applied sciences

MDPI

Article

Fusarium Fungi and Mycotoxins in Bee Pollen Collected in Lithuania

Jolanta Sinkevičienė [1,*], Živilė Tarasevičienė [2] and Vytautas Tamutis [3]

[1] Department of Agroecosystems and Soil Sciences, Faculty of Agronomy, Agriculture Academy, Vytautas Magnus University, Donelaičio Str. 58, LT-44248 Kaunas, Lithuania
[2] Department of Plants Biology and Food Science, Faculty of Agronomy, Agriculture Academy, Vytautas Magnus University, Donelaičio Str. 58, LT-44248 Kaunas, Lithuania
[3] Division of Dendrology and Phytopathology Sciences, Vytautas Magnus University Botanical Garden, Žiliberio Str. 6, LT-46324 Kaunas, Lithuania
* Correspondence: jolanta.sinkeviciene@vdu.lt; Tel.: +370-614-88501

Abstract: This paper presents the results of a study which was aimed at determining the concentration of *Fusarium* fungi and their mycotoxins in fresh bee pollen, stored for different periods. The analysed parameters included palynological analysis, moisture content, fungal counts, identification and toxigenic profiles. In this study, 45 bee pollen samples collected from the same apiary families were investigated. Palynological analysis determined six plant families, among which *Brassicaceae* prevailed. The number of detected isolates in the bee pollen during the study period ranged from 3.5×10^3 to 9.1×10^4 cfu g^{-1}. During the study, the most prevalent fungal genera of *Alternaria*, *Cladosporium* and Yeasts were found in fresh bee pollen. The significantly highest amounts of fungal colonies were determined after 3 days of storage of undried pollen. *Fusarium* fungal genera were detected in 46% of all studied samples, with levels ranging from 10^1 cfu g^{-1}. After 3 days of storage, the most significant *Fusarium* spp. increase (17.03%) was detected. *F. graminearum* and *F. sporotrichioides* prevailed during the whole period of the study. The highest concentrations of mycotoxins ZEN (280 µg kg^{-1}) and DON (120 µg kg^{-1}) were found after 3 days of pollen storage. The results of the present study report the importance of microbiological and mycotoxicological analyses in monitoring bee pollen from the initial stages of its production process.

Keywords: fresh bee-collected pollen; contamination; ELISA; fungi; *Fusarium*; mycotoxins

Citation: Sinkevičienė, J.; Tarasevičienė, Ž.; Tamutis, V. *Fusarium* Fungi and Mycotoxins in Bee Pollen Collected in Lithuania. *Appl. Sci.* **2023**, *13*, 1571. https://doi.org/10.3390/app13031571

Academic Editors: Xiaoming Fang, Hong-Wei Xiao, Yi Li, Hongcheng Zhang, Monica Gallo and Chiara Cavaliere

Received: 18 October 2022
Revised: 2 November 2022
Accepted: 6 January 2023
Published: 26 January 2023

1. Introduction

Fresh bee-collected pollen is a favourable substrate for the growth and development of microscopic fungi [1,2]. As a result of the presence of microscopic fungi, there exists a potential production of mycotoxins. The fungi that produce them are dangerous because the synthesis of mycotoxins is a part of their metabolic pathways [3]. Fungal toxins are secondary metabolites of different fungal species, and they can cause dangerous diseases—mycotoxicoses [4]. The mycotoxigenic fungi belong mainly to *Aspergillus*, *Penicillium* and *Fusarium* genera [3]. *Aspergillus* and *Penicillium* species are found in foods during storage. *Fusarium* species are plant pathogens producing mycotoxins before or immediately after harvest [5,6]. *Fusarium* is a widely distributed toxigenic fungal genus. It grows at 24–26 °C and at a minimum of 0.90 a_w. The production of toxins largely mirrors growth conditions [3]. Fresh bee-collected pollen contains high levels of moisture and increased water activity, a_w, which is conducive to the rapid development of fungi and enables the synthesis of mycotoxins [1,3].

Toxins produced by *Fusarium* spp. are receiving increasing attention. Almost all fusariotoxins are harmful for humans [7,8]. Trichothecenes and fumonisins are some of the most important mycotoxins produced by *Fusarium* species [9]. Trichothecenes are potential inhibitors of protein synthesis, and fumonisins are considered to be potentially

carcinogenic [10]. Zearalenone (ZEN) is produced by various *Fusarium* species: *F. equiseti*, *F. graminearum*, *F. culmorum*, *F. crookwellense*, *F. proliferatum*, *F. moniliforme* and *F. verticillioides* are the most important producers of fumonisin (FBs) [7]. Deoxynivalenol (DON) is produced by *F. culmorum* and *F. graminearum*. The T-2 toxin is much more toxic and less common than DON. It is produced by *F. tricinctum*, *F. sporotrichioides* and *F. poae* [10,11].

Bee pollen is a valuable apitherapeutic product greatly appreciated in natural medicine because of its potential medical and nutritional applications [12]. Pollen can be a good food supplement, as it contains different polyphenolic compounds [13], carotenoids [14] and vitamins [15] and possesses substantial antioxidant properties [12]. As a nutritional enrichment product, bee pollen is used fresh, dried or frozen. In the literature [16–19], we usually find data on fungal contamination of already dried pollen. Microbiological testing of fresh bee pollen would help to clarify the initial mycological condition of bee pollen and allow further pollen quality control measures. As contamination of food raw materials with mycotoxins is difficult to avoid, data on the occurrence of mycotoxins are of major importance for food safety. However, data on the distribution of *Fusarium* mycotoxins in bee pollen are rather limited. Therefore, this study was aimed at determining the contamination of fresh bee pollen by microscopic fungi during a three-day storage period and the potential ability of *Fusarium* fungi to produce mycotoxins in fresh bee pollen.

2. Materials and Methods

2.1. Experimental Site

The experiment was carried out during 2019–2020 at the Vytautas Magnus University Agriculture Academy (VMU AA) Open Access Joint Research Centre of Agriculture and Forestry (Lithuania).

2.2. Bee Pollen Samples

A total of 45 pollen samples (250 g) were supplied directly by a local beekeeper in the district Kaunas, Lithuania. Bees had collected pollen from different plant species. On the same day it was purified from different impurities (the plant particles, insect residues, etc., were removed). Secondly, moisture content (%) of undried pollen was determined. The collected undried bee pollen was stored for different durations (from 1 to 3 days) at 22–25 °C room temperature in plastic food containers.

2.3. Research Methods

Palynological analysis. Two grams of pollen samples were homogenized in 13 mL 70% ethanol, and after vortexing until homogenized they were centrifuged at 3500 rpm for 20 min. After centrifugation, the supernatant was discarded and 13 mL of 50% glycerine–water mixture (1:1) was added and centrifuged at 3500 rpm for 20 min. After centrifugation, the supernatant was poured, the tubes were inverted on blotter paper and the pellet was allowed to dry. The sediment of the tube was taken with the glycerine–gelatine matrix with the help of a dissecting needle and put on the slide, melted on the hot plate and covered with the coverslip. Three preparations were prepared for each sample [20]. Pollen was identified by optical microscopy, using previously published data [21,22].

Moisture content. Moisture content analysis was determined by drying 5 g of a sample in an oven at 105 °C temperature until constant weight.

Isolation and morphological characterization of fungi. The dilute plate method was used for isolation of fungi from bee pollen. Ten grams of each bee pollen sample were homogenized into 90 mL of physiological saline solution (NaCl, 8.5g L^{-1}) and shaken on a horizontal shaker for ca. 15 min. at 400 rpm. For each sample, 1 mL aliquots of each of the four dilutions were inoculated (in triplicate) in the Potato Dextrose Agar (PDA) (Sigma-Aldrich) culture media.

The plates were incubated at 26 °C for 5 to 7 days and then fungi were counted and recorded as colony-forming units (cfu) per gram of sample. Separate fungal colonies were transferred onto appropriate identification media. The Czapek Dox agar (Biolife, Italy) and

Potato Dextrose Agar (PDA) (Sigma-Aldrich) medium were used to isolate and identify individual *Fusarium* genera and species. The morphological characteristics of isolated colonies were identified based on macroscopic (colony appearance) and microscopic (spores forming) features in accordance with various descriptors [23–27].

Mycotoxins analysis. In this study, the following mycotoxins were investigated by enzyme-linked immunosorbent assay (ELISA): deoxynivalenol (DON), zearalenone (ZEN) and the T-2 toxin (T-2) [28]. The ELISA method is totally quantitative, simple, sensitive and fast; therefore, it can be used for the detection of mycotoxins in various foods [29]. The RIDASCREEN® ELISA kits produced by R-Biopharm AG (Darmstadt, Germany) approved by the AOAC Research Institute (Certificate No. 9001) were used for the analysis No R5906, No R5502. Mycotoxin analysis was performed following the manufacturer's instructions.

For the preparation of the extracts, pollen samples were ground and then 5 g fraction were weighed. Three extracts were prepared: for the quantification of DON, extraction was carried out in 100 mL distilled water; in the others 100 mL of a methanol: water solution (70:30 v/v) was added for the quantification of ZEN and 50:50 v/v for T-2. Then, samples were stirred at 150 rpm at 25 °C in an orbital shaker (Certomat®, BS-1, Sartorius, Goettingen, Germany) for one hour and the samples were filtered using Whatman #1 filter. The filtrate was used directly for the quantification of T-2. For DON and ZEN, an additional dilution was required. Dilutions were 1:4 in distilled water for DON and 1:5 in 70% methanol for ZEN.

The method's limits of detection (LoD) were the following: DON—18.5 μg kg^{-1}, ZEN—17.0 μg kg^{-1} and T-2—5.0 μg kg^{-1}. Samples with results lower than the LoD values were considered not contaminated.

The absorbance was determined using an ELISA reader (Stat Fax®303 Plus Microstrip Reader, Awareness Technologies, Westport, CT, USA) with a 450 nm absorbance filter. The optical densities (OD) of the samples were compared to the OD of standards through a linear regression, thus obtaining the concentration of mycotoxin present in each sample. Only calibration curves with R^2 greater than 0.99 were considered. Analyses were performed in duplicate for each sample.

2.4. Statistical Analysis

The results were statistically processed using one-way analysis of variance. Statistical analysis of the experimental data was performed using the software ANOVA from the statistical analysis package SELEKCIJA [30]. Differences between averages of treatments marked with different letters are significant at 95% confidence level ($p < 0.05$). There are no significant differences when $p > 0.05$.

For the evaluation of the relationships between the bee pollen storage time, spread of different fungi species and amount of fungi, the principal component analysis (PCA) was performed using XLSTAT software version 2019.3.02 (Addinsoft, Paris, France).

3. Results

3.1. Palynological Analysis

During the study, different botanical origins of bee pollen samples were analysed (Table 1). Analysis of the floral sources of the samples revealed six pollen types belonging to six different botanical plant families. Pollen families are classified as dominant (≥45%), secondary (16–44%), minor (3–15%) and trace (<3%) according to their density [20].

The *Brassicaceae* type was the dominant class (54.65%), *Rosaceae* was secondary (16–44%), *Fabaceae*, *Poaceae* and *Asteraceae* were minor (3–15%) and *Apiaceae* was represented in small amounts (<3%). In terms of floral diversity, 55% of pollen showed only one floral source (in the class of dominant pollen), whereas the remaining presented a composition originating from five pollen types.

Table 1. Origins of bee pollen.

Plant Sources (Family)	Percentages	Frequency of Pollen Types
Brassicaceae	54.65	Dominant (≥45%)
Rosaceae	15.83	Secondary (16–44%)
Fabaceae	11.24	Minor (3–15%)
Poaceae	10.18	Minor (3–15%)
Asteraceae	5.41	Minor (3–15%)
Apiaceae	2.69	Trace (<3%)

3.2. Levels of Fungal Contamination and Moisture Content of Fresh Bee Pollen

During the study, 10 genera of fungi were isolated from 45 samples in fresh pollen, which are presented in Table 2. Two genera of isolated fungi *Mucor* and *Rhizopus* according to their morphology were attributed to Phylum *Zygomycota*, class *Zygomycetes*; Phylum *Ascomycota* belonged to *Chaetomium* (class *Pyrenomycetes*, order *Shaeriales*) and Yeasts (class *Archiascomycetes*, order *Schizosaccharomycetales*). *Acremonium*, *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epiccocum*, *Fusarium*, *Mycelia sterilia*, *Penicillium* and *Trichoderma* belonged to anamorphic fungi (*Deuteromycota*, class *Hyphomycetes*, order *Moniliales*). Anamorphic fungi dominated among the isolated fungal taxa, and they comprised 73.57% from all isolated fungi.

Table 2. Occurrence of fungi in bee pollen.

Genus	Frequency of Occurrence (%)			
	5 h	1 Day	2 Day	3 Day
Acremonium	2.14	1.33	1.69	1.41
Alternaria	26.79	29.5	29.88	29.3
Aspergillus	1.79	0.49	0.73	1.22
Botrytis	4.29	3.8	3.51	3.2
Chaetomium	0.71	0.11	0.35	0.29
Cladosporium	13.21	14.64	9.89	12.5
Epiccocum	1.79	1	1.2	0.85
Fusarium	9.31	7.93	10.91	10.49
Yeast	12.14	14.2	16.5	16.71
Mucor	6.79	5.91	6.73	7.12
Mycelia sterilia	6.43	6.79	6.94	7.33
Penicillium	7.5	7.34	9.61	10.5
Rhizopus	1.43	1.9	2.2	2.61
Trichoderma	3.93	4.5	2.95	3.75

After 5 h in fresh pollen *Alternaria*, *Cladosporium* and Yeasts prevailed. *Alternaria* fungi were common in 26.79% of the samples (32 samples out of 45) with levels ranging from 10^3–10^4 cfu g^{-1}; *Cladosporium* fungi were isolated in 28 samples, which comprised 20.21%, with levels ranging from 10^2–10^3 cfu g^{-1}. Yeasts comprised 12.14%, isolated from 12 samples, with levels ranging from 10^2–10^3 cfu g^{-1}. Fungi of *Fusarium* genera were isolated from 46% of the studied samples (21 samples out of 45), with levels ranging from 10^1 cfu g^{-1}. *Fusarium* frequency comprised 9.31%. According to frequency, they were the fourth most abundant fungi isolated.

After 1–3 days of storage, the genera of the predominant fungi quantity did not change, but the total amount of isolates increased by 0.7%, compared to 5 h. *Alternaria* fungi were predominant: their amount increased by 2.51% in the period of 1–3 days, and fungi were identified in 37 samples on average. The amount of *Cladosporium* decreased from 0.71%, but the number of positive samples remained unchanged (27 samples on average). Yeast frequency increased by 4.57% as the pollen storage time increased. *Fusarium* frequency increased by 1.18% after 3 days.

During the study period (5 h—3 days), the total amount of microscopic fungi in fresh pollen ranged from 3.5×10^3 cfu g^{-1} to 9.1×10^4 cfu g^{-1} (Table 3). On the day of pollen collection, fungal contamination averaged 6.4×10^2 cfu g^{-1}, and after three days it comprised 7.2×10^3 cfu g^{-1}. A statistically significant reduction in the amount of fungi was found in pollen stored for 1 day.

Table 3. The levels of fungal contamination and moisture content of bee pollen.

Storage Duration	Fungi		Moisture Content (%)
	Levels of Fungal Contamination (cfu g^{-1})	Average (cfu g^{-1})	
Control (after 5 h)	3.9×10^1–7.7×10^3	6.4×10^2 b	22.8a
After 1 day	3.5×10^1–7.8×10^3	6.2×10^2 b	22.7ab
After 2 days	3.8×10^1–8.4×10^3	6.6×10^2 b	22.7b
After 3 days	4.5×10^1–9.1×10^4	7.2×10^3 a	22.6b

Note: cfu g^{-1}—colony forming units per gram in bee pollen; the differences between the averages of treatments, marked by different letter (a, b) are significant ($p < 0.05$).

The highest moisture content (22.8%) in fresh pollen was found on the day of its collection—after 3 days the moisture content decreased significantly by 0.2%.

3.3. Fusarium Fungi and Mycotoxins

The total amount of *Fusarium* fungi in pollen stored for 3 days with moisture content at 22.6%, as compared to the control, significantly increased on an average up to 17.03%. The diversity of *Fusarium* species in fresh pollen was not high. Six *Fusarium* species were identified (Table 4). After 5 h in the pollen studied, *F. graminearum* and *F. sporotrichioides* dominated, which were identified among 30.06% and 27.07% of all isolated *Fusarium* genera, respectively. *F. culmorum* (14.38%) and *F. oxysporum* (13.11%) were the most common species and *F. poae* (7.69%) and *F. verticilioides* (7.69%) were classified as the least common species. During the study period, the number of isolates of most *Fusarium* species were increasing with a longer time of pollen storage. After 3 days, increases in *F. graminearum* (19.23%) and *F. sporotrichioides* (15.92%) isolates were observed compared to the control. These species dominated throughout the study period (Figure 1). A positive strong correlation was found ($r = 0.99$, $p < 0.05$) between *F. graminerum* and pollen storage time, and a weak ($r = 0.95$, $p < 0.05$) correlation was found between *F. sporotrichioides* and storage time. The amounts of *F. poae*, *F. oxysporum* and *F. verticilioides* species increased by 4.82%, 2.17% and 0.8%, respectively. A very strong correlation ($r = 0.95$, $p < 0.01$) was found between *F. culmorum* and storage time.

Table 4. The levels of *Fusarium* species in bee pollen samples (cfu g^{-1}).

Fungi	Storage Duration			
	After 5 h	After 1 Day	After 2 Days	After 3 Days
F. culmorum	1.1×10^1 A ^{b}	1×10^1 A ^{bc}	1×10^1 A ^{b}	1.1×10^1 A ^{b}
F. gramineraum	1.6×10^2 B ^{a}	1.6×10^2 B ^{a}	2×10^2 A ^{a}	2.3×10^2 A ^{a}
F. oxysporum	1×10^1 A ^{b}	1.2×10^1 A ^{b}	1.3×10^1 A ^{b}	1.2×10^1 A ^{b}
F. poae	1×10^1 B ^{b}	1×10^1 C ^{c}	1.3×10^1 AB ^{b}	1.3×10^1 A ^{b}
F. sporotrichioides	1.3×10^2 B ^{a}	1.4×10^2 B ^{a}	1.8×10^2 AB ^{a}	2×10^2 A ^{a}
F. verticilioides	1×10^1 A ^{b}	1×10^1 A ^{bc}	1×10^1 A ^{b}	1×10^1 A ^{b}

Note: cfu g^{-1}—colony forming units per gram in bee pollen; the averages of the variants in the uppercase (rows, $^{a, b, c}$) and lowercase (columns, A, B, C) are significant ($p < 0.05$).

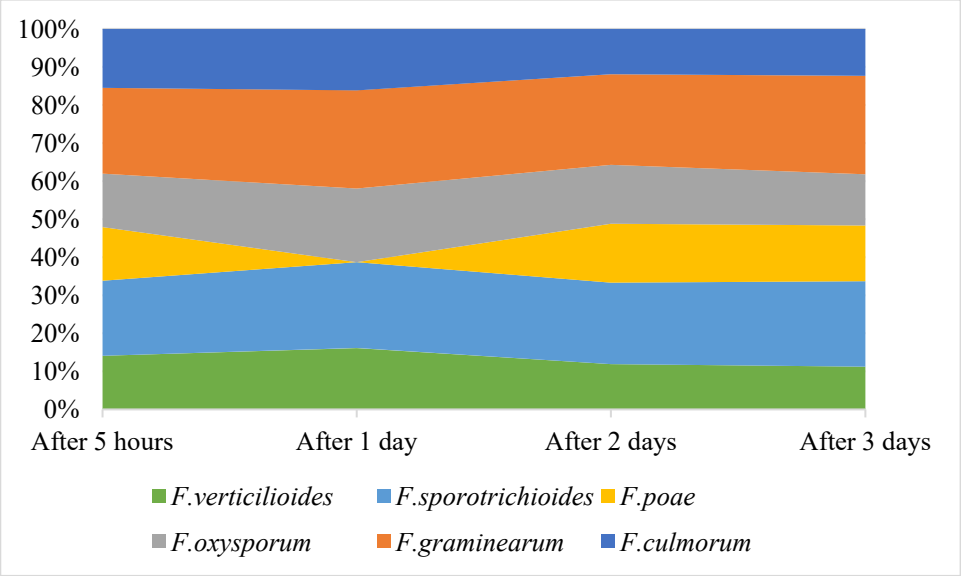

Figure 1. The dynamics of fungi in bee pollen during storage, %.

The results of mycotoxins (Table 5) indicate that the samples of bee pollen were contaminated by ZEN and DON mycotoxins. The average concentration of the toxins zearalenone (ZEN) and deoxynivalenol (DON) varied considerably, ranging from 65 to 280 μg kg^{-1} and from 47 up to 120 μg kg^{-1}, respectively. A storage duration of 3 days influenced the highest concentrations of mycotoxins ZEN and DON. Contamination by the T-2 toxin in all bee pollen samples was found to be below the detection limit.

Table 5. The concentration of mycotoxins (μg kg^{-1}).

Storage Duration	Number of Positive Samples	ZEN	DON	T-2
5 h	21	67b	47b	<LoD
After 1 day	19	65b	<LoDc	<LoD
After 2 days	24	67b	48b	<LoD
After 3 days	26	280a	120a	<LoD

Note: <LoD—below limit of detection; means not sharing common letters are significant ($p < 0.05$).

On the day of pollen collection, 67 μg kg^{-1} of mycotoxin zearalenone (ZEN) was detected. The pollen was most heavily contaminated with zearalenone (ZEN) after 3 days of storage, at 280 μg kg^{-1}, and it was the highest concentration of this mycotoxin found in all study variants.

Mycotoxin deoxynivalenol (DON) at 47 μg kg^{-1} in the pollen was found on the day of pollen collection. A significant increase in the concentration of deoxynivalenol (DON) was found after 3 days, comprising 120 μg kg^{-1}.

A positive correlation ($r = 0.55$, $p < 0.05$) was ascertained between moisture content of the pollen and ZEN concentration. No correlation was found between ZEN and storage duration or the total amount of fungi. No correlation was found between DON and storage period or the total amount of fungi with prolonged storage period.

PCA was used to determine the grouping possibilities of bee pollen samples, considering their storage duration and fungal and mycotoxin contamination (Figure 2). The first and second PCA components were composed of 87.28%.

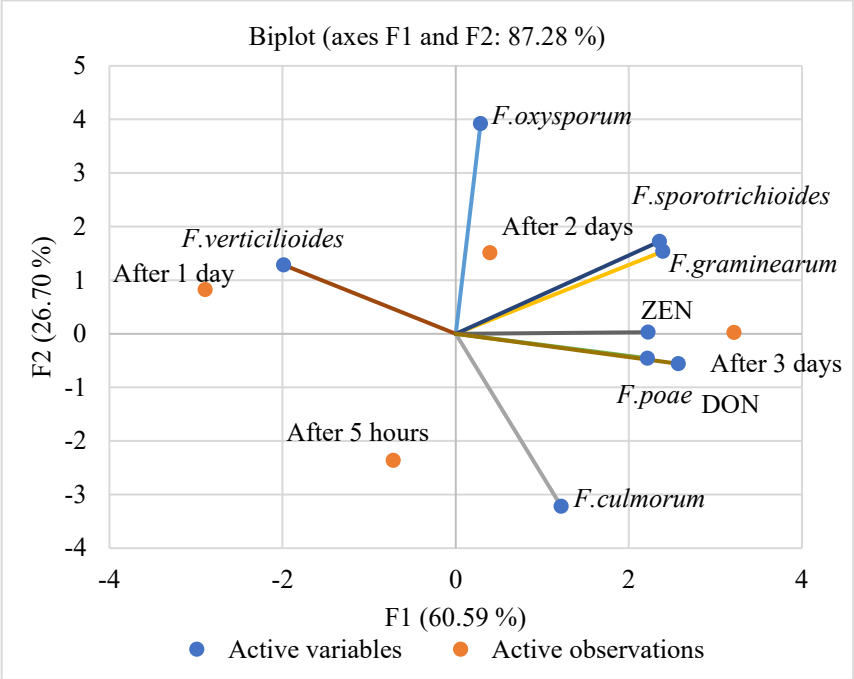

Figure 2. Principal component analysis (PCA) for bee pollen stored for three days (DON—deoxivalenol; ZEN—zearalenone).

All bee pollen samples were completely separated depending on their storage duration. The samples before storage were situated at negative PC2 values, while longer storage duration samples were situated at positive PC2 values.

One storage day samples were closely related to the *F. verticilioides*, while three-day samples were related to *F. poae*, *F. graminearum*, zearalenone and deoxivalenol.

4. Discussion

Microbiological contamination of bee pollen arises from different sources. The primary sources of contamination of bee pollen by microscopic fungi are very difficult to control, and they can come from plants, from the digestive tract of bees or enter with dust from the air or soil. Possible sources of contamination may be the type of collector or the impurity of pollen traps [4,19]. The botanical origin of the pollen is revealed by floral species, which varies depending on the climatic conditions during flowering and the region where the apiary is located [31]. In this study, the pollen of *Brassicaceae* plants dominated, as bees collected the pollen from *Rosaceae Fabaceae*, *Poaceae* and *Asteraceae* family plants.

Some fungi can spread together with pollen [32]. Reports of fungi associated with pollen grains on plants are rare. The intricacy of the surface of many pollen grains could provide ample opportunities for the attachment of fungal spores [33]. During the flowering and pollination process, spores can germinate on female flower parts. The pollen from some *Asteraceae* species is an optimal substrate for fungal growth due to its high lipid content [18]. The dominant endophytes such as *Alternaria*, *Cladosporium* and *Epicoccum* seem not to be host-specific and are opportunistic colonizers of many plants [34]. According to researchers [35,36], *Alternaria*, *Cladosporium* and *Fusarium* are some of the dominant mycobiota in fodder legumes. If bees visit these plants, the contaminated pollen grains will be transferred into the hives [3]. *Fusarium* fungi producing trichothecenes were found in all species of leguminous grasses, the duration of vegetation of which is directly related to the amount of *Fusarium* fungi, and with increasing duration increases the amount of *Fusarium* fungi [37].

In the study, the moisture content of fresh bee pollen 5 h after collection was high (22.8%). Other researchers also obtained similar results: 14.6–37.7% [6], 21–30% [38] and

20% [39]. The moisture content of fresh bee pollen in this study varies slightly—between 0.01 and 0.02%—and was sufficient for fungi growth. The high moisture content can lead to an increase in microscopic fungal contamination which causes the production of mycotoxins [15,40].

Fungi belonging to 14 genera were detected in the samples of fresh bee pollen. The highest number of colonies among fungi comprised *Alternaria*, *Cladosporium* and Yeasts. Fungi of this genera are classified as saprotrophic field fungi derived from soil or plant residues [2]. Fungi of the genera *Alternaria* and *Cladosporium* are sufficiently resistant to the negative effects of the environment due to the accumulation of melanin [41], and their development and growth require high relative air humidity and low availability of food resources [2]. Studies conducted by Gonzales et al. [16] have revealed that the amount of prevailing *Alternaria* fungi in bee pollen comprised 86.6%. According to Deveza et al. [31], *Cladosporium* in bee pollen was singled out as the second most common fungi. Kačaniová et al. [17] found that *Alternaria alternata* and *Cladosporium cladosporoides* were the most frequent isolates found in Slovakia bee pollen samples.

Yeasts are not classified as microorganisms dangerous to human health, and they usually indicate the sanitary condition of hives and hygienic conditions during pollen collection and transportation [42]. Twelve analysed samples were contaminated with Yeasts, and their amount was abundant. The most abundant Yeasts were isolated in the pollen with the highest moisture content (22.8%). The abundance of Yeasts decreases rapidly in the first days of fresh pollen storage [43]. This is partly reflected by the data of our study.

The total amount of microscopic fungi in bee pollen on the day of collection (after 5 h) was similar to the previous results of studies by Sinkevičienė and Amšiejus [44], in which the contamination of fresh pollen after 9 h was from 4.5×10^3 to 9.5×10^3 cfu g^{-1}. Beev et al. [45] also performed a fungal colony count in fresh bee pollen, which varied from 560 to 37,000 cfu g^{-1} and was significantly ($p < 0.01$) higher than the fungal colony count in dried pollen. In our study, the amount of fungi in different periods varied depending on storage duration time. Fresh bee pollen stored for different time periods showed that the amount of fungi started to increase with longer duration of pollen storage. The highest amount of fungi (7.2×10^3 cfu g^{-1}) was determined after 3 days of storage duration of fresh pollen, however, according to scientists from the International Honey Commission (IHC), this amount of fungi failed to exceed the limits of recommended quality criteria ($<5 \times 10^4$ cfu g^{-1}) [46].

In this study, the genus *Fusarium* was detected in less than half of fresh pollen samples tested. *Fusarium* fungi can survive as saprotrophs and require more than 20% substrate moisture to develop and multiply [5]. According to frequency, among all identified fungi *Fusarium* spp. was the fourth. Beev et al. [45] found *Fusarium* spp. to be the second most frequently encountered microscopic fungi in dried bee pollen, but no *Fusarium* fungi were detected in fresh bee pollen. According to the data by Gonzalez et al. [16], the *Fusarium* spp. was rarely detected in dried bee pollen. In the study by Brindza et al. [2], *Fusarium* occurred mostly in dried bee pollen.

Fusarium is the main toxin-producing genus among fungi [10]. Many toxic species of the genus *Fusarium* are widespread in all European cereal growing areas, including Lithuania [47]. Isolates of *Fusarium* species were isolated in the pollen in order to elucidate the producers of fusariotoxins. Six species of the genus *Fusarium* were detected in 21 samples. Similar results (five species) were obtained by other researchers [17].

In this study, *F. graminearum* was clearly predominant in the period of all studies (1–3 days). *F. graminearum* is known as the most common *Fusarium* species and the producer of deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEN) [19,48]. The next dominant species were *F. sporotrichioides.* [4], *F. sporotrichioides* and *F. verticilioides*, which dominated in the study of Kačaniová et al. [17]. However, in our study the contamination level of *F. verticilioides* was generally low, especially at the end of the storage (Figures 1 and 2). Based on other research [17], *F. oxysporum* was one of the prevailing

species, comprising 55.6%. In our study, the amount of *F. oxysporum* isolates was low, and on average comprised 14.19%. Other species, *F. culmorum* (11.18%) and *F.poae* (10.78%), were also less common. In the PCA it was found that *F. poae* was closely related to three storage days duration when at that time the amount of *F. poae* increased. (Figure 2).

The production of mycotoxins depends on a number of factors, such as temperature, humidity, substrate composition and the quantity of fungal inoculum. A long period between collection and drying of pollen can also affect the growth of toxigenic fungi and the production of mycotoxins [49]. The increase in mycotoxins after 3 days showed that the temperature of 22–25 °C and the moisture of the substrate may be favourable for the growth and development of *Fusarium* fungi. The increases found in *F. graminearum*, *F. sporotrichioides* and *F. culmorum* during this period are possibly related to the highest levels of zearalenone (ZEN) and deoxynivalenol (DON) released. PCA analysis indicates the strongest correlation between the colony forming units of *F. graminearum* the concentration of DON and ZEN at the end of the storage (Figure 2). Our previous investigation [50] examined dried pollen samples contaminated with ZEN and DON; the highest DON concentration (185 μg kg^{-1}) was ascertained after 4 months of storage at 8–9 °C temperature, and the highest ZEN concentration (830 μg kg^{-1}) was found after 1 month of storage at 20–22 °C temperature. It was these research results that encouraged us to investigate the mycological and mycotoxicological contamination of fresh pollen collected only from plants. The fungal and toxigenic load detected in bee pollen raises questions on how contamination occurs during pollen production and collection. Kostic et al. [18] states that the source of pollen pollution with mycotoxins can be the hive, and they also mention that the growth of toxin-producing fungi can be stimulated by infected flowering plants. *Trichoderma*, *Myrothecium*, *Phomopsis*, etc., have been also mentioned as DON sources. *Fusarium* spp. are field fungi and produce mycotoxins pre-harvest [47]. Therefore, the detection of *Fusarium* toxins is only a retrospective indicator for the conditions on field and is not directly related to the toxin content during storage [51].

Due to climatic changes, which extensively influence weather conditions in Europe, the presence of toxins is becoming more frequent. DON and ZEN mycotoxins were the most dominant quantified mycotoxins in the pollen samples from Spain [52]. DON, ZEN were the most dominant quantified mycotoxins in the bee pollen samples from Slovakia [3]. Prevailing in pollen, *F. sporotrichioides* and *F. poae* are co-producers of the T-2 mycotoxin [7], but this mycotoxin has not been detected in pollen. However, the amount of mycotoxins may not depend on the number of fungi present [53]. The production of toxins largely mirrors growth conditions [19]. Therefore, according to Petrovic et al. [19] and Kačaniova et al. [17], fresh bee pollen is recommended to be stored at low temperatures for no more than 48 h, and mycotoxin levels should be monitored regularly [4].

5. Conclusions

This study found high levels of contamination of fresh bee pollen by microscopic fungi. The most prevalent fungal genera of *Alternaria*, *Cladosporium* and Yeasts were found in fresh bee pollen. Among them, six *Fusarium* species and ZEN and DON mycotoxins produced by them were isolated, the amounts of which were increasing with a longer storage period. The study found that the pollen on the day of collection was already contaminated by *Fusarium* mycotoxins. Therefore, in controlling the contamination of fresh bee pollen by toxigenic fungi and mycotoxins, it is very important that the pollen is dried as quickly as possible to reduce the levels of fungal contamination. The obtained results emphasize the importance of microbiological analysis in monitoring bee pollen from the initial stages of their production process. Information on the presence of mycotoxins in bee pollen can be very important for risk assessment on human health.

Author Contributions: Conceptualization, J.S. and Ž.T.; methodology, J.S.; validation, V.T.; formal analysis, J.S.; investigation, J.S. and V.T.; resources, Ž.T.; data curation, J.S., Ž.T. and V.T.; writing—original draft preparation, J.S., Ž.T. and V.T.; writing—review and editing, J.S.; visualization, J.S.; supervision, J.S.; project administration, J.S.; funding acquisition, J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bogdanov, S. Pollen: Collection, harvest, composition, quality. In *Bee Product Science (The Pollen Book)*; Muehlethurnen, Switzerland, 2012; Chapter 1; Available online: http://www.bee-hexagon.net (accessed on 20 September 2022).
2. Brindza, J.; Grof, J.; Bacigalova, K.; Ferianc, P.; Toth, D. Pollen microbial collonization and safety. *Acta Chim. Slov.* **2010**, *3*, 95–102.
3. Kostić, A.Ž.; Milinčić, D.D.; Petrović, T.S.; Krnjaja, V.S.; Stanojević, S.P.; Barać, M.B.; Tešić, Ž.L.; Pešić, M.B. Mycotoxins and Mycotoxin Producing Fungi in Pollen: Review. *Toxins* **2019**, *11*, 64. [CrossRef] [PubMed]
4. Hani, B.; Dalila, B.; Saliha, D.; Daoud, H.; Mouloud, G.; Seddik, K. Microbiological sanitary aspects of pollen. *Environ. Biol.* **2012**, *6*, 1415–1420.
5. Pitt, J.I.; Basilico, J.C.; Abarca, M.L.; Lopez, C. Mycotoxins and toxigenic fungi. *Med. Mycol. J.* **2000**, *1*, 41–46. [CrossRef]
6. Nardoni, S.; D'Ascenzi, C.; Rocchigiani, G.; Moretti, V.; Mancianti, F. Occurrence of moulds from bee pollen in Central Italy—A preliminary study. *Ann. Agric. Environ. Med.* **2016**, *23*, 103–105. [CrossRef]
7. Bennett, J.W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, *16*, 497–516. [CrossRef]
8. Hussein, H.S.; Brasel, J.M. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology* **2001**, *167*, 101–134. [CrossRef]
9. Kokkonen, M.; Ojala, L.; Parikka, P.; Jestoi, M. Mycotoxin production of selected *Fusarium* species at different culture conditions. *Int. J. Food Microbiol.* **2010**, *143*, 17–25. [CrossRef]
10. Ji, F.; He, D.; Olaniran, A.O.; Mokoena, M.P.; Xu, J.; Shi, J. Occurence, toxicity, production and detection of *Fusarium* mycotoxin: A review. *Food Prod. Process. Nutr.* **2019**, *1*, 6. [CrossRef]
11. Bianchini, A.; Bullerman, L.B. Mycotoxins: Classification. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Academic Press: Oxford, UK, 2014; Volume 2, pp. 854–861.
12. Komosinska-Vassev, K.; Olczyk, P.; Kaźmierczak, J.; Mencner, L.; Olczyk, K. Bee pollen: Chemical composition and therapeutic application. *Evid. Based Complement. Altern. Med.* **2015**, *2015*, 297425. [CrossRef]
13. De–Melo, A.A.M.; Estevinho, L.M.; Moreira, M.M.; Delerue-Matos, C.; da Silva de Freitas, A.; Barth, O.M.; Almeida-Muradian, L.B. Phenolic profile by HPLC-MS, biological potential and nutritional value of a promising food: Monofloral bee pollen. *J. Food Biochem.* **2018**, *42*, e12536. [CrossRef]
14. Màrgàoan, R.; Màrghitas, L.A.; Dezmirean, D.S.; Dulf, F.V.; Bunea, A.; Socaci, S.A.; Bobis, O. Predominant and secondary pollen botanical origins influence the carotenoid and fatty acid profile in fresh honeybee-collected pollen. *J. Agric. Food Chem.* **2014**, *62*, 6306–6316. [CrossRef]
15. De Arruda, V.A.S.; Santos Pereira, A.A.; Estevinho, L.M.; de Almeida-Muradian, L.B. Presence and stability of B complex vitamins in bee pollen using different storage conditions. *Food Chem. Toxicol.* **2013**, *51*, 143–148. [CrossRef]
16. Gonzalez, G.; Hinojo, M.J.; Mateo, R.; Medina, A.; Jimenez, M. Occurence of mycotoxin producing fungi in bee pollen. *Int. J. Food Microbiol.* **2005**, *105*, 1–9. [CrossRef]
17. Kačaniová, M.; Juraček, M.; Chlebo, R.; Knazovická, V.; Kadasi-Horakova, M.; Kunova, S.; Lejkova, J.; Haščik, P.; Mareček, J.; Šimko, M. Mycobiota and mycotoxins in bee pollen collected from different areas of Slovakia. *J. Environ. Sci. Health* **2011**, *46*, 623–629. [CrossRef]
18. Kostić, A.Ž.; Petrović, T.S.; Krnjaja, V.S.; Nedić, N.M.; Tešić, Ž.L.; Milojković-Opsenica, D.M.; Barać, M.B.; Stanojević, S.P.; Pešić, M.B. Mold/aflatoxin contamination of honey bee collected pollen from different Serbian regions. *J. Apic. Res.* **2017**, *56*, 13–20. [CrossRef]
19. Petrović, T.; Nedić, N.; Paunović, D.; Rajić, J.; Matović, K.; Radulović, V.; Krnjaja, V. Natural mycobiota and aflatoxin B1 presence in bee pollen collected in Serbia. *Biotechnol. Anim. Husb.* **2014**, *30*, 731–741. [CrossRef]
20. Mayda, N.; Keskin, M.; Keskin, S.; Özkök, A. Determination of botanic origins, total phenolic and flavonoid contents of bee pollen collected from the Bilecik province. *Uludag Aricilik Derg.* **2019**, *19*, 152–160.
21. Burmistrov, A.N.; Nikitina, V.A. *Melliferous Plants and Their Pollen*; Rosagropomizdat: Moscow, Russia, 1990; pp. 165–189.
22. Straka, H. *Pollen–und Sporenkunde*; Gustav Fischer Verlag: Stuttgart, Germany, 1975; pp. 30–65.
23. Domsch, K.H.; Gams, W.; Anderson, T.H. *Compendium of Soil Fungi*; Academic Press: London, UK, 1980; p. 860.
24. Ellis, M.B. *Dematiaceous Hyphomycetes*; Commonwealth Mycological Institute Kew: Surrey, UK, 1976; p. 507.
25. Leslie, J.F.; Summerell, B.A. *The Fusarium Laboratory Manual*; Blackwell Publishing: Ames, IA, USA, 2006; p. 400.
26. Pitt, J.I.; Hocking, A.D. *Fungi and Food Spoilage*, 3rd ed.; Springer: New York, NY, USA, 2009; p. 519.

27. Samson, R.A.; Hoekstra, E.S.; Frisvad, J.C. *Food and Indoor Fungi*; Centraalbureau voor Schimmelcultures: Ultrecht, The Netherlands, 2010; p. 389.
28. Wilkinson, A.P.; Ward, C.M.; Morgan, M.R.A. Immunological analysis of mycotoxins. In *Plant Toxin Analysis*; Lins-Kens, H.F., Jackson, J.F., Eds.; Springer: Berlin, Germany, 1992; pp. 185–225.
29. Krska, R.; Schubert-Ullrich, P.; Molinelli, A.; Sulyok, M.; MacDonald, S.; Crews, C. Mycotoxin analysis: An update. *Food Addit. Contam.* **2008**, *25*, 152–163. [CrossRef]
30. Raudonius, S. Application of statistics in plant and crop research: Important issues. *Zemdirb. Agric.* **2017**, *104*, 377–382. [CrossRef]
31. Deveza, M.V.; Keller, K.M.; Lorenzon, M.C.A.; Nunes, I.M.T.; Sales, E.O.; Barth, O.M. Mycotoxicological and palynological profiles of commercial brands of dried bee pollen. *Braz. J. Microbiol.* **2015**, *46*, 1171–1176. [CrossRef] [PubMed]
32. Card, S.D.; Pearson, M.N.; Clover, G. Plant pathogens transmitted by pollen. *Australas. Plant Pathol.* **2007**, *35*, 455–461. [CrossRef]
33. Edlund, A.F.; Swanson, R.; Preuss, D. Pollen and stigma structure and function: The role of diversity in pollination. *Plant Cell* **2004**, *16*, 84–97. [CrossRef] [PubMed]
34. Rodríguez-Carrasco, Y.; Font, G.; Mañes, J.; Berrada, H. Determination of mycotoxins in bee pollen by gas chromatography – tandem mass spectrometry. *J. Agric. Food Chem.* **2013**, *61*, 1999–2005. [CrossRef]
35. Lager, R.; Johnsson, L. Seed-borne fungi affect field emergence in red clover. *J. Plant Dis. Prot.* **2002**, *109*, 350–356.
36. Kononenko, G.P.; Burkin, A.A.; Gavrilova, O.P.; Gagkaeva, T.Y. Fungal species and multiple mycotoxin contamination of cultivated grasses and legumes crops. *Agric. Food Sci.* **2015**, *24*, 323–330. [CrossRef]
37. Orina, A.S.; Gavrilova, O.P.; Gagkaeva, T.Y. Analyses of contamination of annual and perennial *Leguminosae* grasses with fungi using quantitative PCR. *Plant Prot. News* **2018**, *2*, 35–41. [CrossRef]
38. Bobis, O.; Marghitas, I.A.; Dezmirean, D.; Morar, O.; Bonta, V.; Chirila, F. Quality parameters and nutritional value of different commercial bee products. Bulletin of University of agricultural sciences and veterinary medicina Cluj-Napoca. *J. Anim. Sci. Biotechnol.* **2010**, *67*, 1–2.
39. Fatrcová-Šramková, K.; Nôžková, J.; Máriássyová, M.; Kačániová, M. Biologically active antimicrobial and antioxidant substances in the *Helianthus annuus* L. bee pollen. *J. Environ. Sci. Health* **2016**, *51*, 176–181. [CrossRef]
40. Estevinho, M.L.; Afonso, S.E.; Feris, X. Antifungal effect of lavender honey against *Candida albicans*, *Candida krusci* and *Cryptococcus neoformans*. *J. Food Sci. Technol.* **2011**, *48*, 640–643. [CrossRef]
41. Klich, M.A. Health effects of *Aspergillus* in food and air. *Toxicol. Ind. Health* **2009**, *25*, 657–667. [CrossRef]
42. Vasconcelos, M.R.S. Polen Apicola do Estado de Alagoas: Composicao-Fisico-Quimica, Origem Botanica e Atividade Antioxidante. Ph.D. Thesis, Universidade Federal de Alagoas, Maceió, Brazil, 2009.
43. Detry, R.; Simon-Delso, N.; Bruneau, E.; Daniel, H.D. Specialisation of Yeast Genera in Different Phases of Bee Bread Maturation. *Microorganisms* **2020**, *8*, 1789. [CrossRef]
44. Sinkevičienė, J.; Amšiejus, A. Mycobiota in bee pollen collected by different types of traps. *Zemdirb. Agric.* **2019**, *106*, 377–382. [CrossRef]
45. Beev, G.; Stratev, D.; Vashin, I.; Pavlov, D.; Dinkov, D. Quality assessment of bee pollen: A cross sectional survey in Bulgaria. *J. Food Qual. Hazards Control* **2018**, *5*, 11–16. [CrossRef]
46. De–Melo, A.A.M.; Estevinho, M.I.M.F.; Satler, J.A.G.; Souza, B.R.; da Silva Freitas, A.; Barth, O.M.; Almeida-Muradian, L.B. Effect of processing conditions on characteristics of dehydrated bee-pollen and correlation between quality parameters. *LWT–Food Sci. Technol.* **2016**, *65*, 808–815. [CrossRef]
47. Mačkinaitė, R.; Kačergius, A.; Lugauskas, A.; Repečkienė, J. Contamination of cereal grain by *Fusarium* micromycetes and their mycotoxins under Lithuanian climatic conditions. *Ekologija* **2006**, *3*, 71–79.
48. Patriarca, A.; Pinto, V.F. Prevalence of mycotoxins in foods and decontamination. *Curr. Opin. Food Sci.* **2017**, *14*, 50–60. [CrossRef]
49. CAST–Council for Agricultural Science and Technology. *Mycotoxins, Risk in Plant, Animal and Human Systems*; Task Force Report 139; CAST: Ames, IA, USA, 2003; pp. 1–199.
50. Sinkevičienė, J.; Burbulis, N.; Baliukonienė, V. The influence of storage conditions on bee pollen contamination by microscopic fungi and their mycotoxins. *Zemdirb. Agric.* **2021**, *108*, 159–164. [CrossRef]
51. Tenbrink, K.M.; Schulz, J.; Kemper, N. Mycotoxin contamination of selected organic enrichment materials used in pig farming. *Agriculture* **2020**, *10*, 565. [CrossRef]
52. Rodríguez, R.J.; White, J.F.; Arnold, A.E.; Redman, R.S. Fungal endophytes: Diversity and functional roles. *New Phytol.* **2009**, *182*, 314–330. [CrossRef]
53. Tarr, B. *Managing the Effects of Molds and Mycotoxins in Ruminants*; Shur-Gain, Nutrecko; Canada Inc.: Lakeside, ON, Canada, 2006.