activity of BCP extract

Not many researchers study antimicrobial effect of bee collected pollen extract, but Morais et al. [19] study antimicrobial activity of bee pollen extract to some selected bacteria. In their study they tested more bacteria against bee collected pollen extract and they determined that the strongest effect of bee collected pollen extract was against *E. coli* and *S. typhi*, *Enterobacteriaceae* respectively. Also they determined lower antimicrobial activity of bee collected pollen extract against *Bacillus cereus* and *Staphylococcus aureus*. Very similar results had Fatrcová-Šramková et al. [20], who studied antimicrobial effect of monofloral bee collected pollen extract against some pathogenic bacteria. They determined that bee collected pollen extract had an antimicrobial effect to *E. coli* CCM 3988 and to

Salmonella enterica CCM 4420. They used the same ethanolic extract (70 %) as in our experiment. Their results were: diameter of inhibition zone was 3 ± 1 mm after application of poppy bee collected pollen extract to *E. coli* CCM 6988 and 2 ± 1 mm to *S. enterica* CCM 4420. In their experiment similar effect had rape bee collected pollen extract. Also Kačániová et al. [21] researched antimicrobial properties of bee collected pollen in their study. They determined that bee collected pollen extract had antimicrobial effect to some selected pathogenic bacteria, yeasts and fungi. Similar as in our study, they used 70 % ethanolic bee pollen extract and they determined followed values. Diameter of inhibition zones was: 1.83 ± 0.29 mm in *E. coli* CCM 3988 strain, 2.17 ± 0.76 mm in *S. enterica* CCM 4420 strain.

Antibiotic resistance profile

Conversely, antibiotic resistance of *Enterobacteriaceae* genera isolated from chicken was a subject or research area from many authors or researchers. For example, Miranda et al. [22] determined ampicillin resistance in *Enterobacteriaceae* genera isolated from chicken. Also they found antibiotic resistance to another used antibiotic. In our experiment, we found ampicillin resistance only. In contrast with Hleba et al. [23], who researched antibiotic resistance in ubiquitous bacterial cenose isolated from chicken and who don't found ampicillin resistance. They found resistance to another used antibiotics. During recent years, several studies have reported the antimicrobial resistance of some *Enterobacteriaceae* genera isolated from poultry [24-29]. The several researchers like Lira et al. [30], Picozzi et al. [31], Caro et al. [32] and Čížek et al. [33] who researched antibiotic resistance in *Enterobacteriaceae* genera isolated from different products have argued that the results of antibiotic resistance vary from study to study.

5. Conclusions

From obtained results we could be conclude that bacteria isolated from chicken after application of bee collected pollen extract had more resistance to bee collected pollen extract in *in vitro* experiment as *E. coli* CCM 3988, which did not be in contact with BCP extract. Equally, we determined that different percentage of BCP extract had not effect to size of inhibition zones around the discs. We think that will need the next experiments for determining of bee collected pollen extract antimicrobial activity.

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