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MICROBIAL PROPERTIES, NUTRITIONAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF *Brassica napus* subsp. *napus* L. BEE POLLEN USED IN HUMAN NUTRITION

SKŁAD MIKROFLORY, SKŁADNIKI ODŻYWCZE I AKTYWNOŚĆ ANTYOKSYDACYJNA W PYŁKU PSZCZELIM POCHODZĄCYM Z *Brassica napus* subsp. *napus* L. UŻYWANYM W ŻYWIENIU LUDZI

Abstract: An aim of this work was to characterize microbial properties, a nutritional composition and an antioxidant activity of *Brassica napus* subsp. *napus* L. bee pollen sample, which can be possibly used in human nutrition. A plate diluting method was applied for quantitative cfu (*colony forming units*) counts determination. The mean number of mesophilic aerobic sporulating microorganisms ranged 3.78–4.56 log cfu · g^{-1}, the number of mesophilc anaerobes sporulating microorganisms ranged 2.54–4.63 log cfu · g^{-1}, the number of coliforms bacteria 0–3.74 log cfu · $g^{-1}$and the cells number of *Escherichia coli* 0–3.71 log cfu · g^{-1}. The mean number of microscopic fungi ranged from 2.48 to 4.20 log cfu · g^{-1}. The antioxidant activity of bee pollen ranged from 1.25 to 1.93 I/I_0 (in case of the freeze-dried and frozen bee pollen, respectively). The highest total flavonoids content (128.33 mg · kg^{-1}) was occurred in the frozen pollen. The highest value of the flavonoid kaempferol achieved in the dried bee pollen, whereas the freeze-dried form contains the most of other three flavonoids (quercetin, luteolin, apigenin). The sum of proteins (average 251.13 ± 33.06 g · kg^{-1}) decreased in the order: freeze-dried > dried > frozen bee pollen. The freeze-dried form of pollen was characterized with the highest value of the calcium concentration, and the frozen treatment

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with the lowest content (2040 mg · kg^{-1} versus 1800 mg · kg^{-1}). The zinc was presented in amount 36.97 ± 4.15 mg · kg^{-1}. The most of the zinc was contained in the freeze-dried pollen.

Keywords: *Brassica napus* subsp. *napus* L. bee pollen, microbiological quality, bacteria, microscopic fungi, antioxidative properties, nutritional composition

Honeybee-collected pollen is recognized as a well balanced food [1]. Bee pollen, ie a floral pollen collected by a honey bee for its protein content, has been used as a nutrient rich health food for many centuries [2], and its benefits have been widely lauded [3–6]. The German Federal Board of Health has recently officially recognized pollen as a medicine [4]. More specifically, the ingestion of bee pollen by rats has been shown to decrease the level of the lipid oxidation products, malondialdehyde and conjugated dienes, in the erythrocytes [7], thus suggesting the antioxidant role for bee pollen. The same workers also demonstrated the immunostimulation activity on primary and secondary levels of IgM and IgE in rabbits fed on bee pollen-containing diet for 1 month [8]. But there are no official international pollen standards, yet. The pollen is collected by special pollen traps. Fresh, bee collected pollen contains about 20–30 g water per 100 g. This high humidity is an ideal culture medium for microorganisms like bacteria and yeast. For prevention of spoilage and for preservation of a maximum quality the pollen has to be harvested daily and immediately placed in a freezer [9].

The aim of this work was to characterize the microbial properties, the nutritional composition and the antioxidant activity of *Brassica napus* subsp. *napus* L. bee pollen sample, which can be possibly used in human nutrition.

Material and methods

A. Bee pollen samples preparation

Samples of bee-collected pollen (*Brassica napus* subsp. *napus* L.) were obtained from beekeepers, which respected qualitative criteria for gathering, drying and storing as proposed by Bogdanov [9]. The samples were collected during the spring season 2007 from different regions of western Slovakia. The fresh bee pollen was stored at –18 ^{o}C, 20 % moisture, approximately six months until analysed. The pollen samples were dried (9–11 % moisture) approximately 8 hours at maximum temperature 35 ^{o}C. The moisture was tested by thermogravimetric analyzer WPS 50SX/1 by RADWAG. The lyophilized samples of bee pollen were dried in the table laboratory lyophilizator LYOVAC GT 2 by Amsco/Finn-Aqua, 80 hours without heating, until 2 % moisture. The drying process was realized without heating so the nutritive compounds of the pollen were not changed.

B. Microbiological analysis

Determination of colony forming units (cfu) counts in pollen samples. The plate diluting method was applied for quantitative cfu counts determination of respective groups of microorganisms in 1 g of *Brassica napus* subsp. *napus* L. bee pollen sample.

The gelatinous nutritive substrate in Petri dishes was inoculated with 1 cm^3 of the pollen samples by flushing on a surface, in three replications. The basic dilution (10^{-1}) was prepared as follows: 5 g of the pollen content was added to the test tube containing 45 cm^3 of distilled water.

Media and culture conditions. The composition of nutritive substrates, for the total mesophilic sporulating anaerobes and the aerobes bacteria, the coliforms bacteria, and *Escherichia coli*, was according to the directions for use declared by the producer (Biomark laboratories). The total mesophilic sporulating anaerobes bacteria were grown on Meat Peptone agar (anaerobiosis), at 37 ^oC during 72 hours. The total mesophilic sporulating aerobes bacteria were grown in Meat Peptone agar (aerobiosis), at 37 ^oC during 72 hours. The coliforms bacteria were grown on Mac Conkey agar (aerobiosis), at 37 ^oC during 24 hours. *Escherichia coli* were grown on Violet red bile agar (aerobiosis), at 37 ^oC during 24 hours. The composition of these nutritive substrates was according to the directions for use declared by the producer (Biomark laboratories). The bacteria were determined according to Holt et al [10].

Isolation and morphological characterization of fungi. For determination of the fungi colony-forming units (cfu) 5 g of the sample was soaked in 45 cm^3 sterile tap-water, containing 0.02 % Tween 80 and then 30 min shaken. The dilutions (from 10^{-1} to 10^{-5}) in sterile tap-water with 0.02 % Tween 80 were prepared and 1 cm^3 aliquots were inoculated on each of three plates of Czapek-Dox agar with streptomycin (to inhibit the bacterial growth). Petri dishes were inoculated using the spread-plate technique and incubated at 25 ^oC. The total fungi cfu $\cdot$ g^{-1} counts in the samples were determined after 5 days of incubation.

Malt agar and Czapek-Dox agar were used to isolate and identify individual genera and species. After isolation, or in some cases monosporic isolation, individual species were identified on the basis of their macro- and micromorphology in accordance with other scientific reports [11–13].

C. Antioxidant activity

The antioxidative properties were evaluated using the voltammetric procedure based on the protective effect of antioxidants against the oxidative DNA damage. The method was employed using a disposable DNA biosensor fabricated as a screen-printed electrode chemically modified by calf thymus double stranded (ds) DNA.

Preparation of the DNA biosensor: the working carbon electrode (SPE with 25 mm^2 geometric surface area) of the three-electrode screen-printed assembly (including also silver/silver chloride reference electrode and carbon counter-electrode, FACH, Prešov, Slovakia) was chemically modified in the laboratory by covering it with 5 mm^3 of the DNA stock solution and leaving it to dry overnight.

Use of the DNA-Based Biosensor: the procedure reported previously [14] was exploited. Briefly, the new DNA sensor (DNA/SPE) was pretreated by immersion into 10 mmol/dm^3 phosphate buffer, pH 7.0, for 15 min and rinsed with water. Then, the $[Co(phen)_3]^{3+}$ marker was accumulated from 5 cm^3 of its 5×10^{-7} mol/dm^3 solution in 0.010 mol/dm^3 phosphate buffer under stirring for 120 s at an open circuit. The

differential pulse voltammogram (DPV) was recorded immediately from +0.4 to –0.5 V at the pulse amplitude of 100 mV and the scan rate of 25 mV/s using a computerised voltammetric analyser ECA pol, model 110 (Istran, Bratislava, Slovakia), fitted with the DNA/SPE assembly. With the software used, the current was measured with 2 mV scan step at this scan rate. The marker DPV peak current (I_0) at –0.130 V was evaluated against the base-line using the standard software and was corrected by the subtraction of the mean marker DPV peak current measured at the unmodified SPE (n = 10) under identical conditions. Subsequently, the DNA/SPE was regenerated by the removal of the accumulated $[Co(phen)_3]^{3+}$ ions from the DNA layer treating the sensor in the solution of a high ionic strength (0.100 mol/dm^3 phosphate buffer pH 7.0) under stirring during 120 s. The negligible marker peak current was checked by the DPV record in blank. The peak current I_0 was obtained in triplicates.

The DNA damage and the antioxidative effects of the plant extracts were detected after 5 min incubation of the sensor in the cleavage mixture (2×10^{-4} mol/dm^3 $FeSO_4$, 4×10^{-4} mol/dm^3 EDTA, 9×10^{-3} mol/dm^3 H_2O_2, in 0.010 mol/dm^3 phosphate buffer solution with 10 % methanol without or with the addition of the plant extract under the application of the electrode potential of –0.5 V in aerobic conditions at room temperature). The marker peak current I was obtained in triplicates using the marker accumulation/measurement/sensor regeneration scheme as described above and employing the same DNA/SPE for the given composition of the cleavage mixture. The average signals I_0 and I were calculated from the second and the third measurements. To compensate for the differences in the properties of the individual strips of the DNA-biosensor, the normalised (relative) signal value I/I_0 was obtained which represents the survived portion of the original DNA.

D. Total flavonoids and selected flavonoids analysis

HPLC determination of flavonoids. The chromatographic separation were performed on a Purospher Star RP-18e (Merck) column (250 × 4 mm I.D., 5 μm), protected by a Merck Purospher Star (4 × 4 mm, 5 μm) guard column. The HPLC system consisted of Shimadzu LC 10ADvp series pumping system, SPD 10AV/VP UV/VIS detector set at 360 nmand C-R6A chromatography data station software. Two solvents were used with constant flow rate 1 cm^3/min.

The injection volume was 20 mm^3. Solvent A consisted 0.05 % of TFA/methanol (95:5, V/V), solvent B included methanol/0.05 % TFA (95:5, V/V). For the elution program, the following proportions of solvents B were used: 0–15 min, 40 % B; 15–30 min, 40–55 % B; 30–35 min, 55–70 % B. The ethanolic extracts were injected under this conditions as well as the mixture of authentic samples of quercetin, luteolin, kaempferol and apigenin.

The correlation between antioxidant activity and content of flavonoids was analysed using SAS 9.1.3 software.

E. Minerals and proteins analysis

Minerals – calcium, zinc and total proteins were determined by standard methods in the accredited analytic laboratories – BEL/NOVAMANN International Ltd. Nove Zamky and EL Ltd. Spisska Nova Ves. The samples of pollen were homogenized, and then they were further processed according to determined chemical compounds.

Zinc concentration was determined using electrothermal atomizer atomic absorption spectrometry (ETA-AAS). Determination of calcium in tested pollen samples was realized by the inductively coupled plasma-atomic emission spectrophotometry (ICP-AES) method.

For quantitative determination of proteins content in analysed pollen samples was used Kjeldahl method.

Results and discussion

Microbiological analysis

The mean number of mesophilic aerobic sporulating microorganisms ranged 3.78––4.56 log cfu · g^{-1}, the number of mesophilc anaerobes sporulating microorganisms ranged 2.54–4.63 log cfu · g^{-1}, the number of coliforms bacteria 0–3.74 log cfu · g^{-1} and the cells number of *Escherichia coli* 0–3.71 log cfu · g^{-1}. The mean number of microscopic fungi ranged from 2.48 to 4.20 log cfu · g^{-1} (Fig. 1). Five species of microscopic fungi were isolated from *Brassica napus* subsp. *napus* L. bee pollen. The most frequently microscopic fungi species were *Alternaria alternata* and *Cladosporium cladosporoides* (Fig. 2).

From the ecological point of view should be mentioned, that microscopical fungi of genus *Cladosporium* and *Alternaria*, which were mostly isolated from examined

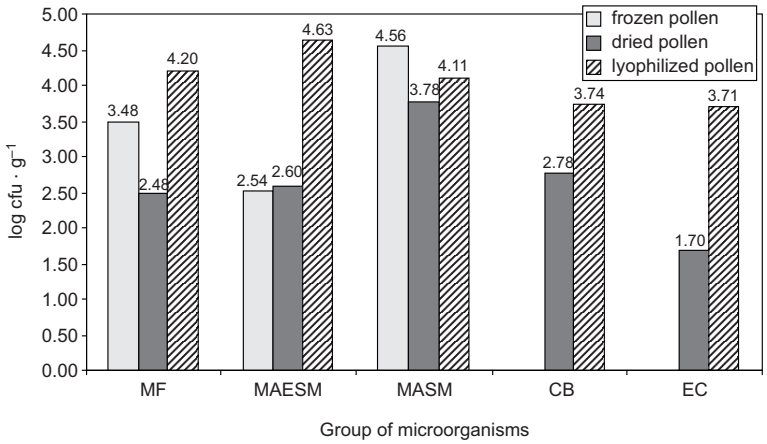

Fig. 1. The number of microorganisms in *Brassica napus* subsp. *napus* L. bee pollen [log cfu · g^{-1}]: MF – microscopic fungi, MAESM – mesophilc anaerobes sporulating microorganisms, MASM – mesophilc aerobes sporulating microorganisms, CB – coliforms bacteria, EC – *Escherichia coli*

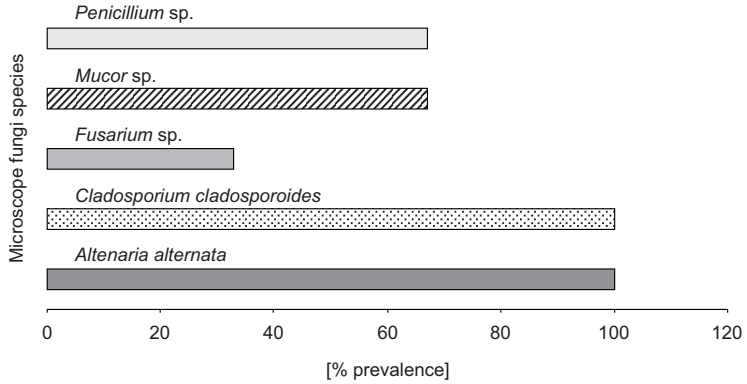

Fig. 2. The prevalence of microscopic fungi in *B. napus* subsp. *napus* L. bee pollen samples [%]

samples, very often saprophyte on sweet products and honeydew. When these microscopics fungi have suitable surroundings, they can multiply on these substrates as much as possible, and then they cover them with sensorial visible black sediment coatings [15].

The freshly collected bee pollen contains approximately 20 % of water, and this is the reason why it can get mouldy. How big attention beekeeper gave to storage of bee pollen, it is possible to find out from laboratory analysis. We expect that microscopic fungi of genus *Penicillium*, *Aspergillus*, *Mucor*, *Rhizopus* and other can participate on mouldy bee pollen. Therefore, amount of these microscopic fungi in degraded bee pollen will be substantial higher. Dry bee pollen contains just a little water what averts expansion of unwelcomed microscopic fungi.

Antioxidant activity, total flavonoids and selected flavonoids analysis

The antioxidant activity of the bee pollen samples ranged from 1.25 to 1.93 I/I_0 (in case of freeze-dried and frozen bee pollen, respectively) (Table 1).

Table 1

The antioxidant activity of bee pollen compounds (I/I_0)

Pollen	Antioxidant activity (I/I_0)
Frozen	1.93 ± 0.02
Dried	1.83 ± 0.02
Freeze-dried	1.25 ± 0.02

The dietary antioxidants, flavonoids and other components have been investigated extensively [16–18], and it has been demonstrated that each constituent of floral pollen possesses its own distinct flavonoid/phenolic HPLC profile [16, 18]. On the basis of the findings by Campos et al [19], it is concluded that the free radical scavenging effectiveness of a bee pollen is determined by its constituent pollens, and that the free radical scavenging effectiveness of a bee pollen values for the constituent pollens are

consistent (always the same) for each pollen species. Differences in the nature and levels of the flavonoids and other phenolics would suggest that the effectiveness of various floral pollens (and therefore of the bee pollen mixes) as antioxidants/free radical scavengers may vary widely [19]. The free radical scavenging effectiveness values are in large part determined by the free radical scavenging activity of the flavonoid/phenolic constituents, although other constituents, perhaps proteins can contribute up to half the activity. This activity can decrease significantly on storage (ageing), and it is therefore proposed that the freshness of bee pollen may be determined from its free radical scavenging capacity relative to that of fresh bee pollen with the same floral pollen mix.

The highest total flavonoids content (128.33 mg · kg^{-1}) was occurred in the frozen pollen. The highest value of the flavonoid kaempferol achieved in the dried bee pollen, whereas the freeze-dried form contains the most of other three flavonoids (quercetin, luteolin, apigenin) (Table 2).

Table 2

The content of flavonoids in pollen [mg · kg^{-1}]

Flavonoids [mg · kg^{-1}]	Pollen		
	Frozen	Dried	Freeze-dried
Quercetin	7.67 ± 0.03	11.52 ± 0.04	16.89 ± 0.02
Luteolin	33.60 ± 0.52	37.59 ± 0.09	40.15 ± 0.14
Kaempferol	57.13 ± 0.82	61.16 ± 0.39	57.08 ± 0.61
Apigenin	29.76 ± 1.33	17.70 ± 0.47	32.09 ± 0.69
Flavonoids	128.33 ± 2.05	115.33 ± 3.86	121.33 ± 3.30

There were observed the statistically significant strong dependence between antioxidant activity and individual flavonoids, namely quercetin, ($p < 0.001$), luteolin ($p < 0.01$), but not between kaempferol, apigenin, and total flavonoids (Table 3).

Table 3

Correlation matrix for antioxidant activity and flavonoids content

	AA (DNA biosenzor)	Flavonoids	Quercetin	Luteolin	Kaempferol	Apigenin
AA (DNA biosenzor)	1	$p \geq 0.05$ NS	$p < 0.001$ $r = -0.95499$	$p < 0.01$ $r = -0.86507$	$p \geq 0.05$ NS	$p \geq 0.05$ NS
Flavonoids		1	$p \geq 0.05$ NS	$p \geq 0.05$ NS	$p \geq 0.05$ NS	$p \geq 0.05$ NS
Quercetin			1	$p < 0.001$ $r = 0.96886$	$p \geq 0.05$ NS	$p \geq 0.05$ NS
Luteolin				1	$p \geq 0.05$ $r = 0.95397$	$p \geq 0.05$ NS
Kaempferol					1	$p < 0.001$ $r = -0.97444$
Apigenin						1

AA – antioxidant activity.

Minerals and proteins analysis

The sum of proteins (average 251.13 ± 33.06 g · kg^{-1}) decreased in the order: freeze-dried > dried > frozen bee pollen. Somerville and Nicol [20] investigated the crude protein levels in pollen pellets. Pollens collected from species of the same genus demonstrated similar protein profiles.

The freeze-dried form of pollen was characterized with the highest value of calcium concentration, and the frozen treatment with the lowest content (2040 mg · kg^{-1} versus 1800 mg · kg^{-1}). The zinc was presented in amount 36.97 ± 4.15 mg · kg^{-1}. The most of zinc was contained in the freeze-dried pollen.

In the study by Szczesna [21] calcium content of *Brassicaceae* honey bee-collected pollen ranged from 542 to 1080 mg · kg^{-1} dry matter (d.m.) (average 782 ± 23 mg · kg^{-1} d.m.), content of *Artemisia* ranged from 798 to 827 mg · kg^{-1} d.m. (812 ± 21 mg · kg^{-1} d.m.), content of multifloral pollen ranged from 648 to 796 mg · kg^{-1} d.m. (718 ± 63 mg · kg^{-1} d.m.). The zinc content of *Brassicaceae* honey bee-collected pollen ranged from 31.9 to 39.9 mg · kg^{-1} d.m. (average 35.7 ± 3.6 mg · kg^{-1} d.m.), the content of *Artemisia* ranged from 25.6 to 31.2 mg · kg^{-1} d.m. (28.4 ± 4.0 mg · kg^{-1} d.m.), the content of multifloral pollen ranged from 34.1 to 53.6 mg · kg^{-1} d.m. (41.5 ± 7.6 mg · kg^{-1} d.m.). The differences in the contents of individual elements as reported by different authors can be explained by differences in geographic and botanical origin of the pollen samples tested. The high concentration of the tested elements, especially in the pollen of *Brassicaceae* family, makes that variety of pollen an important potential source of macro- and micronutrients. In comparison with recommended dietary intakes of elements, the obtained contents of bee pollen point to a high nutritive value of the product, which can be recommended as a natural source of macro- and micronutrients [22]. The product can be successfully used as different dietary formulas and supplements in order to enrich our food rations with valuable nutrients performing important functions in the human body.

Conclusions

According to microbiological analyses the bee pollen is the ideal cultural medium for microorganisms like bacteria and yeast. The drying (moisture 10–11 %), freezing (–18 to –20 ^{o}C) and lyophilization were not enough efficient ways to preserve hygienic quality of bee pollen. It is important to look for the treatment which will protect bee pollen from another microorganisms spreading, and it allows decreasing of number microorganisms as much as possible.

There have to be other studies to determine the quality parameters of bee pollen with different botanical origin. Microbial properties, nutritional composition and antioxidant activity of bee pollen were largely investigated, but enormous botanical diversity in pollen pellets causes that there are still many gaps to be solve to standardize quality of bee pollen as food supplement or medicine.

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SKŁAD MIKROFLORY, SKŁADNIKI ODŻYWCZE I AKTYWNOŚĆ ANTYOKSYDACYJNA W PYŁKU PSZCZELIM POCHODZĄCYM Z *Brassica napus* subsp. *napus* L. UŻYWANYM W ŻYWIENIU LUDZI

Abstrakt: Celem pracy była charakterystyka mikroflory, składników odżywczych i aktywności antyoksydacyjnej w pyłku pszczelim wyprodukowanym z *Brassica napus* subsp. *napus* L. Oznaczono wskaźnik cfu (jednostki tworzenia kolonii). Średnia liczebność mezofilowych aerobowych sporulujących mikroorganizmów wynosiła 3,78–4,56 log cfu · g^{-1}. Liczebność mezofilowych anaerobowych sporulujących mikroorganizmów wynosiła 2.54–4.63 log cfu · g^{-1}. Liczebność bakterii coli wynosiła 0–3,74 log cfu · g^{-1}. Średnia liczebność grzybów wynosiła 2.48 do 4.20 log cfu · g^{-1}. Aktywność antyoksydacyjna w pyłku pszczelim wynosiła 1,25–1,93 I/I_0 (odpowiednio dla pyłku liofilizowanego oraz pyłku zamrażanego). Największa całkowita zawartość flawonoidów (128,33 mg · kg^{-1}) występowała w pyłku zamrażanym. Największa zawartość

flawonoidu kemferolu występowała w suszonym pyłku pszczelim, natomiast pyłek liofilizowany zawierał największe ilości pozostałych flawonoidów (kwercetyny, luteoliny, apigeniny). Sumaryczna zawartość białka (średnia 251,13 ± 33,06 g · kg^{-1}) zmniejszała się w kolejności: pyłek liofilizowany > suszony > zamrażany. Liofilizowana postać pyłku charakteryzowała się największą zawartością wapnia (2040 mg · kg^{-1}), natomiast najmniejszą zawartość wapnia stwierdzono w pyłku zamrażanym (1800 mg · kg^{-1}). Zawartość cynku w badanym pyłku wynosiła 36,97 ± 4,15 mg · kg^{-1}. Najwięcej cynku znajdowało się pyłku liofilizowanym.

Słowa kluczowe: *Brassica napus* subsp. *napus* L., pyłek pszczeli, mikrobiologiczna jakość, bakterie, mikroflora grzybowa, właściwości antyoksydacyjne, składniki odżywcze