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DIVERSITY OF BACTERIA IN SLOVAK AND FOREIGN HONEY, WITH ASSESSMENT OF ITS PHYSICO-CHEMICAL QUALITY AND COUNTS OF CULTIVABLE MICROORGANISMS

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ABSTRACT

The aim of the study was to assess microbial microbiome of 30 honey samples and compare potential differences between the samples from apiaries and commercial trade in Slovakia and foreign countries (Latvia, Switzerland, India, Japan and Tanzania) as well as to indicate their physico-chemical and basic microbiological quality. Study of each sample consisted of physico-chemical analysis (water content, pH, free acidity and electrical conductivity), basic microbiological analysis performed by dilution plating method (total plate count, sporulating aerobic microorganisms, bacteria from *Enterobacteriaceae* family, preliminary lactic acid bacteria and microscopic fungi) and metagenomic analysis for bacterial diversity evaluation. Seven samples did not meet with legislative limits of physico-chemical parameters. Average values of cultivable microbial groups ranged at level 1-2 log CFU.g^{-1}, while bacteria from *Enterobacteriaceae* family were not detected in any samples. Sporulating microorganisms occurred most often, in 77% of samples and yeasts were proven in 60% of samples. Bacterial diversity, determined by metagenomic analysis, was varied. We distinguished 2 groups – group A and group B. Group A contained mainly fresh (produced in 2018) Slovak and Swiss honey and we found mainly genus *Lactobacillus* followed by genus *Bombella* in them. Group B contained mainly older Slovak honey (produced in 2017) and commercial foreign samples, in which production year is difficult to know. Group B samples were interesting because of human bacteria presence with genus *Prevotella* dominance. Redundancy analysis showed significant connection of the electric conductivity and microbial assemblage, that indicates important influence of botanical origin to microbial representation in honey.

Keywords: Bee product, Electrical conductivity, Lactic acid bacteria, Metagenomics, *Prevotella* sp.

INTRODUCTION

Honey is a natural product of bees traditionally used as food product and medicine all over the world including Slovakia, Romania and Russia (**Guziy *et al.*, 2017; Šedík *et al.*, 2019**). Consumption of honey almost doubled in last decade in Slovakia. Comparing with other food, ripe high quality honey is considered to be a food with a minimal level of microbial contamination with many beneficial effects in human nutrition and with no or limited risks for human health. Most of the potential microorganisms relevant for food safety are expected to be in inactive forms as they cannot survive in honey because of its properties including hyperosmolarity **(Bovo *et al.*, 2018)**. Essentially, honey is a supersaturated solution comprising approximately 80% sugars by weight, predominantly fructose and glucose, with sucrose, maltose, and many other sugars at much lower concentrations (**Cooper, 2014). Israili (2014)** concluded antimicrobial activity of honey as follows: A large number of *in vitro* and limited clinical studies have confirmed the broad-spectrum antimicrobial (antibacterial, antifungal, antiviral and antimycobacterial) properties of honey, which may be attributed to the acidity (presence of acids, low pH), osmotic effect, high sugar concentration, presence of bacteriostatic and bactericidal factors (hydrogen peroxide, antioxidants, lysozyme, polyphenols, phenolic acids, flavonoids, methylglyoxal and bee peptides) and increase in cytokine release and to immune modulating and anti-inflammatory properties of honey.
In general, sporulating bacteria and yeast belong to common inhabitants of honey. **Martins *et al.* (2003)** tested 80 multifloral honey samples from retail public markets in Portugal; they were centred on *Bacillaceae* spores and fungi; spores of *Clostridium perfringens* were not detected in any sample, *Bacillus cereus* were identified in 13.7%; yeasts and microscopic filamentous fungi (MFF) were detected in 88.8% samples with identification of 3 MFF genera: *Aspergillus*, *Penicillium* and *Mucor* and 2 genera of yeasts: *Saccharomyces* and *Candida*. **Tolba *et al.* (2007)** identified bacilli in 7 honey samples mainly from Northern Ireland. They found *Bacillus pumilus*, *B. licheniformis*, *B. subtilis*, *B. fusiformis* and *Paenibacillus motobuensis*. **Amir *et al.* (2010)** analysed occurrence of MFF in 19 honeys of blossom, blended and honeydew origin from Algeria. Microscopic filamentous fungi were found mainly in samples with low water content (16.2 and 17%) and included it that this fact was influenced by xerophilic properties of honey and MFF. Honey, especially in the fresh state, is interesting by presence of lactic acid bacteria (LAB) presence. For the first time **Olofsson *et* Vásquez (2008)** found LAB in the honey stomach (crop) and in the fresh honey as well, and they suggested that honey can be considered a fermented food product because of the LAB involved in honey production. In the last time, numerous studies about microorganisms in bees were published with main focus to bee intestinal microbiome. **Maes *et al.* (2016)** performed bee-cage experiments with different diet and demonstrated that typically occurring alterations in diet quality play a significant role in colony health and establishment of a dysbiotic gut microbiome. According to **Bonilla-Rosso *et* Engel (2018)**, simple sugars such as glucose and fructose present in nectar and pollen, and complex polysaccharides such as pectin from the pollen wall are apparent substrates for bacterial fermentation.

Properties as well as quantity and diversity of microorganisms of honey are influenced by many factors. Some of them can be partially or fully managed during the production process while others not. **Snowdon *et* Cliver (1996)** described primary sources of microorganisms in honey, including pollen, the digestive tract of honey bees, dust, air, dirt and flowers as well as secondary contamination, which can be taken place during and after honey extraction, including humans, equipment, containers, wind, dust, insects, animals or water. Blossom honey comes from nectar of plants and honeydew honey is originated from honeydew produced mainly by aphids. **Gilliam *et al.* (1983)** found that nectar is not a major source of microorganisms for honey bees, but nectars of some flowers, they tested, contained *Staphylococcus* sp. as well as gram-positive and gram-negative rod-shaped bacteria, actinomycetes and fungi. Bacteria are probably added during the process by which nectar becomes honey and the nectar sugars probably act as inducers for the resident microbiota in honey stomach (crop), with enhancing their numbers (**Olofsson *et* Vásquez, 2008**). Honeybees collect honeydew, which is product from aphids inhabiting green parts of plants and at the same time, with honeydew, they may collect other attached structures, such as the hyphae or fungal spores of plant pathogens and microalgae (**Escuredo *et al.*, 2012**).

According to **Snowdon *et* Cliver (1996)**, bacteria or yeasts are principally found in comb honey and sometimes also filamentous fungi are present, while information about presence and persistence of viruses and parasites are not available. However some human enteric viruses, such as hepatitis A, sustain dry conditions and could be expected to persist in honey.

Traditionally number of microorganisms particularly bacteria and microscopic fungi were assessed by cultivation of them on artificially prepared media in laboratory. However such conditions are not responded to requirement of many microbial species. Microbiology has experienced a transformation during the last decade that has altered microbiologists' view of microorganisms and how to study them **(Handelsman, 2004)**. At present, methods based on DNA analysing are more available for scientists. Metagenomic analysis, particularly 16S rRNA gene sequencing on high throughput sequencing platform Illumina became the most common and accurate analyses. These techniques were previously used to asses gut microbiomes of bees. **Bovo *et al.* (2018)** analysed 2 honey samples (orange tree blossom and eucalyptus honey from beekeepers in Sicily) by shotgun metagenomics and surprisingly they noted that the largest number of reads assigned to 5 organism group (Viruses, Bacteria, Plants, Fungi and Arthropods) matched virus sequences in both honey samples (67.55 – 98.56%). Microbiology of honey is still full of secretes and it indicates, that honey is probably able to keep DNA or RNA of various organisms.

The aim of the study was to analyse the honey samples in term of their basic physico-chemical properties, counts of cultivable microorganisms and metagenomic analysis to detect diversity of bacteria.

MATERIAL AND METHODS

Material

Totally, we tested 30 honey samples: 24 were available in Slovakia (18 with Slovak origin and 1 with origin in EU and non-EU countries) and 6 were obtained from foreign countries: from Latvia (mix EU and non-EU countries honey), Switzerland India, Japan and Tanzania. From botanical point of view, 24 samples were blossom (4 false acacia, 1 rape, 1 sunflower, 1 buckwheat and 17 multifloral), 5 samples were honeydew and 1 sample was blended. According to way of obtaining, 24 samples were from apiaries (22 directly from beekeepers and 2 from farmers' market), and 6 samples were from commercial trade. Detailed characterization is in the table 1.

Physico-chemical analysis

The physico-chemical analysis consisted of water content, pH, free acidity and electrical conductivity. These parameters were measured according **IHC (2009)**: water content by refractometric method, potentiometric pH measurement by pH-meter, free acidity by titration to pH 8.3 and electrical conductivity (EC) by conductometric method. Individual measurements were performed at least 2 times with average expression.

As the electrical conductivity is one of main descriptive criteria to differentiate blossom and honeydew honey where honeydew (and nectar only of some plants, e. g. chestnut) is linked to higher conductivity we sorted samples to the three groups as follows (table 1):

- **Low EC** – honeys with EC **to 0.29 mS.cm^{-1}** (n = 13, EC: 0.11 - 0.29 mS.cm^{-1}),
- **Middle EC** - honeys with EC **from 0.30 to 0.69 mS.cm^{-1}** (n = 10, EC: 0.32 - 0.69 mS.cm^{-1}),
- **Higher EC** - honeys with EC **from 0.70 mS.cm^{-1}** (n = 7, EC: 0.72 - 1.32 mS.cm^{-1}).

Table 1 Characterization of analysed samples

Sample code	Botanical origin	Geographical origin	Obtaining	Year of production	Note
BloSCo1	blossom (false acacia – *Robinia pseudoacacia*)	Slovakia	commercial trade (Slovakia)	2018	
BloSBee1	blossom (rape – *Brassica napus*)	western Slovakia (Male Krstenany)	directly from beekeeper	2017	creamed
BloSBee2	blossom	middle Slovakia (Dolny Pajer)	directly from beekeeper	2018	
BloSBee3	blossom (with dominance of false acacia)	western Slovakia (Dezerice)	directly from beekeeper	2018	1st extraction
BloFCo1	blossom	mix EU and non-EU honey	commercial trade (Slovakia)	best before 2020	
BloSBee4	blossom (false acacia)	western Slovakia (Nitra-castle, Cerman)	directly from beekeeper	2018	1st extraction
BloFCo2	blossom	mix EU and non-EU honey	commercial trade (Latvia)	best before 2019	
BloSBee5	blossom (with dominance of false acacia)	eastern Slovakia (Sobrance)	directly from beekeeper	2017	
BloSBee6	blossom	middle Slovakia (Smrecany)	directly from beekeeper	2018	1st extraction
BloSBee7	blossom	middle Slovakia (Horna Orava)	directly from beekeeper	2018	
BloFCo3	blossom	India (Himalaya)	commercial trade (India)	durability 2014 - 2017	
BloSBee8	blossom	middle Slovakia (Babin)	directly from beekeeper	2018	
BloSBee9	blossom	middle Slovakia (Smrecany)	directly from beekeeper	2018	2nd extraction
BloSBee10	blossom (sunflower – *Helianthus annus*)	western Slovakia (Dunajska Luzna)	directly from beekeeper	2018	
BloFCo4	blossom	Japan (labelled as Mexican Orange honey)	commercial trade (Japan)	best before 2024	
BloHoSBee1	blended	middle Slovakia (Smrecany)	directly from beekeeper	2017	4th extracting
BloSBee11	blossom (buckwheat – *Fagopyrum esculentum*)	western Slovakia (Risnovce)	directly from beekeeper	2018	

BloFBee1	blossom	Switzerland	directly from beekeeper (farmers' market)	2018	slightly fermented
BloSBee12	blossom (spring)	eastern Slovakia (Nova Lubovna)	directly from beekeeper (Switzerland)	2018	
HoSBee1	honeydew	eastern Slovakia (Kosice)	directly from beekeeper	2017	suspected of adulteration
BloSBee13	blossom	western Slovakia (Dezerice)	directly from beekeeper (farmers' market)	2018	2nd extraction
BloSBee14	blossom (raspberry – *Rubus idaeus*, linden – *Tilia* sp.)	eastern Slovakia (Orlov)	directly from beekeeper	2018	
BloSBee15	blossom	western Slovakia (Nitra-Zobor)	directly from beekeeper	2018	3rd extraction
BloSBee16	blossom (dandelion – *Taraxacum officinale*, willow – *Salix* sp.)	eastern Slovakia (Orlov)	directly from beekeeper	2018	
BloFBee2	blossom	Switzerland	directly from beekeeper	2018	slightly fermented
HoSBee2	honeydew	eastern Slovakia (Orlov)	directly from beekeeper (Switzerland)	2018	
HoSBee3	honeydew	middle Slovakia (Sutovo)	directly from beekeeper	2017	
HoSBee4	honeydew	eastern Slovakia (Nova Lubovna)	directly from beekeeper	2018	
HoSBee5	honeydew	western Slovakia (Nitra)	directly from beekeeper	2018	5th extraction
BloFCo5	blossom (October blossom)	Tanzania (Kasalu)	directly from beekeeper	durability 2016 - 2019	

sample code (according to botanical, geographical origin and obtaining): first part: Blo – blossom, Ho – honeydew, BloHo – blended (blossom-honeydew); second part: S – Slovakian, F – foreign; third part: Bee – from beekeeper, Co - from commercial trade; EU – European union, western Slovakia - Bratislava Region, Nitra Region, Trnava Region, Trencin Region; middle Slovakia – Banska Bystrica Region, Zilina Region, eastern Slovakia – Kosice Region, Presov Region

Microbiological analysis – dilution plating method

We used dilution plating method to quantify the microorganisms in honey. The target microbial groups were: TPC (total plate count), SAM (sporulating aerobic microorganisms), bacteria from *Enterobacteriaceae* family, preliminary LAB (lactic acid bacteria) and MF (microscopic fungi, i. e. yeasts and MFF – microscopic filamentous fungi). The basic dilution (10^{-1}) was performed by homogenizing 5 g honey and 45 ml saline solution (0.85% NaCl, 0.10% peptone). Specific conditions of microbiological analysis are listed in the table 2.

Table 2 Quantitative microbial examination of honey

Microbial group	Medium	Inoculation	Cultivation		
			temperature	time	O_2 requirement
TPC	GTY	pouring	30 °C	2-3 days	aerobic
SAM	NA 2	pouring*	25 °C	3 days	aerobic
Enterobacteriaceae	VRBG	pouring	30 °C	1-2 days	aerobic
preliminary LAB	MRS	pouring**	37 °C	3 days	aerobic**
MF	DG 18	pouring	25 °C	5-7 days	aerobic

TPC – total plate count, cultivated on GTY – agar with glucose, tryptone and yeast extract (HiMedia®, India); SAM – sporulating aerobic microorganisms, inoculated by *pouring – after heat shock (at 80 °C for 10 min), cultivated on NA 2 – nutrient agar no. 2 (HiMedia®, India); *Enterobacteriaceae* family, cultivated on VRBG – violet red bile glucose agar (HiMedia®, India); LAB – lactic acid bacteria, inoculated by **double-pouring (decrease of oxygen in medium), cultivated on MRS – de Man, Rogosa and Sharpe agar (HiMedia®, India); MF – microscopic fungi, cultivated on DG 18 – Dichloran Glycerol agar - with chloramphenicol (HiMedia®, India)

DNA extraction

We weighted 20 g of honey to sterile 50 ml tube and added sterile distilled water to total volume of 45 ml. The solution was heated at 75 °C and rotated in hybridization chamber to solve the honey. Then, samples were centrifuged at 8000 rpm for 10 min. Supernatant was removed and pellet was resuspended in 1 x PBS solution (pH 7.4), which was added to total volume 30 ml. Then, samples were centrifuged at 8000 rpm for 5 min. Most of supernatant was removed. We left only approximately 2 ml and transferred it to 2 ml tubes. These tubes were again centrifuged at 13000 rpm for 5 min and supernatant was removed. Pellets were stored at -80 °C to next analysis. To pellet we added glass beads and 250 µl PrepMan™ Ultra Sample Preparation Reagent (ThermoFisher Scientific) and then homogenized by bead homogenizer BeadBug™ 3 (Benchmark Scientific) at highest speed for 1 min. We heated the samples at 110 °C for 5 min. After heating, the tubes were centrifuged and lysates were transferred to new tube and used for PCR reaction.

Metagenomic analysis

Barcoded primers 515F and 806R **(Caporaso *et al.*, 2011)** which amplify V4 section of the 16S gene were used for PCR reaction. The composition of the PCR mixture was as follows: 15 µl KAPA HIFI HotStart mix 2X (Kapa Biosystems), 8 µl of each primer with a concentration of 2.5 µM, and 1µl of isolated DNA. Amplification was performed using SureCycler 8800 Thermal Cycler (Agilent) and thermal profile was following: Initial denaturation for 90 s at 98 °C followed by 35 cycles of denaturation for 15 s at 98 °C, annealing for 15 s at 62 °C and extension for 15 s at 72 °C. Final extension was 2 min at 72 °C. PCR products were purified using a PCR purification kit (Jena Bioscience), quantified by qubit (Invitrogen), diluted to the same concentration and pooled together. Illumina sequencing library was prepared by TruSeq LT PCR free kit (Illumina) with a modification involving omission of the DNA fragmentation and size selection. NebNext Quantification kit (New England Biolabs) was used for the library quantification then the library was diluted to 4 nM concentration, and denatured. The sequencing reaction was performed on Illumina MiSeq using the MiSeq Reagent Kit v3 (600-cycle).

Acquired sequencing data was processed in SEED environment **(Větrovský *et al.*, 2018)**. Forward and reverse reads were joined with minimum 100 base overlap. Only sequences with quality higher than Q30 were used in further analysis. Sequences were assigned to samples according used barcodes and then barcoded primers were removed. Sequences were checked for chimeras and clustered to operational taxonomic unit (OTUs) using Vsearch **(Rognes *et al.*, 2016)** at a similarity level of 97%. From each cluster (OTU) the most abundant sequence was found and identified using RDP classifier **(Wang *et al.*, 2007)**. Chloroplast sequences originated from pollen were removed.

Statistical analysis

Data from microbiological analysis were calculated as log $CFU.g^{-1}$. Significant difference was assessed if it was at least 1.00 log $CFU.g^{-1}$. Data from cultivation analysis as well as metagenomic data were descriptively processed in MS Excel 2007.

Shannon and Chao1 diversity indices were calculated using ComEcolPaC **(Drozd, 2010)**. For analysis of microbial communities and their connection to physic-chemical parameters RDA was derived and significance were analysed by

permutation test in package Vegan **(Oksanen *et al.*, 2013)** in R statistical environment **(Team R, 2013)**. OTUs with an only single member were removed prior these analyses.
Heatmaps were made using Heatmap3 **(Zhao *et al.*, 2014)** in R.

RESULTS AND DISCUSSION

Physico-chemical and microbiological quality

In the table 3, there are results of basic physico-chemical parameters and microbial counts for each sample. Water content ranged from 15.0 to 20.7% with average value 17.5 ± 1.3%. Similar results were published by **Bušová *et* Kouřimská (2018)**, who found average water content 18.3% in honey directly from Czech beekeepers and 18.6% in commercial samples. Water is a parameter, which is important for evaluation of honey maturity and its low value relates directly with long durability. According to **Council Directive 2001/110/EC**, honey has to contain less than 20% of water. Only one sample (BloFBee1) exceeded this limit. The sample was gently fermented before analysis. According to **Bogdanov *et* Martin (2002)** the water content of honey can naturally be as low as 13.6% and as high as 23.0% depending on source of the honey, climatic conditions and other factors. Water in honey is related to honey preservation and storage, as high water content can lead to a growth of yeasts and microscopic filamentous fungi, causing fermentation, flavour loss and low shelf life **(Al-Farsi *et al.*, 2018)**. Fermentation does not usually a problem in honey with water content less than 18% **(Bogdanov *et* Martin, 2002)**.

Table 3 Physico-chemical parameters, microbial counts and diversity of microbial assemblage of tested honey samples

Sample code	Physico-chemical quality				Microbiological quality (log CFU.g^{-1})						Diversity
	Water (%)	pH	FA (meq.kg^{-1})	EC (mS.cm^{-1})	TPC	SAM	E	pLAB	yeasts	MFF	Shannon's index
BloSCo1	16.3	4.1	13.2	0.11	3.15	2.57	< 1.00	< 1.00	< 1.00	< 1.00	0.459
BloSBee1	16.1	3.8	19.1	0.13	1.85	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	6.659
BloSBee2	17.7	3.8	20.2	0.16	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	5.514
BloSBee3	18.5	3.8	18.2	0.17	2.28	2.30	< 1.00	< 1.00	1.00	< 1.00	5.198
BloFCo1	17.4	4.7	7.65	0.18	1.60	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	6.325
BloSBee4	17.6	3.8	18.8	0.20	< 1.00	1.60	< 1.00	< 1.00	1.30	1.00	4.662
BloFCo2	17.8	4.4	17.2	0.20	2.00	1.30	< 1.00	< 1.00			7.098
BloSBee5	16.9	4.1	28.5	0.22	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	7.077
BloSBee6	18.6	3.9	18.0	0.23	4.17	1.70	< 1.00	3.57	2.00	2.04	4.007
BloSBee7	19.9	3.5	48.0	0.26	1.00	1.00	< 1.00	< 1.00	1.48	1.00	4.760
BloFCo3	16.4	4.2	10.0	0.26	2.19	2.10	< 1.00	1.60	1.00	< 1.00	6.632
BloSBee8	18.6	3.3	31.3	0.28	1.48	< 1.00	< 1.00	< 1.00	2.00	1.00	3.719
BloSBee9	18.0	3.8	20.6	0.29	2.37	1.48	< 1.00	3.08	2.07	1.30	5.760
LOW EC	n = 13	n = 13	n = 13	n = 13	n = 10	n = 8	**ND**	n = 3	n = 7	n = 5	n = 13
mean ± SD	**17.7 ± 1.1**	**3.9 ± 0.4**	**20.8 ± 10.4**	**0.21 ± 0.06**	**2.21 ± 0.90**	**1.76 ± 0.53**		**2.75 ± 1.03**	**1.55 ± 0.47**	**1.27 ± 0.45**	**5.22 ± 1.81**
BloSBee10	15.0	3.7	29.4	0.32	< 1.00	1.48	< 1.00	< 1.00	< 1.00	< 1.00	4.261
BloFCo4	18.6	4.3	52.0	0.35	1.90	1.60	< 1.00	< 1.00			5.761
BloHoSBee1	18.3	3.8	54.5	0.36	3.58	1.00	< 1.00	3.54	1.30	< 1.00	6.956
BloSBee11	16.0	3.6	40.0	0.37	2.11	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	6.046
BloFBee1	20.7	4.0	25.2	0.42	1.48	1.48	< 1.00	< 1.00	1.00	< 1.00	2.127
BloSBee12	16.9	4.4	19.1	0.43	1.30	1.30	< 1.00	< 1.00	1.00	< 1.00	3.179
HoSBee1	16.2	3.8	42.7	0.49	1.00	1.00	< 1.00	< 1.00	< 1.00	< 1.00	6.989
BloSBee13	17.6	3.7	40.9	0.51	2.26	1.70	< 1.00	< 1.00	< 1.00	< 1.00	3.729
BloSBee14	18.2	3.7	42.3	0.64	1.95	< 1.00	< 1.00	< 1.00	1.70	< 1.00	1.774
BloSBee15	16.6	3.8	41.3	0.69	2.48	1.30	< 1.00	< 1.00	2.53	1.30	3.294
MIDDLE EC	n = 10	n = 10	n = 10	n = 10	n = 9	n = 8	**ND**	n = 1	n = 5	n = 1	n = 10
mean ± SD	**17.4 ± 1.6**	**3.9 ± 0.3**	**38.7 ± 11.2**	**0.46 ± 0.13**	**2.01 ± 0.76**	**1.36 ± 0.26**		**3.54**	**1.51 ± 0.64**	**1.30**	**4.41 ± 1.92**
BloSBee16	17.6	3.6	57.0	0.72	2.24	1.30	< 1.00	< 1.00	3.07	1.30	2.067
BloFBee2	18.2	3.8	74.3	0.80	1.60	2.21	< 1.00	< 1.00	1.00	< 1.00	3.355
HoSBee2	17.1	4.2	45.1	0.88	2.30	1.30	< 1.00	< 1.00	2.21	< 1.00	1.857
HoSBee3	15.2	4.7	16.0	1.00	1.30	1.60	< 1.00	< 1.00	1.00	< 1.00	3.357
HoSBee4	17.2	4.3	48.2	1.04	1.70	1.00	< 1.00	< 1.00	1.95	< 1.00	2.438
HoSBee5	16.4	4.5	68.0	1.20	2.10	1.70	< 1.00	< 1.00	2.00	< 1.00	4.838
BloFCo5	18.0	4.6	25.2	1.32	2.26	1.60	< 1.00	1.70			5.835
HIGHER EC	n = 7	n = 7	n = 7	n = 7	n = 7	n = 7	**ND**	n = 1	n = 6	n = 1	n = 7
mean ± SD	**17.1 ± 1.0**	**4.2 ± 0.4**	**47.7 ± 21.3**	**0.99 ± 0.21**	**1.93 ± 0.39**	**1.53 ± 0.38**		**1.70**	**1.87 ± 0.79**	**1.30**	**3.39 ± 1.48**

sample code (according to botanical, geographical origin and obtaining): first part: Blo – blossom, Ho – honeydew, BloHo – blended (blossom-honeydew); second part: S – Slovakian, F – foreign; third part: Bee – from beekeeper, Co - from commercial trade;
FA – free acidity, EC – electrical conductivity; TPC – total plate count, SAM – sporulating aerobic microorganisms, E – bacteria from *Enterobacteriaceae* family, pLAB – preliminary lactic acid bacteria, MFF – microscopic filamentous fungi, SD – standard deviation, n – number of samples with detected value, ND – not detected

Values of pH ranged from 3.3 to 4.7 with average value 4.0 ± 0.4. Similarly, **Amir *et al.* (2010)** found pH of Algerian honey from 3.7 to 4.7, with average pH 4.0. All honeys are acidic with a pH-value generally lying between 3.5 and 5.5, due to the presence of organic acids that contribute to honey flavour and stability against microbial spoilage **(Bogdanov *et al.*, 2004)**. Organic acids constitute 0.5% of honey and include gluconic acid which is bye product of enzymatic break down of glucose **(Olaitan *et al.*, 2007)**. According to **Escuredo *et al.* (2012)** pH and electrical conductivity are two parameters widely used to distinguish between nectar and honeydew honeys, but they found pH from 3.5 to 5.0 without significant differences between blossom and honeydew honeys from Galicia (Nortwest Spain). **Al-Farsi *et al.* (2018)** tested 58 Omani honey samples and found wider range of pH – from 3.46 to 7.51, while they stated that in Oman there are two main sources for honey – summer plant *Acacia tortilis* (from *Fabaceae* family) and winter plant *Ziziphus spina-Christi*, L., called Sidr (from *Rhamnaceae* family). Value of pH as well as total acidity and free acidity have some classification power for the discrimination between unifloral, while lactones, due to their strong variability, do not provide useful information **(Bogdanov *et al.*, 2004)**. Overall, free acidity of tested samples ranged from 7.7 to 74.3 meq.kg^{-1}. According to **Council Directive 2001/110/EC**, honey has to contain not more than 50 meq.kg^{-1}. Five samples (2 from middle EC group and 3 from higher EC group) exceeded the

limit. Average values of free acidity were different depending on electrical conductivity (EC) dividing: 20.8 ± 10.4 meq.kg^{-1} in honeys with low EC, 38.7 ± 11.2 meq.kg^{-1} in honeys with middle EC and 47.7 ± 21.3 meq.kg^{-1}. In general, blossom honey posses lower free acidity comparing with honeydew honey, except lime, buckwheat, eucalyptus honey or some other blossom honey. **Zielińska *et al.* (2014)** found free acidity 14.7 ± 4.6 meq.kg^{-1} in rape (*Brassica napus*), 15.4 ± 6.6 meq.kg^{-1} in multifloral, 30.7 ± 17.4 meq.kg^{-1} in lime (*Tilia* sp.), 29.8 ± 11.8 meq.kg^{-1} in goldenrod, 35.6 ± 8.5 meq.kg^{-1} in honeydew and 45.0 ± 6.4 meq.kg^{-1} in buckwheat (*Fagopyrum esculentum*) Polish honey.

Electrical conductivity (EC) in tested honey ranged from 0.11 to 1.32 mS.cm^{-1}. Low EC was 0.21 ± 0.06 mS.cm^{-1} on average and these honeys were mainly from false acacia, rape or blossom – multifloral honey. Middle EC was 0.46 ± 0.13 mS.cm^{-1} on average and these honeys were from sunflower, buckwheat, raspberry, linden, blossom - multifloral or blended. Sample HoSBee1 was labelled as honeydew honey. We found EC 0.49 mS.cm^{-1}, what is not sufficient for EC of honeydew honey. According to **Council Directive 2001/110/EC**, EC of honeydew honey has to be not less than 0.8 mS.cm^{-1}. EC correlates well with the mineral content of honey **(Bogdanov *et al.*, 2004)**. Minerals are present in honey in very small quantities (0.17%) with potassium as the most abundant **(Olaitan *et al.*, 2007)**. Minerals in honeydew as well as chestnut honey are higher comparing with blossom honey. **Escuredo *et al.* (2012)** determined average EC of blossom honey 0.540 ± 0.200 mS.cm^{-1} (with range: 0.224 - 0.920 mS.cm^{-1}) and total minerals of blossom honey 148.6 ± 55.9 mg.kg^{-1} (with range: 47.9 - 280.3 mg.kg^{-1}); and average EC of honeydew honey 0.830 ± 0.200 mS.cm^{-1} (with range 0.482 - 1.168 mS.cm^{-1}) and total minerals 235.4 ± 80.8 mg.kg^{-1} (with range 95.2 - 387.4 mg.kg^{-1}). **Amir *et al.* (2010)** found of Algerian honey - ash content 0.13-1.02%, with average 0.4% and electrical conductivity 0.3 - 1.2 mS.cm^{-1}, with average 0.6 mS.cm^{-1} and indicated the honeys as blossom (63%), honeydew (16%) and blended (21%). The electrical conductivity is good criterion related to botanical origin of honey and thus is very often used in routine honey control instead of the ash content (**Gulfraz *et al.*, 2011**).

We detected viable microbial counts as follows: TPC (87% of samples) > SAM (77% of samples) > yeasts (60% of samples) > MFF (23% of samples) > pLAB (17% of samples) > bacteria from *Enterobacteriaceae* family (0% of samples). Target microbial groups ranged from ND (not detected, i. e. less than 1.00 log CFU.g^{-1}) to 4.17 log CFU.g^{-1} (table 3). Average values of tested microbial groups were at level 1 – 2 log CFU.g^{-1}. **Tolba *et al.* (2007)** demonstrated total viable counts from <100 to 1700 CFU.g^{-1} (<2.00 to 3.23 log CFU.g^{-1}) and did not find yeasts or MFF. Sporulating bacteria, MFF and yeasts are typically found in honey, often at low numbers, while spores can persist indefinitely **(Snowdon *et* Cliver, 1996)**.

Diversity of bacteria

Totally 155310 of high quality and chimera-free sequences were obtained representing average 5177 sequences per sample. Sequences were clustered into 11926 clusters and 1743 of them had at least 5 members and while 5630 OTUs were presented by single sequence. A part of sequences, 323 OTUs comprising 11517 (7.5%) sequences were identified as chloroplast 16S rRNA genes. Chloroplast co-amplification is common issue when plant tissue samples are analysed **(Medo *et al.*, 2018)**. In our samples chloroplasts sequences were linked to pollen grains naturally occurring in honey.

There were found occurrence of microbial species from 18 phyla (figure 1). The most common phylum was *Firmicutes* followed by *Proteobacteria* and *Bacteroidetes* and *Actinobacteria*. Other phyla were significantly less common. There was apparent difference between samples in occurrence of *Bacteroidetes*. Differences between samples were most apparent on genus level which is the lowest taxonomic level with fully reliable identification for the used method. Diversity of bacteria expressed as values of Shannon's index shoved values in very wide range 0.46 to 7.10 (Table 3). Dominance of single or few species in a sample resulted to very low diversity indices. Analysis of sequences identified 492 genera however only 52 had more than 2% share in any sample (Figure 2).

According to **Bovo *et al.* (2018)**, bacteria from honey could be group combining their main role/action or prevalent ecological niche and their putative origin: Predominant group consists of bacteria typical for the hive micro-environment (also common microbiota of the bee gut), e. g. *Lactobacillus kunkeei* (obligate fructophilic LAB), *Parasaccharibacter apium*, *Gilliamella apicola*, *Frischella perrara*. Bee pathogens, e. g. *Melissococcus plutonius* or *Paenibacillus larvae* represent the second group. The third abundant group of microbes in honey are plant associated species, e. g. *Pseudomonas syringae*, *Erwinia amylovora*, *Spiroplasma citri*. Ubiquitous and specialized species, e. g. *Escherichia coli*, *Bacillus cereus* as well as antagonistic bacteria like *Pseudomonas agglomerans* (with action against *E. amylovora*) are also common in honey.

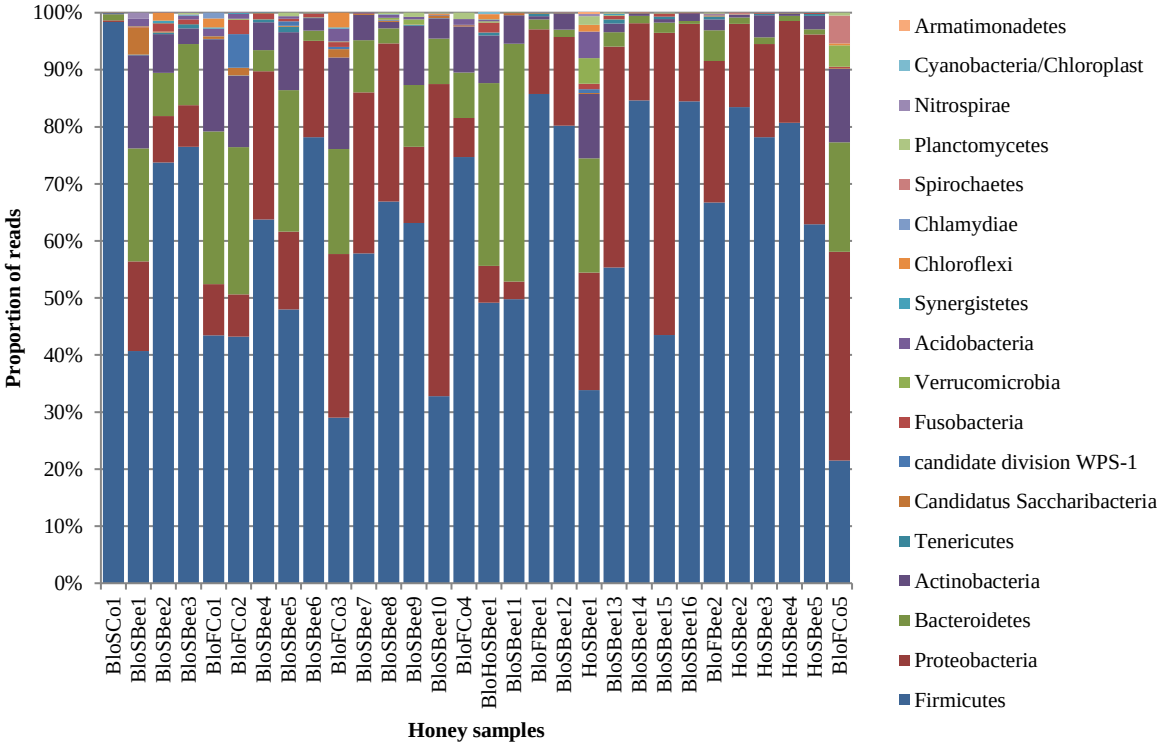

Figure 1 Proportion of reads assigned to bacterial phylum in honey samples
sample code (according to botanical, geographical origin and obtaining): first part: Blo – blossom, Ho – honeydew, BloHo – blended (blossom-honeydew); second part: S – Slovakian, F – foreign; third part: Bee – from beekeeper, Co - from commercial trade

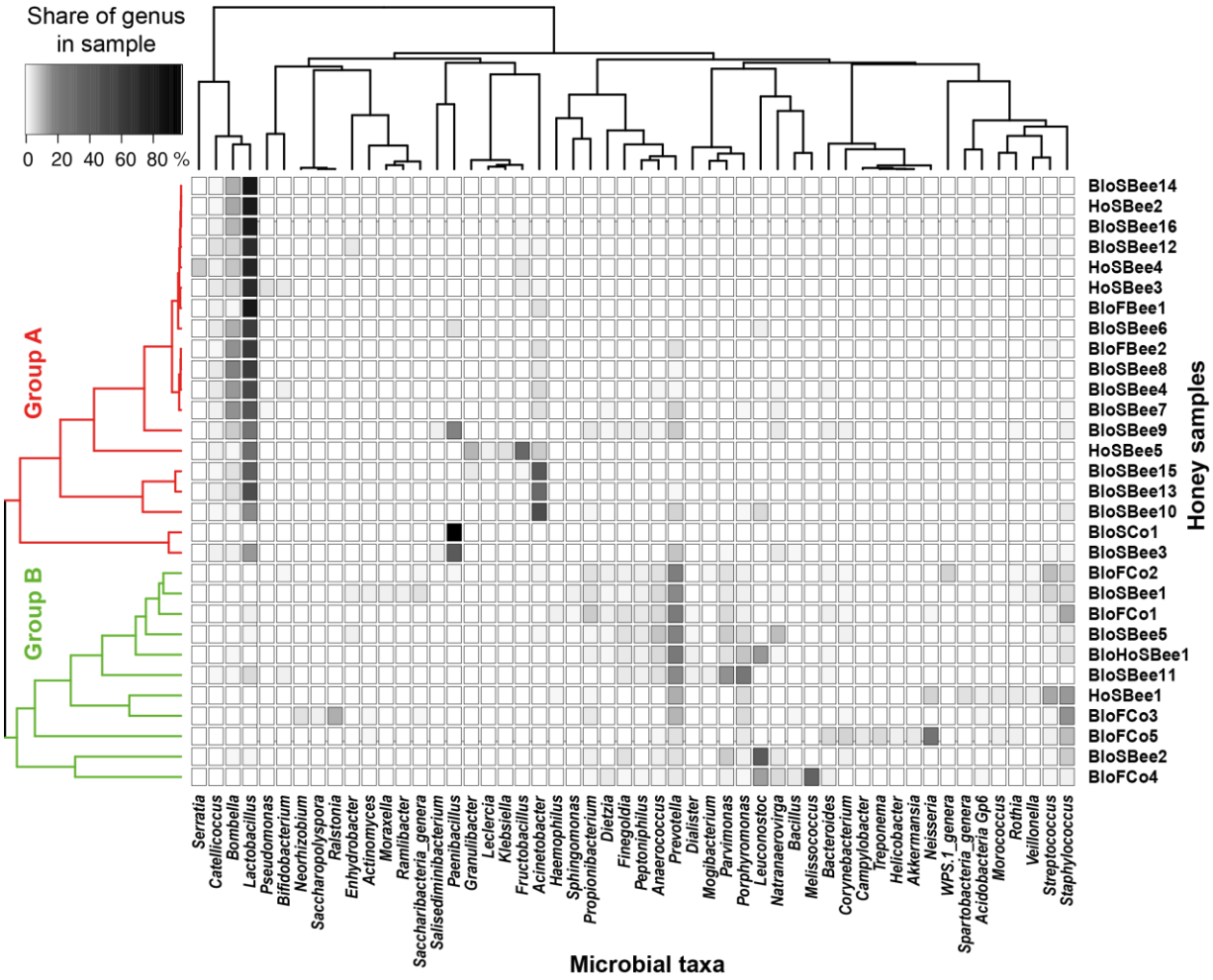

Figure 2 Bacterial genera with at least 2% share in any sample of honey and grouping of samples according bacteria occurrence
sample code (according to botanical, geographical origin and obtaining): first part: Blo – blossom, Ho – honeydew, BloHo – blended (blossom-honeydew); second part: S – Slovakian, F – foreign; third part: Bee – from beekeeper, Co - from commercial trade

At presented heatmap (figure 2), honey samples were clustered to two big groups (clusters) – A and B - according to most common bacterial genera in samples.

The main factor of dividing into the group A and group B seems to be the freshness of the sample, because the first 19 samples are blossom/honeydew honeys from beekeepers and commercial trade from the year 2018, except HoSBee3, which is from 2017 and BloSCo1, which is commercial. Next 11 samples originated from beekeepers from the year 2017 (except BloSBee 2, 11) and from commercial trade. Production year of commercial samples can be uncertain, because only packaging and/or expiration date are present at the label and in fact honey could be produced several years before packaging.

In group A consist of 19 honey samples, genera *Lactobacillus* was dominant followed by *Bombella*. In detailed analysis lactobacilli OTUs highest sequence similarity was found with *Lactobacillus apinorum*. *Lactobacillus kunkeei* is dominant LAB in honey stomach (crop) and fresh honey **(Olofsson *et* Vásquez, 2008)**. *L. apinorum*, originated from the honey bee gut, is the second fructophilic LAB within the genus *Lactobacillus* **(Maeno *et* al., 2017)**. *Bombella apis* was detected in midgut, crop and hive of honey bees and bumble bees **(Bonilla-Rosso *et* Engel, 2018)**. Preliminary LAB were found in samples BloSBee 6, 9; BloHoSBee 1 and BloFCo 3, 5 by dilution plating method, but presence of LAB by lactobacilli detection was only in samples BloSBee 6 and 9. Although LAB do not survive in stored honey, it is likely that humans have been consuming viable LAB in fresh honey during and directly after honey hunts throughout human history **(Olofsson *et* Vásquez, 2008)**. In 3 member subgroup (BloSBee 10, 13, 15) *Fructobacillus* were very abundant while *Paenibacillus* were apparent in some samples. However, in sample BloSCo1, genus *Lactobacillus* was not detected, only genus *Paenibacillus*, which was dominant and in detailed analysis, it was identified as *Paenibacillus alvei* (from honey DNA followed by confirmation of culture DNA from the NA2 plate). *Paenibacillus* sp. were separated from the bacilli, they originated mainly from the soil and are common in the honey, but *P. alvei* is one of the secondary invaders of European Foulbrood (EFB) and *P. larvae* is the causative agent of American Foulbrood (AFB) **(Generch, 2010)**.

Composition of microbial assemblage in group B (11 honey samples) samples was significantly different. Besides genera *Leuconostoc* and *Melissococcus* which were reported in honey, few other detected genera are not often in honeys or bees. Bacteria *Melissococcus pluton* causes the brood disease EFB, while some other organisms, like *Paenibacillus alvei*, *P. apiaries*, *Brevibacillus laterosporus*, *Enterococcus faecalis* or *Bacterium eurydice* are present as secondary invaders of dead brood **(Shimanuki *et* Knox, 2000)**. Presence of genera like *Prevotella, Staphylococcus* or *Porphyromonas* suggests contamination of these samples, during the extracting, processing and/or storage of the samples. On the other side, last time, presence of *Neisseria* sp., which mostly known as human pathogen, were confirmed in gut of bees and bumblebees **(Kwong *et* Moran, 2013)**.

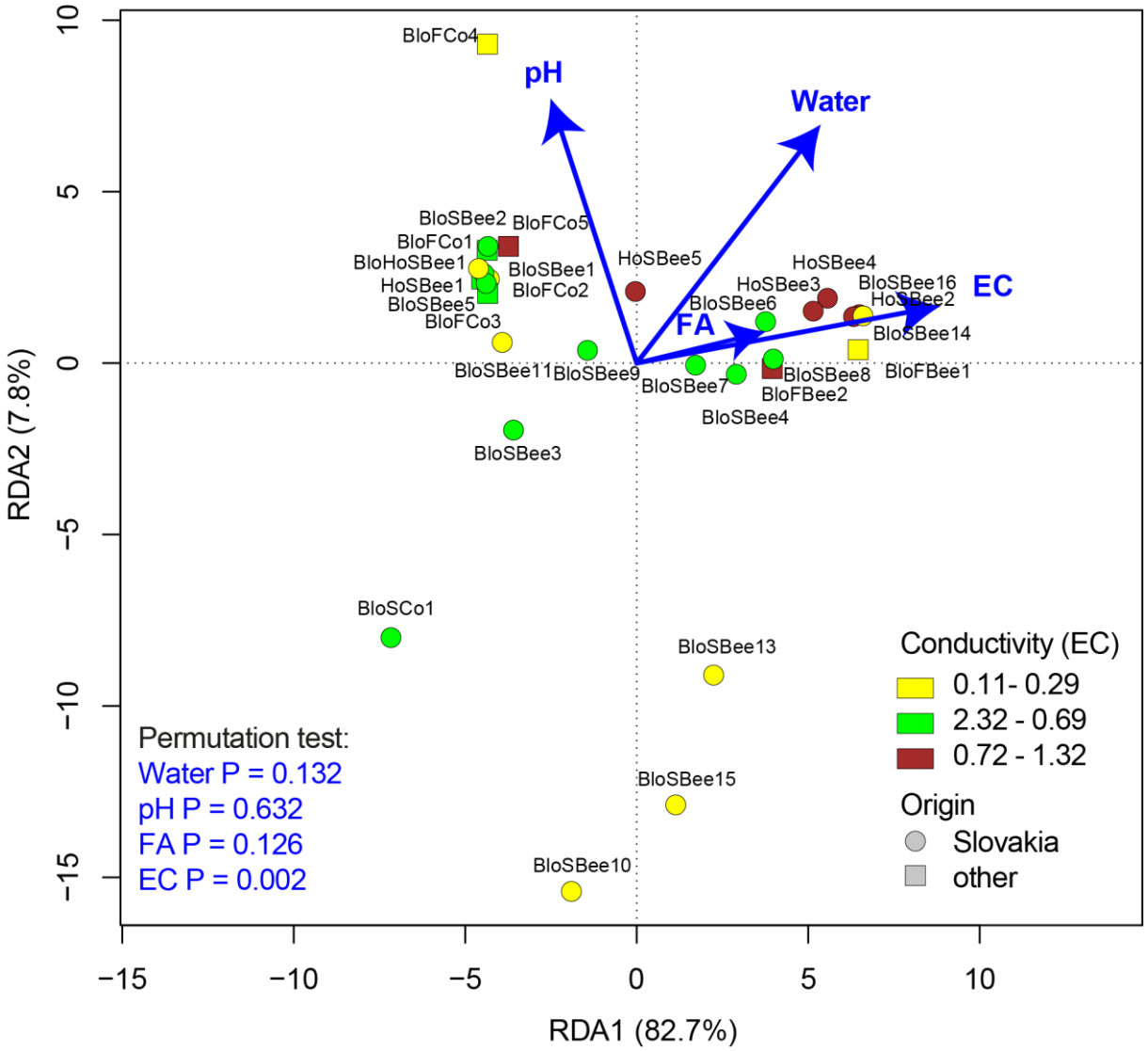

Figure 3 RDA scatterplot from analysis of physico-chemical properties and microbial assemblage of honey sample code (according to botanical, geographical origin and obtaining): first part: Blo – blossom, Ho – honeydew, BloHo – blended (blossom-honeydew); second part: S – Slovakian, F – foreign; third part: Bee – from beekeeper, Co - from commercial trade; FA – free acidity, EC – electrical conductivity

Microbial assemblage of honey is connected mainly to the microbiome of bees. Honey seems to be a suitable indicator of surrounding during the honey production, processing and storage. Relation of physic-chemical attributes and microbial assemblage using redundancy analysis showed electrical conductivity as main factor related to variation in honey microbiome (figure 3). First RDA factor explained majority of variance (82.7%) in microbial assemblage. This factor was in strong correlation (R2 = 0.69) with EC. Permutation test approved EC as the only one significant physico-chemical attribute (P = 0.003) among tested. According to **Bogdanov *et* Martin (2002)**, EC is suitable parameter for evaluation differentiation between blossom and honeydew honeys as well as for unifloral honeys. As EC is strongly depended on source of honey, probably the source is important determinant of microbial assemblage at least the same that bees themselves.

CONCLUSION

Overall, electrical conductivity was the main parameter, which was connected with the botanical origin. Viable counts of microorganisms could be influenced by the water content as well as freshness of the sample. We found sporulating aerobic microorganisms and yeasts as the most often occurring viable microbes. However, metagenomic analysis gave us interesting view into the presence of bacterial DNA in the honey. Bacterial diversity in the honey samples indicated main differences between the Slovak and Swiss fresh samples (produced in 2018) and the older ones (produced in 2017) together with commercial samples from Slovakia and foreign countries. In general, lactobacilli were dominant in Slovakian and Swiss fresh honeys. In older samples, *Prevotella* sp. and other representatives probably originating in human contamination during the honey extracting and processing were dominant. Surprisingly, the spectrum of identified bacterial genera was broad. Probably, most of them are not in viable state as honey represents a suitable environment for keeping of DNA and RNA intact. Subsequently, data from metagenomic analysis can provide us various information about surrounding during honey production, processing and storage.

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