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Quality Assessment of Bee Pollen: A Cross Sectional Survey in Bulgaria

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HIGHLIGHTS

- Mean water activity value for fresh and dried bee pollen samples were 0.717 and 0.359, respectively.
- Mean pH values of fresh bee pollen samples (4.23) was significantly lower than that of dried ones (5.21).
- The fresh Bulgarian bee pollen samples had poor microbial quality compared to the dried ones.

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Acronyms and abbreviations
a_w=Water activity
TTA=Total Titratable Acidity
CFU=Colony Forming Unit

ABSTRACT

Background: Due to its nutrient content, a variety of spoilage microorganisms can grow in bee pollen, especially when handling practices are not appropriate. So, this survey was designed to assess the physicochemical and microbiological properties of bee pollen collected from Bulgaria.

Methods: In June 2014, 13 fresh and 19 dried bee pollen samples were collected from Bulgaria. Water activity (a_w), pH, Total Titratable Acidity (TTA), and bacteriological and fungal counts were evaluated. Data were analyzed using Statistica ver. 10.

Results: Mean a_w value for fresh and dried bee pollen samples were 0.717 and 0.359, respectively showing significant (p<0.01) difference. The mean pH values of fresh bee pollen samples (4.23) was significantly (p<0.01) lower than that of dried ones (5.21). Also, there is statistically significant difference (p<0.01) between mean TTA value of fresh bee pollen (3.69 g/100 g) and mean TTA value of dried samples (2.09 g/100 g). The mean total viable count in fresh bee pollen samples was 182153.8 Colony Forming Unit (CFU)/g which was significantly (p<0.01) higher than that of dried samples (30352.6 CFU/g); whereas statistical analysis demonstrated that there were no significant differences (p>0.01) among the fresh and dried pollen for Enterobacteriaceae, lactic acid bacteria, and *Staphylococcus* spp. counts. The fungal colony count in the fresh bee pollen samples varied from 560 to 37000 CFU/g which were significantly (p<0.01) higher than that in the dried ones.

Conclusion: The fresh bee pollen analyzed in this survey had poor microbial quality compared to the dried bee pollen. There is also need to adopt appropriate manufacturing practices to prevent possible contamination by equipment or handling of Bulgarian bee pollen.

Introduction

Pollen grain is a tiny body with various structures, produced in the male apparatus of seed-bearing plants and conveyed in different ways such as wind, water, insects, birds, etc. to the female structure, where fertilization occurs. Unlike other widely known bee products such as honey and beeswax, pollen is not secreted but gathered. It

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is an important source of macro- and micro-nutrients (Bastos et al., 2004; Isidorov et al., 2009), and substantial amounts of polyphenols and primarily flavonoids (Carpes et al., 2009; Leja et al., 2007).

Due to its high nutritional composition, particularly in terms of its energy supply, bee pollen is considered as a food source for man (Atanassova and Lazarova, 2010; Block et al., 1997; Campos et al., 2008). Besides being a food source, pollen is also be used for therapeutic purposes (Kacaniova et al., 2011). However, due to its nutrient content, a variety of spoilage microorganisms can grow in bee pollen, especially when the collection, drying, storage, and marketing practices are not appropriate (Gonzalez et al., 2005; Medina et al., 2004). Also, contamination can be attributed to various factors and sources, weather, plant materials, insects, agricultural devices, etc. (Hani et al., 2012). In this context, humans may possibly be exposed to the negative influence of different fungal species, fungal spores, other invasive microorganisms or toxic fungal metabolites such as mycotoxins, directly by consumption of contaminated foodstuffs. Number of studies have revealed significant contamination levels of bee pollen by pathogenic microorganisms (Belhadj et al., 2014; Brindza et al., 2010), which compromise its safety for consumers. Bulgaria and other countries of the world, including Brazil, Poland, and Switzerland have established national standards for bee pollen (Campos et al., 2008). So, this survey was designed to assess the physicochemical as well as microbiological properties of bee pollen collected from different regions of Bulgaria.

Materials and methods

Sample collection and preparation

In June 2014, a total of 32 bee pollen samples (weighting 45-59 g per sample) consisting of 13 fresh and 19 dried ones were collected. They represented mixed pollen loads from different plants of eight different regions in Bulgaria from beekeepers as follow: Strandzha (n=4 fresh and 4 dried), Shumen (n=6 fresh and 6 dried), Sliven (n=1 fresh and 1 dried), Stara Zagora (n=2 dried), Karlovo (n=2 dried), Vratsa (n=2 fresh), Veliko Tamovo (n=3 dried), and Lovech (n=l dried). The samples were placed in sterile plastic zip-lock bags, vacuumed, packed, and stored at 4 °C until analysis. All the samples were handled aseptically to prevent secondary contamination.

Physicochemical analysis

The Water activity (a_w) of the pollen samples was determined using a dew-point a_w meter (Wert-Messer Meter, Germany) with an auto-temperature control system. Five g of each sample was placed in the sample holder and a_w value was recorded at 20±0.2 ºC.

The pH measurements were done according to the modified method of Obadina et al. (2008). Briefly, 10 g of each sample was dissolved in 90 ml deionized water, homogenized, and filtered through a Whatman no. 1 filter paper. The pH level of each sample was then determined using a pH meter (Eutech pH 510 meter, Eutech Instruments, Singapore).

Measurement of Total Titratable Acidity (TTA) was performed following the method described by Xu et al. (2012) with little modification. One g of each sample was dissolved in 20 ml deionized water, homogenized, and filtered through a Whatman no. 1 filter paper. Two-drop phenolphthalein indicator was added to the filtrate and titrated against 0.1 M NaOH to a pH 8.2 (Eutech pH 510 meter, Eutech Instruments). The TTA (g/100g) was calculated as follows:

$$\mathrm{TTA} = \frac{(\mathrm{titre})\ \mathrm{x}\ (\mathrm{N})\mathrm{x}\ \mathrm{Ew}}{\mathrm{Sw}}$$

Where titre is volume of NaOH consumed during titration (ml), N is the Normality of base (mol/l), E_w is milli-equivalent weight of tartaric acid (0.075), and S_w is the sample weight.

Microbiological analysis

One g of each pollen sample was added to nine ml sterile ringer solution and vortexed. Serially dilutions to 10^{-4} were done. One ml of each aliquot concentration was inoculated onto McConkey agar (Sigma, USA), plate count agar (Hi Media Laboratories, Mumbai, India), mannitol salt agar (Hi Media Laboratories, Mumbai, India), de Man, Rogosa and Sharpe agar (Biolab SA, Switzerland), as well as Salmonella-Shigella agar (Merck-Darmstadt, Germany) using spread plate technique as described by Adebayo et al. (2013) with some modifications. The plates were incubated at 37 °C for 24 h, and then colonies were counted and recorded as Colony Forming Unit (CFU) per g of sample.

Using the method of Njobeh et al. (2009) with little modifications, 1 g from each pollen sample was weighed into a sterile test tube and diluted by 9 ml sterile ringer solution and vortexed, then serially diluted down to 10^{-4}. Then, an aliquot of one ml from each sample was inoculated onto potato dextrose agar (Biolab SA, Switzerland) following the pour plate technique. The plates were incubated at 25 °C in the dark for seven days, and then colonies were counted and recorded in CFU/g. Thereafter, single colonies were sub-cultured on potato dextrose agar to obtain a pure fungal culture and incubated at 25 °C for 4-7 days followed by macro-morphological characterization and identification. The distinct fungal species were done based on macro- and

micro-morphologic identification keys of Pitt and Hocking (2009).

Statistical analysis

Data were analyzed using Statistica ver. 10 (StatSoft, Inc., USA). All analytical measurements were carried out in two replicates. Significant levels were considered as $p<0.01$.

Results

Physicochemical characteristics of 32 Bulgarian bee pollen samples are shown in Table 1. Mean a_w value for fresh and dried bee pollen samples were 0.717 and 0.359, respectively showing significant ($p<0.01$) difference. The mean pH values of fresh bee pollen samples (4.23) was significantly ($p<0.01$) lower than that of dried ones (5.21). Also, there is statistically significant difference ($p<0.01$) between mean TTA value of fresh bee pollen (3.69 g/100 g) and mean TTA value of dried samples (2.09 g/100 g).

As described in Table 2, the mean total viable count in fresh bee pollen samples (182153.8 CFU/g) was significantly ($p<0.01$) higher than that of dried samples (30352.6 CFU/g); whereas statistical analysis demonstrated that there were no significant differences ($p>0.01$) among the fresh and dried pollen for Enterobacteriaceae, lactic acid bacteria, as well as *Staphylococcus* spp. counts.

The fungal colony count in the fresh bee pollen samples varied from 560 to 37000 CFU/g with a mean of 10512.3 CFU/g which were significantly ($p<0.01$) higher than fungal colony count in the dried ones varying from 150 to 11000 CFU/g with a mean of 2418.4 CFU/g. Overall, 136 fungal isolates representing 49 fungal species were detected. Broadly, the genus *Penicillium* was the most frequently occurring that consisted of 49 isolates which among them *P. brevicompactum* was the most dominant species isolated from 14 different samples; also, *Fusarium* (5 isolates) was the least fungal contaminant (Table 3).

Table 1: Physicochemical characteristics of Bulgarian fresh and dried bee pollen samples

		Water activity	pH	Total Titratable Acidity (g/100 g)
Fresh (n=13)				
	Mean	0.717	4.23	3.69
	SD	0.004	0.127	0.394
	Min	0.713	3.98	3.04
	Max	0.725	4.40	4.31
Dried (n=19)				
	Mean	0.359	5.21	2.09
	SD	0.089	0.307	0.464
	Min	0.182	4.59	1.35
	Max	0.450	5.61	2.89

Table 2: Bacterial contamination of Bulgarian fresh and dried bee pollen samples

		Enterobacteriaceae (CFU/g)	Lactic acid bacteria (CFU/g)	*Staphylococcus* spp. (CFU/g)	Total viable count (CFU/g)
Fresh (n=13)					
	Mean	2378	446	337.5	182153.8
	SD	4776.8	437.6	442.2	177478.6
	Min	33	140	100	30000
	Max	12000	1200	1000	430000
Dried (n=19)					
	Mean	1027.5	2704.2	111.8	30352.6
	SD	1035.3	2484.5	75.8	24900.6
	Min	60	130	30	400
	Max	3800	5400	200	67000

Table 3: Fungal contamination in Bulgarian fresh and dried bee pollen

	Number of contaminated bee pollen samples	
	Fresh	Dried
Aspergillus spp.	7	7
Fusarium spp.	-	5
Penicillium spp.	28	21
Alternaria spp.	15	4
Cladosporium spp.	4	5
Other species	25	15

Discussion

In the present survey, we found that some Bulgarian bee pollen samples had not perfect quality characteristics, especially from view point of microbial quality. Pollen grains are highly sterile reproductive gametophytes from flowering plants, therefore any form of contamination is secondary (Shevtsova et al., 2014). Investigations revealed that some plant diseases may be transmitted by pollen (Card et al., 2007; Flores et al., 2005). Contamination of pollen could result from bees activities (foraging and transport), handling by humans, or from its natural habitat (Gilliam, 1979; Serra and Escola, 1997). Other extrinsic factors such as wind, rain-splash, dew or fog drip, and sprinkler irrigation splash can also contribute to pollen contamination (Lacey and West, 2007). Pollen, bacterial, fungal, and several other contaminants all co-exist in the aerospace (Lacey and West, 2007). Bees dampen the pollen with nectar and lug them in corbiculaes on their rearmost legs during transport (Atanassova and Lazarova, 2010); thus, bee pollen is more vulnerable to microbial contamination. Sanitary conditions around the hive, human handling operations such as harvesting, drying, packaging, and storage also have strong implications on pollen contamination (Serra and Escola, 1997).

The a_w is an important factor in food safety and stability (Mathlouthi, 2001; Rahman, 2010). Higher a_w tend to promote the proliferation of microorganisms (Abbas et al., 2009; Mathlouthi, 2001) such as pathogenic bacteria grown at a_w>0.85, whereas fungi grown well at a_w>0.6 (Rahman, 2010). The a_w values seen in our study were too close to the levels that are favour for both bacterial and fungal activities. This agrees with observations by Feas et al. (2012) who obtained considerable microbial load in bee pollen at a_w and pH range of 0.21-0.37 and 4.3-5.2, respectively. Although high a_w affects other aspects of food quality/stability such as enzymatic activities and moisture sorption, more aseptic techniques should be employed in the dehydration and handling of pollen since this might reduce the risk of human-induced contaminations. The pH and TTA values are also suggestive of dynamic microbial activity (Jay et al., 2005). Gram-positive bacteria are known to ferment foods resulting in higher pH and TTA values. However, mixture of endogenous enzymes and other microorganisms such as yeast and mould from the native microflora of food substances are also known to participate in the fermentation processes (Jay et al., 2005).

High contamination of the pollen samples by microorganisms in this study is similar to observations by Shevtsova et al. (2014) on pollen samples from Slovakia. The presence of coliforms in food generally indicates faecal contamination, which causes various illnesses in humans (Jay et al., 2005). It is well known that some Enterobacteriaceae members are opportunistic pathogens rarely cause disease in healthy individuals (Sanders and Sanders, 1997). It is also important that in all dry bee pollen samples, the total aerobic plate count possessed less than 100000 CFU/g (Campos et al., 2008). Lactic acid bacteria are known to cause food spoilage such as milk fermentation (Zavisic et al., 2012). The growth of Gram-positive, facultative anaerobes such as *Lactobacilli* in the pollen samples of the current survey is similar with the findings of Belhadj et al. (2014) who isolated *Lactobacillus plantarum*, *L. fermentum*, *Lactococcus lactis*, *L. ingluviei*, *Pediococcus pentosaceus*, *L. acidipiscis*, and *Weissella cibaria* from raw bee pollen grains collected in some Algerian areas.

The present survey revealed biodiversity in fungal species in the bee pollen samples such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. known to be major producers of mycotoxins which have adverse health implications on humans and animals alike (Beev et al., 2013; Jay et al., 2005). In this regard, Serra and Escola (1997) detected a high number of molds in bee pollen collected from Spain. Also, Kacaniova et al. (2011) found some mycotoxins in bee pollen sampled from Slovakia. Similar to our results, Gonzalez et al. (2005) established that *Penicillium* spp. is the predominant fungi in ready-to-eat bee pollen samples. In a similar work carried out in Italy, Nardoni et al. (2016) also identified variety of fungi in bee pollen, including

Cladosporium spp., *Alternaria* spp., *Humicola* spp., *Mucoraceae* spp., *Acremonium* spp., *Penicillium* spp., and *Aspergillus* spp. The results of Petrović et al. (2014) on Serbian bee pollen samples showed presence of *Acremonium, Alternaria, Aspergillus, Cladosporium, Epiccocum, Fusarium, Mucor, Penicillium*, and *Rhizopus* ranging from 10^3 to 10^5 CFU/g which is comparable to our results.

Conclusion

It can be concluded that the fresh bee pollen analysed in this survey had poor microbial quality compared to the dried bee pollen. There is also need to adopt appropriate manufacturing practices to prevent possible contamination by equipment or handling of Bulgarian bee pollen. It would be imperative in the future to do additional investigations for precise identification of isolated Enterobacteriaceae from dried bee pollen. Also, effective monitoring and control is necessary for the safety of bee pollen intended for consumption in Bulgaria.

Conflicts of interest

The authors have no conflict of interest.

Acknowledgments

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