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NATURAL MYCOBIOTA AND AFLATOXIN B_1 PRESENCE IN BEE POLLEN COLLECTED IN SERBIA

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Abstract: Total fungal count, incidence of fungi and aflatoxin B_1 (AFB_1) concentration were studied in 33 samples of bee pollen randomly collected from beekeepers in Serbia. The total number of fungi was determined by dilution method whereas AFB_1 was detected using the Enzyme-Linked Immuno-Sorbent Assay (ELISA). The mycological estimation showed the presence of nine genera of fungi as followed: *Acremonium, Alternaria, Aspergillus, Cladosporium, Epiccocum, Fusarium, Mucor, Penicillium* and *Rhizopus*, with total number ranging from 1×10^3 to 1×10^5 CFU g^{-1}. The results have shown the predominance of the fungi from the genera *Aspergillus* and *Alternaria*. Among *Aspergillus* species it was observed that the most frequent species was *A. flavus* with incidence of 27.27 %. Mycotoxin AFB_1 was detected as 100% positive in all samples (100%) with an average concentration of 8.61 μg kg^{-1}. The obtained results indicated that honey bee pollen must be strictly controlled during its manipulation in the harvesting and manufacturing. Therefore, the implementation of good manufacturing (beekeeping) practice to define procedures for honeybee products could be crucial to reduce the risk of possible contamination and provide natural and safety product without risk on the human health**.**

Key words: bee pollen, fungi, aflatoxin B_1

Introduction

Serbia posess excellent prerequisities for the development of beekeeping, due to heterogeneous relief and climatic conditions and various honey bee pasture *(Nedić et al., 2011)*. Bee pollen as one of bee product is considered as the most complete food in the nature. It is made of natural flower pollen homogenized with small quantities of nectar and bee saliva and collected at the hive entrance. The consumption of bee pollen is constantly increased due to grooving consumers

demand for the healthier and nutritious diet *(Linskens and Jorde, 1997)*. Nowadays, it is used less as a crude product, but more as dietary supplement added to the honey mixture.

Bee pollen consists of proteins, lipids, sugars, dietary fibers, trace elements, enzymes, fatty acids, vitamins and minerals *(Bonvehí and Jordà, 1997)*. Pollen has also high contents of biological active substances such as polyphenols, mainly flavonoids which possess high antioxidant capacity *(Carpes et al., 2009; Leja et al., 2007)*.

In the fresh collected bee pollen the water content is about 20-30% *(Bogdanov, 2012)* which combined with highly nutritional ingredients represent a suitable substrate for the growth of variety of microorganisms especially yeasts, moulds, spore forming bacteria and cocci *(Brindza et al., 2010)*. Therefore, the pollen loads has to be dried immediately after harvesting in order to extend its shelf life and prevent deterioration. In the dried bee pollen the moisture content should be in the range of 4 to 8% *(Mutsaers, 2005; Melo et al., 2011)*. The quality of bee pollen is highly depending on the applied methods of its preservation. Unfortunately, a part of beekeepers in Serbia traditionally carry out the draying process outdoors, on the natural flow of air, in the shadow, which could be the reason for the contamination of pollen loads with mycotoxigenic fungi.

Mycotoxins can occur in wide variety of products including maize, rice, cereals, nuts, dried food, green and roasted coffee, cacao beans and spices as a result of fungal contamination before and after harvest *(Magan and Olsen, 2004)*. Human could be exposed to mycotoxins directly by consumption of contaminated foods, or indirectly by consuming animal foods previously exposed to mycotoxins in feed *(Tarr, 2006; Krnjaja et al., 2012)*.

Mycotoxins with the most detrimental impact on animal and human health are aflatoxins. They are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites mainly by the species *Aspergillus flavus* and *Aspergillus parasiticus* which are ubiquitous in air and soil *(Rustom, 1997)*. Among aflatoxins B_1 (AFB_1) is consider as the most toxic and can cause liver cancer in humans *(Groopman et al., 1988)*.

The maximum recommended values of mycotoxins have been established in many countries for the number of food products, but there are no permissible limits for the mycotoxins in bee pollen. The scientific literature about the occurrence of mycotoxins in bee pollen is scarce. It was reported that 28.6% of *A. flavus* and *A. parasiticus* isolated from Spanish bee pollen was able to produce AFB_1 *(González et al., 2005)*. In the study of *Medina et al. (2004)* it was performed that bee pollen could be a suitable substrate for the production of ochratoxin A (OTA). Moreover, it was observed that the species from the genera *Aspergillus* and *Penicillium* isolated from Slovakian pollen should be consider as the most important producers of mycotoxins *(Kačaniová and Fikselová, 2007)*. AFB_1 was not detectable in the batch samples of Greek bee pollen with natural

mycobiota, but if the bee pollen substrate was inoculated with *A. parasiticus*, AFB_1 was detected *(Pitta and Markaki, 2010)*.

Considering that the consumption of bee pollen could be a potential risk for the human health if it is contaminated with the mycotoxigenic fungi, the main objective of this study was to determine the occurrence of natural mycobiota in bee pollen originated from Serbia, as well as to estimate the level of AFB_1 in those samples. As far as we know, this is the first study about natural mycobiota and AFB_1 presence in bee pollen from Serbia.

Materials and Methods

Bee pollen samples. Thirty three samples of bee pollen were randomly purchased from the beekeepers from Serbia. The samples originated from different region in Serbia (regional geographic map used by *Lazarević et al. (2012)*: 4 samples being from Region Vojvodina, 9 samples originated from Belgrade Region, 3 sample being from Western Region, 2 samples being from Eastern Region, 9 samples being from Central Region and 6 samples being from Southern Region were collected during the period of 2010-2012 (Table 1). After collection the bee pollen samples were stored frozen, prior to the analyses.

Determination of moisture content and water activity. The moisture content was determined by drying 3-5 g of samples in an oven at 105ºC, until a constant weight (AOAC, 1997). Water activity was performed at 22ºC using a Instrument Testo 650, Germany.

Isolation and identification of fungi. Mycological analysis was performed according to the standard methods. For each bee pollen, 10 g of sample were homogenized into 90 ml of saline solution (NaCl, 8.5 g/l). A serial dilution method was done and 1 ml of dilution of 10^{-3} and 10^{-4} were poured over the surface of Sabouraud maltose agar. After 5-7 days of incubation at 25°C, the total fungal count were identified and expressed as colony-forming units per gram of bee pollen (CFU g^{-1}). The morphological characteristics of isolated colonies were identified based on macroscopic (colony appearance) and microscopic (spores forming) investigations *(Watanabe, 1994)* to the genera level, except for potent producers of AFB1, *A. flavus* which was determined to the species level.

The mycotoxins analyses. The presence of AFB_1 was detected by ELISA according to the instructions Tecna S.r.l. (Italy) ELISA kits on an ELISA reader (Biotek EL x 800TM, USA) with detection limit of 1 μg kg^{-1} for AFB_1.

Statistical analysis. The incidence of fungal species (%) was calculated as number of samples with fungal species x 100/ total number of samples. Correlation between individual values obtained for moisture content, total fungal count and AFB_1 was determined using Pearson's correlation coefficient.

Results and Discussion

Moisture content and water activity. The water content is consider as an important parameter for the quality control of dehydrated foods like bee pollen because it play an important role in organoleptic properties and maintaining shelf life. The parameters officially established for the maximum water content in dry pollen are 10, 8, 6 and 4% in Bulgaria, Argentina, Switzerland and Poland and Brazil, respectively *(Melo et al., 2011)*. According to Serbian Official Gazette the maximum established value for water content of bee pollen is 8% *(Official Gazette, 2003)*. In the tested samples the water content varied in the ranged from 7.00 to 10.56%, of which 51.51% of the samples had content above the limit set by Serbian legislation. However, these samples were within the established limits of Bulgaria (10%), for commercial bee pollen with an exception of thre samples (18, 25 and 30) that exceeded this level (Table 1).

The most important environmental factors for the fungal growth are the water activity (measures of amount of free water) and temperature *(Lacey and Magan, 1991)*. It has been recommended that the water activity should be lower than 0.30 for the good storage stability of dehydrated foods like bee pollen *(Bonvehí and Jordà, 1997)*. The mean water activity in the tested samples was 0.34 a_w which could be considered as low enough to provide the microbiological stability of the product.

Microbial contamination of bee pollen. The total fungal count was in the range of $1x10^3$ to $1x10^5$ CFU g^{-1} (Table 1). According to morphological appearance by microscopic examination nine genera of fungi were determined, such as: *Acremonium, Alternaria, Aspergillus, Cladosporium, Epiccocum, Fusarium, Mucor, Penicillium* and *Rhizopus*. The predominant fungi were from the genera *Alternaria* (48.48) and *Aspergillus* (39.39%), followed by *Penicillium* spp. (24.24%) and *Mucor* spp. (21.21%) while the most common isolated species was *A. flavus* with an incidence of 27.27% (Table 2). These results are similar to those obtained by *González et al. (2005)* and indicated that the common fungi occurred in bee pollen are related to those usually presented in the grains and cereals before and after harvest, which are also known as “field” and “storage” fungi *(Logrieco et al., 2003)*. Also, in the work of *Brindza et al. (2010)* the most of the isolated species were from the genera *Mucor, Aspergillus, Alternaria* and *Rhizopus*. According to these authors the great majority of isolated fungi from bee pollen

represented the saprophytic microorganisms inhabiting soil and plant residues, indicating that these fungi originated from the microenvironment. On the other hand, *Snowdon and Cliver (1996)* performed that the species from the genera *Aspergillus* and *Penicillium* have also been associated with the intestines of honey bee.

Table 1. The moisture content (W), water activity (a_w) and total fungal count (CFU g^{-1}) in tested bee pollen samples collected in different regions in Serbia during 2010-2012

No. of sample	Year	Sample origin	W (%)	a_w	CFU g^{-1}
1	2010	Central Region	9.21	0.33	$1.8x10^4$
2	2010	Central Region	7.98	0.30	$1.3x10^4$
3	2010	Region Vojvodina	7.42	0.31	$7.x10^3$
4	2010	Central Region	7.96	0.32	$6x10^3$
5	2010	Belgrade Region	8.56	0.33	$2x10^3$
6	2010	Central Region	8.49	0.33	$1x10^3$
7	2012	Central Region	7.92	0.30	$1.1x10^4$
8	2011	Region Vojvodina	8.01	0.30	$1x10^3$
9	2011	Belgrade Region	7.52	0.29	$8x10^3$
10	2011	Eastern Region	7.93	0.31	$2x10^3$
11	2011	Southern Region	9.07	0.33	$1x10^3$
12	2011	Belgrade Region	7.94	0.31	$6x10^3$
13	2011	Belgrade Region	8.00	0.31	$1.3x10^4$
14	2011	Belgrade Region	7.91	0.33	$2x10^3$
15	2011	Eastern Region	7.57	0.30	$1.7x10^4$
16	2011	Belgrade Region	9.47	0.35	$4x10^3$
17	2011	Central Region	9.91	0.32	$1x10^5$
18	2011	Belgrade Region	10.56	0.40	$3x10^3$
19	2010	Region Vojvodina	9.01	0.34	$8x10^3$
20	2012	Region Vojvodina	7.58	0.30	$8x10^3$
21	2012	Southern Region	8.77	0.31	$1x10^4$
22	2012	Belgrade Region	8.76	0.31	$2x10^4$
23	2012	Central Region	7.94	0.30	$2x10^4$
24	2012	Central Region	8.38	0.31	$4x10^3$
25	2012	Central Region	10.19	0.36	$2x10^3$
26	2012	Southern Region	8.44	0.44	$1x10^4$
27	2012	Western Region	9.03	0.43	$2x10^4$
28	2012	Western Region	9.83	0.46	$6x10^3$
29	2012	Belgrade Region	9.29	0.41	$3x10^3$
30	2012	Southern Region	10.26	0.40	$7x10^3$
31	2010	Southern Region	7.01	0.31	$3x10^3$
32	2010	Southern Region	7.00	0.29	$3.2x10^3$
33	2010	Western Region	7.93	0.32	$2x10^3$

Table 2. The incidence (%) of fungal species isolated from tested bee pollen samples

Fungal species	Incidence (%)
Acremonium spp.	3.03
Alternaria spp.	48.48
Aspergillus spp.	39.39
Aspergillus flavus	27.27
Cladosoprium spp.	6.06
Epiccocum spp.	3.03
Fusarium spp.	9.09
Mucor spp.	21.21
Penicillium spp.	24.24
Rhizopus spp.	9.09

Mycotoxicological analysis. Mycotoxicological analysis by ELISA revealed the presence of AFB_1 in all 33 samples, with an average concentration of 8.61 μg kg^{-1} (Table 3). High level of total aflatoxins were found in Slovakian poppy bee pollen (up to 16.20 μg kg^{-1}) and rape bee pollen (up to 5.40 μg kg^{-1}), while this concentration was lower in the samples of sunflower bee pollen *(Kačaniová et al., 2011).* In Greece in the study of *Pitta and Markaki (2010)* AFB_1 was not found in a batch sample of bee pollen with natural mycobiota throughout the 20 days of incubation period, but when bee pollen was used as a substrate for inoculation of *A. parasiticus*, AFB_1 was detected in inoculated samples after the 3rd day of incubation. According to the results of *González et al. (2005)* toxigenic potential of toxigenic *Aspergillus* isolates was in range from 3.5 to 9.3 μg AFB_1 kg^{-1}.

Table 3. The concentration of aflatoxin B_1 (AFB_1) in tested bee pollen samples

Item	AFB_1
Sample size[a]	33/33
Incidence %	100
Range (μg kg^{-1})	3.49-14.02
Mean[b] (μg kg^{-1})	8.61

[a] Number of positive samples/Number of total samples
[b] Mean concentration in positive samples

The positive correlation found between the moisture content and level of AFB_1 (r = 0.05), between the water activity and level of AFB_1 (r = 0.01), as well as between the moisture content and total fungal count (r = 0.23) was not significant. In addition, the established negative correlation between the water activity and total fungal count (r= - 0.07) and between the total fungal count and level of AFB_1 (r = - 0.18) was also not significant.

It is usually accepted that the fungal growth and mycotoxins production are related with an interaction occurring among the strain, substrate and environmental conditions. Likewise, it has already been reported that the presence of fungi in products does not necessarily mean the presence of mycotoxins *(Harley, 1997)* as well as mycotoxins content are not always related to the number of fungi presented *(Tarr, 2006)*.

Bee pollen was a suitable medium for the proliferation of fungi and AFB_1 production, probably not only because it is a rich source of free amino acids, sugars, minerals etc., which could stimulate the production of AFB_1, but also because of improperly handle and store during production. Concerning that the a_w value of the tested samples was in the limit that ensure the microbial stability of the product it could be assumed that the production of AFB_1 was occurred in the period from the gathering of the pollen loads to drying and packaging stages. Growth of fungi and subsequent production of mycotoxins depend on climatic conditions and cultivation techniques or systems. Therefore, the presence of AFB1 in the tested samples could be explained by the drought and high ambient temperatures which contributed the production of AFB_1 *(Bruns, 2003)*. According to Annual Reports by Republic Hydro-meteorological Service of Serbia on agro-meteorological conditions in Serbia during the period from 2010 to 2012 quoted on drought during the period of June-August. The specified period largely coincides with the beekeepers season and collection of honeybee pollen. It has been assumed that the drought in 2012 had also been the reason for the occurrence of mycotoxigenic fungi and high level of AFB_1 in the maize grains, whereas *A. flavus* was isolated as the most common species from the *Aspergillus* genus *(Krnjaja et al., 2013; Lević et al., 2013)*.

The potential reason for the fungal contamination of bee collected pollen could also be the impurity of pollen traps if they are not disinfected before upcoming beekeeping season *(Nedić et al., 2008)*. In the study of *Snowdon and Cliver (1996)* it was discussed that the primary sources of microbial contamination are very difficult to control because they included the epiphyte microflora of pollen, digestive tracts of honey bees, dust, air, etc. On the other hand, the second (after harvest) sources have the strongest effect on the contamination of bee pollen like higienic condition during cleaning, drying and storage of the product, equipment and buildings.

The application of good manufacturing practice is the best way to control contamination of bee pollen. It has been recommended that pollen has to be collected no later than 48 hours after the installation of pollen trap *(Bonvehí and Jordà, 1997)* and should be dried as quickly as possible, to reduce the levels of microbial contamination. The outdoors drying, which is in common practice in one part of beekeepers in Serbia, must be avoided in order to obtain the natural product without the risk on the human health. The drying process should be carried out for 2 h, by artificial heating at the air temperature of 40°C *(Bonvehí and Jordà, 1997)*.

Bee collected pollen could also be frozen and sold in vacuum-sealed packaging, which enable the preservation of more sensitive ingredients. In contrast to this recommendation, bee pollen in Serbia is mainly repacked in polyethylene bags, with the net weight of 100 g of dried pollen and sold on the beekeeping retail exhibitions that are held outdoors.

Consumers, especially in developing countries, continue to use bee pollen, usually as a dietary supplement, despite the fact that currently there are no international microbiological criteria and regulations for its production. More studies are needed about the microbial quality and safety of pollen as well as establishment of international quality standards, although in commercial transactions an analysis of aflatoxins is usually included *(Hani et al., 2012; Nogueira et al., 2012).*

Conclusion

The results obtained in this study confirmed that bee pollen was a suitable substrate for the fungal growth and AFB1 production. The relatively high level of AFB1 in the tested samples indicated that the hygienic condition during processing and storage of bee pollen was not at a satisfactory level. Therefore, the implementation of good manufacturing practice and HACCP approach in all stages of bee pollen production will certainly ensure the high quality of the product. For that purpose it is very important that governments set legal limits for mycotoxins in bee pollen. The information about the presence of mycotoxins in honeybee products could be very important for risk assessment on the human health.

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Prirodna mikobiota i prisustvo aflatoksina B1 u polenu prikupljenom u Srbiji

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Rezime

Ukupan broj gljiva, učestalost (incidenca) gljiva i koncentracija aflatoksina B_1 (AFB_1) ispitivani su u 33 uzoraka polena sakupljenih od pčelara iz različitih

regiona u Srbiji. Ukupan broj gljiva određen je primenom metode razređenja a AFB_1 je određen primenom imunoadsorpcione enzimske metode (ELISA).

Mikološkim ispitivanjima identifikovano je devet rodova gljiva: *Acremonium, Alternaria, Aspergillus, Cladosporium, Epiccocum, Fusarium, Mucor, Penicillium* i *Rhizopus,* sa ukupnim brojem od 1 x 10^3 to 1 x 10^5 CFU g^{-1}. Najučestalije vrste gljiva su u rodovima *Aspergillus* i *Alternaria*. Među *Aspergillus* vrstama najučestalija je vrsta *A. flavus* sa incidencom od 27,27%. AFB_1 je detektovan u svim uzorcima sa prosečnom koncentracijom od 8,61 μg kg^{-1}.

Dobijeni rezultati ukazuju da pčelarski polen mora biti strogo kontrolisan tokom prikupljanja i njegove dalje prerade. Zbog toga, sprovođenje dobre proizvođačke (pčelarske) prakse podrazumeva definisanje procedura za pčelarske proizvode što bi moglo biti presudno za smanjenje rizika od moguće kontaminacije i dobijanje prirodnih i bezbednih proizvoda bez rizika po zdravlje ljudi.

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