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First survey on the presence of mycotoxins in commercial bee pollen sourced from 28 countries

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ABSTRACT

Bee pollen is a natural product valued for its nutritional and medicinal benefits, and it is gaining popularity for its potential use as a feed and supplement for cattle. However, due to the lack of information on the extent of its contamination with dangerous substances, there are still a number of questions regarding the safety of this beekeeping product. Mycotoxins, in particular, are a family of molecules typically found in food that may pose a health risk to consumers and for which no legal restrictions are set in bee pollen.

In this study, the presence of five mycotoxins - aflatoxin B1, ochratoxin A, zearalenone, deoxynivalenol, and toxin T2 - was evaluated in 80 bee pollen samples from diverse climatic areas, organic and conventional beekeeping, with different floral composition, and commercial format - fresh, dry, or bee bread. The analyses were performed by enzyme-linked immunosorbent assays and the obtained results showed the presence of the least one of the analyzed mycotoxins in 100% of the samples, with aflatoxin B1 having the highest incidence rate. Monofloral pollens showed lower total mycotoxins concentration compared with the multifloral samples analyzed, while bee bread was the commercial format with the highest total mycotoxins concentration among the analyzed ones. The obtained results were also used to assess the risk associated with single and multiple mycotoxin exposure due to bee pollen consumption by calculating the respective hazard quotients and margins of exposure. In 28% of the analyzed cases, deoxynivalenol exceeded the safe limits, while aflatoxin B1, because of its generally high concentration, resulted of high public health concern in 84% of the considered cases.

1. Introduction

Bee pollen is one of the most valued natural products, which is greatly appreciated because of its high content in macro-, micronutrients and bioactive compounds including carbohydrates, proteins, lipids, vitamins, minerals, carotenoids, and polyphenols (Kieliszek et al., 2018; Thakur and Nanda, 2020). Since past times, bee pollen has been used in the folk medicine against various diseases and nowadays, modern science has revealed substantial evidence on its therapeutic potential especially due to its antioxidant, antibacterial and anti-inflammatory effects (Cornara et al., 2017; Khalifa et al., 2021). The recommended daily dose for human consumption is around 20 g for adults, but nowadays bee pollen is also attracting increasing interest in the feed market. In fact, it is being marketed as nutritional supplement for livestock or for pests, and as feed for invertebrates such as bumble bees, which are traded on an industrial scale for pollination of crops, or for the growth of edible insects used as a protein source for food and feed (Haefeker, 2021; Kostić et al., 2020).

Due to the health-promoting properties of bee pollen, monitoring its degree of contamination with hazardous substances is important from a food safety point of view (Kostić et al., 2019; Végh et al., 2021). Mycotoxins are toxic metabolites of fungi that can contaminate plant-based products, known to carry-over from animal feedstuff to animal products providing an exposure pathway via consumption of both (CAC, 1995). They are a heterogeneous and toxic class of molecules composed of several hundreds of compounds, of which only a limited number have

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been studied in detail and receive regular attention. Mycotoxins represent a subject of global relevance due to severe health-associated risks and substantial economic implications which stem from the market losses of crop products or reduced livestock production (Eskola et al., 2019; IARC, 2012). This has led many countries to set legislative limits for maximal levels of mycotoxins in feed and food ensuring they are not harmful to human or animal health (CAC, 1995; FDA, 2023; GB, 2017; European Parliament, 2002; EC, 2006a; EC, 2006b; EC, 2013). For instance, in the EU, harmonized limits now exist for 67 mycotoxin–foodstuffs combinations (EC, 2006a). So far, bee pollen is excluded from this decision-making scheme as the consumption of this product by consumers is generally considered negligible (EC, 2018). In their technical regulation for bee pollen quality, Campos et al. (2008) reported a suggestion for maximum levels of AFB1 (2 μg/kg) and total aflatoxins (4 μg/kg) in bee pollen; however, this proposal was never made into an official and widely accepted regulation.

Mycotoxins have been found in several items of foods and feeds across the globe. Amongst naturally occurring mycotoxins, those produced by several genera of filamentous fungi, namely *Aspergillus*, *Fusarium* and *Penicillium*, which produce aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT), fumonisins (FBs), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), citrinin (CIT), T-2 and HT-2 toxins, are of greatest concern to human health and livestock (Biomin, 2021; Eskola et al., 2019; Gruber-Dorninger et al., 2019). Mycotoxins can occur at any stage of the food chain, strongly favored under various environmental factors such as temperature and humidity during production, harvesting, and storage (CAC, 1995; Daou et al., 2021). Furthermore, the spread of molds may be significantly affected by climate change, and by an increasing variable weather that may alter the prevalence of specific fungi and the mycotoxins they produce as well as their geographical distribution (Russell et al., 2010; Sanjiv Chhaya et al., 2022; EFSA et al., 2020). Bee pollen production is a complex process and is also the result of the application of different beekeeping models, which make this product have different characteristics depending on its place and type of production, harvesting and processing. Bee pollen is usually found in the market in three formats: fresh, dry or as bee bread. Fresh pollen refers to the original ball or pellet formed by the bees during collection, generally with a water content between 20 and 30%, and dried to the processed form that allow extends the shelf life of the product at ambient temperatures and makes its commercialization easier. Dry bee pollen is the product undergone to a drying process at temperatures not higher than 42 °C, with water content not higher than 6–8% (Campos et al., 2008). While fresh and dry pollen are harvested in pollen traps outside the hives, bee bread is produced inside the hive by the worker bees via the addition of nectar and bee salivary enzymes, where it is stored and fermented (FAO et al., 2021).

While the body of knowledge on occurrence of mycotoxins in food and feed is continually growing (Biomin, 2021; Eskola et al., 2019; Gruber-Dorninger et al., 2019), research on mycotoxin contamination in bee pollen is sparse. Among the microorganisms found in pollen, fungi belonging to the species of *Aspergillus*, *Fusarium* and *Penicillium* are one of the most serious causes of concern. Some of the studies, conducted in the past to identify the mold communities found in bee pollen revealed the presence of *Aspergillus* sp. (Altunatmaz and Aksu, 2016; Beev et al., 2018; Estevinho et al., 2012; Kačániová et al., 2011; Nardoni et al., 2016; Petrović et al., 2014; Shevtsova et al., 2019), *Fusarium* ssp. (Beev et al., 2018; Estevinho et al., 2012; Petrović et al., 2014; Sinkevičienė et al., 2021), and *Penicillium* ssp. (Altunatmaz and Aksu, 2016; Beev et al., 2018; Estevinho et al., 2012; Kostić et al., 2016; Nardoni et al., 2016; Petrović et al., 2014; Shevtsova et al., 2019; Sinkevičienė et al., 2021), which could potentially produce mycotoxins. A limited number of studies so far aimed at the direct detection and quantification of mycotoxins in bee pollen and the majority of them revealed the presence of these toxic metabolites in the analyzed samples. According to these studies, the most widespread mycotoxins found in bee pollen are aflatoxin B1 (AFB1) (Kačániová et al., 2011; Kostić et al., 2016; Li et al., 2010; Nuvoloni et al., 2021; Petrović et al., 2014), DON, ZEN, OTA and T-2 (Kačániová et al., 2011; Nuvoloni et al., 2021; Sinkevičienė et al., 2021). Among them, AFB1 is the most dangerous mycotoxin for people and animal's health, as it is one of the most potent natural carcinogens known, it can induce DNA damage, chromosomal aberrations, and several liver diseases (EFSA CONTAM Panel, 2020a). Similarly, OTA and ZEN show immunotoxic, hepatotoxic, and neurotoxic effects (EFSA CONTAM Panel, 2020b; Ropejko and Twarużek, 2021), while trichothecenes like DON and T2 can cause metabolic and neurotransmitter's impairment (Cope, 2018).

Currently, there is no comprehensive study that monitored the degree of contamination with mycotoxins considering the place and type of production, harvesting and processing. In this study, five of the most common mycotoxins AFB1, OTA, ZEN, DON and T-2 found in foodstuffs were examined in bee pollen. This work aimed to investigate for first time, the presence of these mycotoxins in commercial bee pollen considering country of origin, organic and conventional beekeeping, bee pollen harvested as mono and multifloral, and commercial format - fresh, dry or bee bread. This study includes commercial bee pollen samples sourced from 28 countries that represent various geographical origins from the main climate groups of the Köppen climate classification (Beck et al., 2018). Mycotoxins were measured in bee pollen samples to evaluate their occurrence and concentration levels based on the enzyme-linked immunosorbent assay (ELISA), widely used in the routine screening of mycotoxin contamination in various agricultural and food products (Hosseini et al., 2018). This study also aims to characterize, for the first time, the risk associated with the exposure of people to single and multiple mycotoxins using HQ, HI, MOE, and MOE_T models to help identify if its consumption is safe.

2. Materials and methods

2.1. Sampling

Bee pollen samples were purchased from herbal stores, grocery stores and mostly from e-commerce providers. Up to 80 samples of 72 commercially available brands of beekeeping products were sourced from 28 countries from the main climate groups of the Köppen climate classification: Group A – tropical (n = 1), Group B – arid (n = 6), Group C – temperate (n = 62), Group D – continental (n = 12). Inside of every group it was possible to attribute the samples to more specific climatic subareas. Group Aw - tropical savanna, wet (n = 1 from India), Group Bsk - arid, cold steppe (n = 1 from USA, n = 1 from Mexico), Group Bwh – arid, hot desert (n = 1 from Saudi Arabia, n = 1 from Jordan), Group Bwk – arid, cold desert (n = 2 from Russia), Group Cfa – temperate, hot summer, without dry season (n = 3 from Bulgaria, n = 2 from China, n = 1 from Italy, n = 1 from USA, n = 1 from Russia), Group Cfb – temperate, warm summer, without dry season (n = 9 from France, n = 4 from Spain, n = 2 from Germany, n = 2 from Hungary, n = 1 from Australia, n = 1 from Bulgaria, n = 1 from Italy, n = 1 from Slovakia, n = 1 from Serbia, n = 1 from Poland, n = 1 from Slovenia, n = 1 from the Netherlands), Group Csa – temperate, hot and dry summer (n = 12 from Spain, n = 2 from Greece, n = 1 from Italy, n = 1 from Portugal, n = 1 from Israel), Group Csb – temperate, warm and dry summer (n = 11 from Spain, n = 1 from Portugal), Group Dfb – continental, warm summer, without dry season (n = 3 from Latvia, n = 2 from Lithuania, n = 1 from Canada, n = 1 from Estonia, n = 1 from Romania, n = 1 from USA), Group Dfc – continental, cold summer, without dry season (n = 1 from Italy, n = 1 from Austria), Dfd – continental, very cold winter, without dry season (n = 1 from Russia). According to the labelling, sixty-two samples come from conventional beekeeping and eighteen from organic beekeeping. Of these, sixty-nine samples were harvested as multifloral type and eleven as monofloral.

With respect to the processing conditions, sixty-four samples are sold as dry pollen, eight as fresh frozen pollen, one as fresh pollen and seven as bee bread. Following collection, the samples were listed, assigned a

sample ID, and stored according to the manufacturers' instructions.

2.2. Sample preparation and analysis

2.2.1. Moisture content

Of the 72 commercial brands, only two indicated the moisture content on the label. Dry bee pollen's moisture determination was made by a gravimetric method, based on the sample weight loss due to desiccation until constant weight was reached by drying under infra-red radiation. Prior to moisture determination, the samples were kept according to storage instructions on the label and in their original bags until the moisture was determined. The equipment used is composed of an electronic precision balance Micronal (B160), adapted with an infra-red dryer Mettler Toledo (LP16), with adjustments to the intensity of radiation emitted, so that the sample reaches 105 °C. For this analysis, a rate of the sample was applied to a holder (small aluminum plate) previously weighed. After that, radiation was reflected on the sample and the percentage of moisture was displayed on the equipment.

2.2.2. Mycotoxins determination

For sample preparation, each package was thoroughly mixed by hand agitation. A portion of each sample (20 ± 1 g) was finely ground using an Oster® Blender base. Homogenized samples were stored in sterile 40 mL amber vials (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at the appropriate storage conditions for every type of sample until analysis.

2.2.2.1. ELISA verification for bee pollen screening. Bee pollen samples were tested for AFB1, OTA, ZEN, DON, and T-2 using the following ELISA kits from Biosystems (Barcelona, Spain): AFB1 kit for feed and food, OTA kit for cereals, ground roasted, instant coffee, green coffee, cocoa powder, wine and must, ZEN kit for cereals, milk, and milk powder, DON kit for cereals, silage, feed, food and beer, and T-2 Toxin kit for cereals and silage. Since there are no specific kits for pollen and these kits had not previously been verified by the manufacturer for use with bee pollen, they were subjected to spike recovery testing prior to use in the market survey to ensure there were no matrix interferences that would yield false results. Bee pollen samples were tested by spiking the amount of ground sample indicated in the kits' protocol with the target mycotoxin at a concentration equal to the quantification limit. Particularly, bee pollen samples were separately spiked with 0.2 ng/g of AFB1, 1.7 ng/g of OTA, 12.5 ng/g of ZEN, 30 ng/g of DON and 20 ng/g of T-2. Spiked samples were left uncovered and dried for 2 h at room temperature. Mycotoxin stock solutions were acquired from Sigma-Aldrich (St. Louis, MO, USA) for AFB1, OTA, ZEN, DON and T-2. Mycotoxin extraction and ELISA assays were then performed as described below. The results were considered acceptable with mycotoxin recovery rates of 80–120%.

2.2.2.2. Mycotoxins extraction and ELISA screening. Mycotoxin extraction was performed on ground bee pollen samples following the manufacturers' specifications with some minor changes. Extractions for AFB1 were conducted using 3.0 g of ground sample, while 2.0 g of sample were used to extract OTA and 1.0 g for ZEN, DON and T-2. Samples were then mixed with 9 mL ACS grade methanol (Honeywell, Hampton, NH, USA) diluted to 80% with deionized water for AFB1 and with 8 mL methanol/PBS buffer and 2 mL of *n*-hexane (Honeywell, Hampton, NH, USA) for OTA. ZEN extraction was conducted adding 4 mL of 60% methanol with deionized water to the samples; DON extractions were carried out with 19 mL deionized water and T-2 extractions were carried out with 10 mL HPLC grade acetonitrile (Honeywell, Hampton, NH, USA) diluted with deionized water (84:16, v/v). All the extractions were conducted by vortexing the sample – solvent mixtures for 10 min at high speed and then centrifuging at 2000 *g* for 15 min. The supernatant extracts were diluted according to the manufacturer's specifications with the buffer solutions supplied in each kit. Previously, for OTA, *n*-hexane was removed. ELISA was performed on all sample extracts following the manufacturers' specifications. The extract from each sample was tested in duplicate in a 96-well ELISA microplate. Absorbance was measured at 450 nm using a Spark 20M multimode microplate reader (TECAN, Switzerland). The averages of the absorption values of the standard solutions were first calculated, then divided by the absorption value of the standard zero solution and multiplied by 100. Quantification of mycotoxins was obtained by interpolating the values measured for each sample with the calibration curve prepared with the standards provided with the kit. From the resulting equations, the working range, the slope, and the point of intersection with the Y-axis (intercept) were determined. The concentration values were obtained from the calibration curve were multiplied by the corresponding factor depending on the sample dilution. According to the manufacturer, the limit of quantitation (LOQ) of the mycotoxins 'kit is from 0.2 to 30 ng/g. For the calculation of LOQ, 3 blank samples of bee pollen were spiked and analyzed, and then the average of the measured concentration and the standard deviation were calculated. The method-LOQ was set as the minimum concentration that can be quantified with acceptable accuracy and precision. Within-laboratory repeatability and reproducibility were both tested over 1 and 5 days, respectively. For the repeatability and reproducibility tests, one of the bee pollen samples was spiked with standard working solutions of mycotoxins at a concentration equal to the calculated limit of quantification. The sample was analyzed five times, intra-day for repeatability and inter-day for reproducibility, and from the results obtained, the mean concentration, the standard deviation and the % relative standard deviation were calculated. The method precision and recovery were studied via recovery experiments at a concentration equal to the double of LOQ. Within-laboratory reproducibility and the u'(bias) component were used to calculate measurement uncertainty (u'). The u′(bias) component was estimated from the recoveries. Expanded uncertainty (U) was then calculated by multiplying the measurement uncertainty u' by a coverage factor k = 2.

2.3. Exposure assessment (probable daily intake estimates) and risk characterization

2.3.1. Exposure assessment

In the present study, exposure assessment was determined based on the estimated intake through food. Eq. (A1) shows the calculation of Probable Daily Intake (PDI) through food, according to Assunção et al. (2015). Occurrence (mycotoxin content ($\mu g \cdot kg^{-1}$) determined in food analysis) and consumption (EFSA Comprehensive European Food Consumption Database, 2022) data were used to determine PDI. For the risk assessment, different factors were taken into account: occurrence according to the type of processing (fresh bee pollen, dry bee pollen, bee bread); population groups (Infants – up to 11 months of age, Toddlers – from 12 to 35 months of age, Other Children – from 36 months to 9 years of age, Adolescents – from 10 to 17 years of age, Adults – from 18 to 64 years, Lactating Women, Elderly – from 65 to 74 of age, Very Elderly – from 75 years of age and older); gender (Male or Female); type of consumption (Chronic or Acute) and European country in which the consumption survey was realized. For the PDI calculations and risk characterization, the results of samples presenting mycotoxin concentration < LOQ were replaced with zero.

2.3.2. Risk assessment of single mycotoxins

Two different approaches were performed for the risk assessment of individual mycotoxins based on their carcinogenicity. For DON and ZEN, risk characterization was performed comparing PDI values with the reference dose for relevant health endpoints, tolerable daily intake (TDI, Eq. (A2)) or the value of the no observed adverse effect level (NOAEL, Eq. (A3)). TDI value was 1.0 $\mu g\ kg^{-1} \cdot bw \cdot day^{-1}$for DON (FAO/WHO, 2011), and 0.25 $\mu g\ kg^{-1} \cdot bw \cdot day^{-1}$ (EFSA CONTAM Panel, 2016) for ZEN. The uncertainty factor (UF) was set as 100 (10 ×

10), which was interpreted as reflecting extrapolation from experimental animals to human (factor 10 for inter-species variability between man and animal) and extrapolation from an average human NOAEL to a sensitive human NOAEL (factor 10 for human or intra-species variability) (EFSA CONTAM Panel, 2011). NOAEL value was 1.0 μg kg^{-1} · bw · $day^{-1}$for DON (EFSA CONTAM Panel, 2017). For ZEN, it has been determined no NOAEL, which value is 40 μg kg^{-1} · bw · day^{-1} (EFSA CONTAM Panel, 2011). Comparisons were performed using hazard coefficients (HQ), which are the ratio between exposure and a reference dose as referred at Eq. (A2 y A3). When the HQ was <1, the exposure was considered to be within safe limits and a HQ > 1 ratio indicated a non-tolerable exposure level (European Food Safety Authority, 2013).

Regarding the carcinogenic potential of aflatoxins and OTA, no level of exposure is considered safe, and the Margin of Exposure (MoE) was calculated for exposure to these toxins (Eq. (A4)) as a ratio of the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$) and the level of exposure (PDI) and to help prioritize risk management actions. $BMDL_{10}$ values are 0.4 μg kg^{-1} · bw · day^{-1} for aflatoxins and 14.5 μg kg^{-1} · bw · day^{-1} for OTA with neoplastic effects (EFSA CONTAM Panel, 2020a; EFSA CONTAM Panel 2020b). The magnitude of the MoE indicates the risk level, a MOE of 10,000 or more is of low concern for public health, and MoE below 10,000 being of high public health concern (European Food Safety Authority, 2013).

2.3.3. Cumulative risk assessment of co-occurring mycotoxins

Considering the co-occurrence of different mycotoxins, two groups were established with regard to toxicological similarity: Group 1, AFB1 and OTA based on neoplastic effects; Group 2, DON and ZEN based on endocrine activity (testicular toxicity and strong estrogenic potency). The MoE_T was used for Group 1 and the HI method for Group 2. The MoE_T was calculated as the reciprocal of the sum of the reciprocals of the individual MoEs (Eq. (A6)), and it was considered of high concern for public health if $MoE_T < 100$. Hazard index (HI) was calculated as the sum of the respective HQs for the individual mixture components of the same toxicity (Eq. (A6 y A7)). Significant adverse effects were speculated if HI > 1.

2.4. Statistical analysis

Data were statistically analyzed using the software GraphPad Prism version 9.4.1 (681) for Windows (GraphPad Software, San Diego, California USA). For data analysis, only positive samples (mycotoxin concentration above the LOQ) were considered and mycotoxin concentrations below the LOQ were replaced by zero. Extreme concentration values that were very distant from the average obtained for a specific group, were also neglected. The comparison between the mycotoxins' content of groups of samples with different characteristics (Organic/Non-Organic, Mono/Multifloral) was performed by unpaired, parametric, two-tailed T-test with a confidence level of 95%. Multiple comparisons among three or more groups of samples (Climatic zones, type of processing) were carried out by ordinary one-way ANOVA tests. In both cases the statistical significance was defined as P value < 0.05.

Correlation analysis between two selected data sets (e.g., moisture content and mycotoxins concentration) were performed assuming that the data were sampled from a Gaussian distribution, so that a Pearson correlation coefficient ($-1 < r < 1$) could be obtained as a result.

3. Results and discussion

3.1. ELISA verification results for screening of mycotoxins in bee pollen

Mean recoveries for mycotoxins in the spiked samples were within the predetermined acceptable range of 80–120% for bee pollen samples tested with the ELISA kits (Table S1 of Supplementary material). The mean recoveries were 88.79% for the AFB1 kit, 84.46% for the DON kit, 81.26% for the OTA kit, 90.24% for the ZEN kit and 85.72% for the T-2 kit with a coefficient of variation (CV) under 20.0%. The recoveries obtained in this study were consistent with the typical recoveries achieved in previous studies using methods such as ELISA, thin layer chromatography (TLC), and HPLC (Maia & Pereira Bastos de Siqueira, 2002; Scudamore et al., 1997; Urusov et al., 2015). The limit of quantification (LOQ) as determined during spike recovery testing to be from 0.02 ng/g to 20 ng/g. Recovery and repeatability were found that satisfied the level studied for every mycotoxin, making it possible to know the uncertainty values. Reproducibility was between 8 and 15%, and most of the values were around 8–12%. Repeatability was found to be below 19%. Most of the values were around 6–10%. Values of measurement uncertainty (u') for individual mycotoxins were obtained in the range of 15%–21%, while expanded uncertainty (U) values were in the range 31%–43%.

3.2. General mycotoxin occurrence

Of the 80 bee pollen samples tested in this study, 100% had quantifiable levels of mycotoxins above the LOQs determined for each mycotoxin (Table 1). AFB1 had the highest incidence rate (98.75%), followed by DON (66.25%), ZEN (55%) and OTA (28.75%). T-2 mycotoxin was not detected in any of the samples. The estimated level of AFB1 in bee pollen ranged between 1.1 and 5.3 ng/g, based on ELISA testing, and in 71,25% of the analyzed samples its concentration overcame the maximum levels suggested by Campos et al. (2008). DON was estimated to be present in the range of 33–135 ng/g and ZEN was quantified from 16 to 622 ng/g. The levels of OTA detected in this study were between 2 and 7.8 ng/g.

Co-contamination of mycotoxins was frequently observed with a rate of 87.5%. Within this percentage, co-occurrence was mostly represented by three major mycotoxins, AFB1, DON, ZEN, and AFB1, DON, OTA, detected in some single samples, with an incidence of 32.5% and 23.75%, respectively.

The only sample in the current study with detectable levels of four mycotoxins was a dry bee pollen sample containing AFB1 (5.2 ng/g), DON (42 ng/g), OTA (2 ng/g) and ZEN (72 ng/g). Considering the similarity of the toxicological properties for each individual mycotoxins, that is, between carcinogens and non-carcinogens, the co-occurrence of the carcinogenic mycotoxins AFB1 and OTA was found in 28.75% of the samples, while the co-occurrence of non-carcinogenic mycotoxins DON and ZEN was detected in 33.75%.

3.2.1. Occurrence in different climatic areas

The analyzed bee pollen samples were purchased from 28 different countries belonging to the main climate areas of the Köppen classification (A: tropical, B: arid, C: temperate, D: continental). Knowing the exact origin of every sample it was possible to attribute the collected bee pollens to more specific climatic subareas, as it can be seen in Table 1. Just one bee pollen (1.25%) could be purchased from a tropical climatic area, while the rest of the samples were distributed in the following way: 7.5% coming from an arid area, 76.25% coming from a temperate climatic region and 15% coming from a continental area. The only available sample from the A area was characterized by the co-occurrence of three different mycotoxins: AFB1, OTA, ZEN. Bee pollens purchased from arid climatic regions were all characterized by the presence of AFB1 and ZEN (100%), while DON was found in 66.6% of them, and OTA in none of them. In samples coming from C area, AFB1 had the highest occurrence (98.4%), followed by DON (68.8%), ZEN (44.3%), and OTA (34.4%). All bee pollens coming from continental climatic regions contained AFB1 (100%); ZEN was found in 83.3% of them, DON in 58.3%, while OTA just in one sample.

From the multiple comparisons carried out among samples coming from different climatic areas and sub-areas, it was not possible to observe any significant difference in terms of single and total mycotoxins concentration.

Although it is known that climatic conditions can have an important

Table 1
Concentration of mycotoxins (AFB1, DON, OTA, ZEN, T2) detected in bee pollen samples.

Sample	Climatic area	Production	Harvesting	Processing	AFB1 ng/g	DON ng/g	OTA ng/g	ZEN ng/g	T2 ng/g	Sum ng/g
1	Cfa	NO	MULTI	DP	2.3	33	ND	ND	ND	35.4
2	Dfb	NO	MULTI	DP	3.1	42	ND	ND	ND	45.1
3	Csb	NO	MULTI	DP	1.8	ND	2.8	ND	ND	4.6
4	Cfb	O	MULTI	DP	3.1	38	ND	ND	ND	40.8
5	Csa	NO	MULTI	DP	1.8	64	2.4	ND	ND	68.4
6	Csb	NO	MONO	DP	3.6	66	ND	ND	ND	69.5
7	Csa	NO	MULTI	DP	2.5	65	2.6	ND	ND	70.6
8	Csb	NO	MONO	DP	2.3	88	2.4	ND	ND	93.1
9	Csa	NO	MULTI	DP	1.9	39	2.2	ND	ND	43.0
10	Cfb	O	MULTI	DP	2.9	37	3.9	ND	ND	43.3
11	Cfb	O	MULTI	DP	3.2	57	2.1	ND	ND	62.6
12	Csb	NO	MULTI	DP	3.1	46	2.8	ND	ND	51.5
13	Csa	NO	MULTI	DP	1.9	66	2.1	ND	ND	69.6
14	Csb	NO	MULTI	DP	2.2	84	2.8	ND	ND	89.0
15	Csa	NO	MULTI	DP	1.9	75	2.8	ND	ND	79.9
16	Csb	O	MULTI	DP	2.3	41	2.7	ND	ND	46.1
17	Csb	O	MULTI	DP	3.2	88	3.5	ND	ND	94.9
18	Cfb	O	MULTI	DP	2.8	106	2.8	ND	ND	111.9
19	Cfa	NO	MULTI	DP	2.7	58	ND	ND	ND	60.6
20	Cfa	O	MULTI	DP	3.0	93	ND	ND	ND	95.6
21	Dfc	NO	MULTI	DP	2.2	49	3.0	ND	ND	54.4
22	Csa	NO	MULTI	DP	2.3	135	2.6	ND	ND	139.6
23	Csb	O	MONO	DP	2.2	75	2.5	ND	ND	79.3
24	Dfd	NO	MULTI	BB	3.4	ND	ND	44	ND	47.3
25	Dfc	NO	MULTI	BB	2.9	ND	ND	19	ND	21.7
26	Csa	NO	MULTI	DP	1.9	37	2.6	ND	ND	41.5
27	Csa	O	MULTI	DP	1.7	43	2.4	ND	ND	47.1
28	Csa	NO	MULTI	FP	3.3	ND	ND	ND	ND	3.3
29	Cfa	O	MULTI	DP	3.0	ND	ND	ND	ND	3.0
30	Cfb	NO	MULTI	FP	2.9	ND	ND	58	ND	60.8
31	Cfb	NO	MONO	FP	5.3	ND	ND	ND	ND	5.3
32	Cfb	NO	MONO	FP	2.1	ND	ND	ND	ND	2.1
33	Cfb	NO	MONO	FP	2.9	ND	ND	ND	ND	2.9
34	Cfb	NO	MONO	FP	4.6	ND	ND	ND	ND	4.6
35	Cfb	NO	MONO	FP	1.9	ND	ND	ND	ND	1.9
36	Cfb	NO	MONO	FP	4.2	ND	ND	37	ND	41.4
37	Cfb	NO	MONO	FP	2.9	ND	ND	ND	ND	2.9
38	Cfb	NO	MONO	FP	1.1	ND	ND	ND	ND	1.1
39	Csa	O	MULTI	DP	1.1	ND	ND	ND	ND	1.1
40	Cfb	O	MULTI	DP	1.9	57	ND	ND	ND	59.1
41	Cfa	O	MULTI	DP	ND	45	ND	50	ND	95.2
42	Bsk	NO	MULTI	DP	2.4	ND	ND	27	ND	29.1
43	Cfa	NO	MULTI	DP	2.1	ND	ND	63	ND	64.8
44	Cfb	NO	MULTI	DP	4.1	ND	7.8	622	ND	634.2
45	Cfb	NO	MULTI	DP	1.7	ND	ND	19	ND	20.3
46	Cfa	NO	MULTI	DP	1.9	ND	ND	66	ND	67.5
47	Cfb	O	MULTI	DP	1.2	ND	ND	54	ND	55.0
48	Cfb	NO	MULTI	DP	1.8	36	ND	18	ND	55.1
49	Cfb	NO	MULTI	DP	2.2	44	ND	55	ND	100.6
50	Dfb	NO	MULTI	DP	2.4	ND	ND	45	ND	47.6
51	Csa	NO	MULTI	FP	5.1	ND	4.7	242	ND	251.3
52	Cfb	NO	MULTI	DP	2.8	40	ND	47	ND	89.3
53	Dfb	NO	MULTI	DP	2.1	ND	ND	112	ND	114.5
54	Csb	NO	MULTI	DP	5.2	42	2.0	72	ND	120.9
55	Cfb	NO	MULTI	DP	1.7	67	ND	17	ND	86.5
56	Dfb	NO	MULTI	DP	1.4	59	ND	20	ND	80.5
57	Cfb	NO	MULTI	DP	1.7	63	ND	67	ND	131.1
58	Csa	NO	MULTI	DP	1.5	81	ND	59	ND	141.3
59	Dfb	NO	MULTI	BB	3.1	58	ND	101	ND	161.9
60	Dfb	NO	MULTI	BB	3.4	73	ND	70	ND	146.0
61	Cfb	NO	MULTI	BB	2.6	71	ND	120	ND	193.5
62	Bwk	NO	MULTI	BB	2.3	45	ND	92	ND	139.6
63	Bwk	NO	MULTI	DP	2.4	73	ND	25	ND	100.4
64	Cfb	O	MULTI	DP	1.4	50	ND	16	ND	66.8
65	Dfb	NO	MULTI	DP	4.0	90	ND	28	ND	122.1
66	Dfb	NO	MULTI	BB	3.5	ND	ND	85	ND	88.4
67	Cfa	NO	MULTI	DP	4.4	36	ND	39	ND	79.6
68	Bsk	NO	MULTI	DP	2.4	39	ND	69	ND	110.1
69	Bwh	NO	MULTI	DP	2.5	ND	ND	43	ND	45.8
70	Csa	NO	MULTI	DP	2.7	55	ND	48	ND	105.6
71	Aw	NO	MULTI	DP	5.1	ND	3.1	112	ND	120.0
72	Dfb	NO	MULTI	DP	2.7	47	ND	50	ND	100.2
73	Bwh	NO	MULTI	DP	1.9	46	ND	32	ND	79.8
74	Csb	O	MULTI	DP	1.3	65	ND	29	ND	95.3

(*continued on next page*)

Table 1 (*continued*)

Sample	Climatic area	Production	Harvesting	Processing	AFB1 ng/g	DON ng/g	OTA ng/g	ZEN ng/g	T2 ng/g	Sum ng/g
75	Csa	O	MULTI	DP	4.0	61	ND	25	ND	90.1
76	Csb	O	MULTI	DP	2.2	77	ND	47	ND	126.3
77	Csa	NO	MULTI	DP	3.7	42	ND	38	ND	83.8
78	Cfb	NO	MULTI	DP	2.2	66	ND	30	ND	98.5
79	Csa	NO	MULTI	DP	2.2	72	ND	25	ND	99.5
80	Csb	NO	MULTI	DP	2.7	51	ND	51	ND	105.0

Climatic area: Aw = tropical savanna; Bsk = arid, cold steppe; Bwh = arid, hot desert; Bwk = arid, cold desert; Cfa = temperate, hot summer, without dry season; Cfb = temperate, warm summer, without dry season; Csa = temperate, hot and dry summer; Csb = temperate, warm and dry summer; Dfb = continental, warm summer, without dry season; Dfc = continental, cold summer, without dry season; Dfd = continental, very cold winter, without dry season.
Production: Organic (O) - Non-Organic (NO).
Harvesting: Monofloral (MONO) – Multifloral (MULTI).
Processing: Bee Bread (BB) – Dry Pollen (DP) – Fresh Pollen (FP).
ND = Not Detected.

impact on the presence of mycotoxins in food and natural products, making accurate predictions and distinctions about their distribution in different areas of the world can be challenging. Due to climatic change, in fact, fungus species that were predominantly found in some climatic regions (e.g., *Aspergillus* spp. in tropical and subtropical regions) are now starting to proliferate in new areas where favorable climatic conditions are occurring, increasing the risk of mycotoxins contamination (Moretti et al., 2019). These new circumstances, together with the other conditions that can occur during the product processing, make it hard to establish any link between geographical origin and the presence of mycotoxins. The fact that mycotoxins were detected in samples coming from each one of the considered climatic regions and that no significant difference among them was revealed is a proof of that but also a cause of concern.

3.2.2. Occurrence in different types of production

Among the 72 brands, 75% produce non-organic pollen and 25% organic. In organic pollen, AFB1 had the highest incidence rate (94.4%), followed by DON (83.3%), OTA (38.8%), and ZEN (33.3%). Co-occurrence of AFB1 and OTA was observed in 38.8%, while DON and ZEN in 27.7%. In non-organic pollen, AFB1 had the highest incidence rate (100%), followed by DON, ZEN (61.3%), and OTA (25.8%). Co-occurrence of AFB1 and OTA was observed in 25.8%, and DON and ZEN in 37% of the samples. No significant differences in terms of single and total mycotoxins' content were found by comparing the two different types of production. Although there are currently no data available on the presence of mycotoxins in organic beekeeping, several studies have been conducted on the occurrence of these toxic compounds in organic produced food. What emerged from these studies is consistent with what could be inferred from the analyzed bee pollen samples. In fact, mycotoxins, as well as other contaminants, could be detected in several organic food items, showing that an organic production does not necessarily lead to healthier products (Gonzalez et al., 2019).

3.2.3. Occurrence in different harvesting types: mono and multifloral pollen composition

Among the 72 brands, the majority (95.8%) provides multifloral pollen and very few (5.5%), monofloral pollen, particularly chestnut, blackberry, broom, hawthorn, rockrose, poplar, dandelion, apple tree, plum tree, and willow. In monofloral pollen, AFB1 was detected in all samples (100%), followed by DON (27.2%), OTA (18.8%) and ZEN, which was detected in one sample. Co-occurrence of AFB1 and OTA was observed in 18.8% of samples. In multifloral pollen, AFB1 was detected in 98.5% of the samples, followed by DON (72.4%), ZEN (62.3%), and OTA (30.4%). Co-occurrence of AFB1 and OTA was observed in 30.4%, while DON and ZEN in 40.57%. The comparison between mono and multifloral samples revealed that the total mycotoxins' concentration was significantly higher in the analyzed multifloral samples than in the available monofloral ones (Fig. 1).

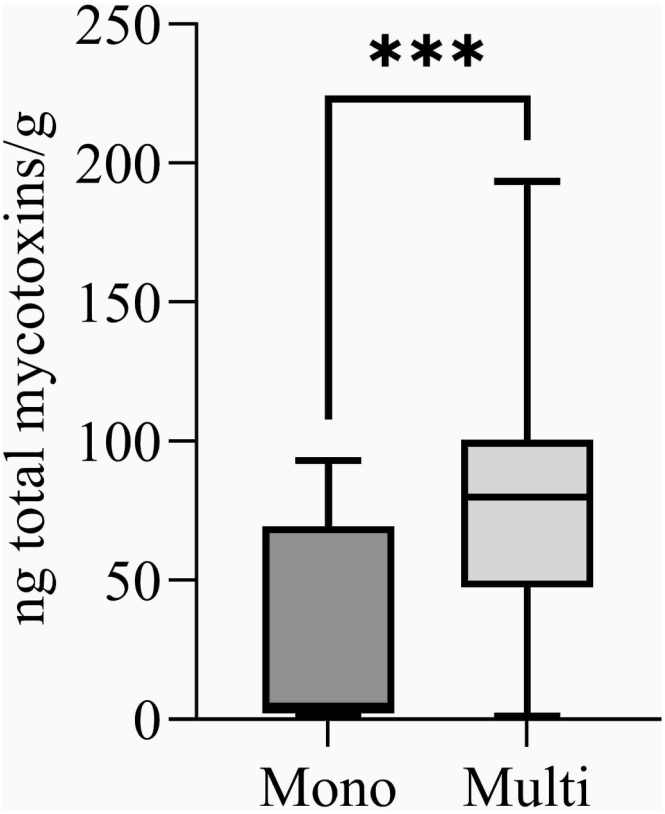

Fig. 1. Graphical T-test results for total mycotoxins in mono and multifloral pollen.

3.2.4. Occurrence in different processing

Among the 72 brands, 86.1% provide dry pollen, 9.7% bee bread and 4.16% fresh pollen. In bee bread, the moisture content is below 6%, ranging between 0.18 and 4.12%. AFB1 and ZEN were detected in all the samples, in the ranges of 2.3–3.5 ng/g and 19–120 ng/g, respectively followed by DON (57.14%) in the range of 45–73 ng/g. In fresh pollen, the moisture content ranges between 9.36 and 19.6%. AFB1 was detected in all the samples, in the range of 1.1–5.3 ng/g. OTA and ZEN were only detected once in two different samples at 4,7 and 37 ng/g respectively. In dry pollen, the moisture content ranges between 1.18 and 15.02%. In 90.3% of the samples was lower than 6% and in 9.67%, was from 6.06 to 15.02%. AFB1 was detected in all dry samples except one in a range of 1.1–45 ng/g, while DON was found in 87% of samples in the range of 33–135 ng/g. OTA and ZEN were respectively detected in 31.9% and 59.4% of dry bee pollen samples in the ranges of 2.0–7.8 ng/g and 16–622 ng/g. Statistical analysis revealed that the concentration of

AFB1 was significantly higher in fresh bee pollen than in dry bee pollen, while ZEN resulted more abundant in bee bread than in dry bee pollen (Fig. 2a and b). From the comparison of the total mycotoxins content found in the three types of processing, it appeared that fresh bee pollen had a significantly lower total mycotoxins' concentration than the other two groups, while bee bread showed a notably higher content of total mycotoxins than both dry and fresh bee pollen (Fig. 3). The analysis of the relationship between samples' moisture content and their total concentration of mycotoxins revealed that the two variables were characterized by a negative correlation ($r = -0.4714$). However, the same statistical analysis separately performed for bee bread, fresh bee pollen, and dry bee pollen samples showed that in all three types of processing there was a positive correlation between moisture content and total mycotoxin concentration ($r = 0.4185$ for BB, 0.2948 for FP, 0.07198 for DP). The obtained results showed that the production of mycotoxins by molds is indeed linked with the humidity of the surrounding environment, but that there are also other factors that must be considered like, for instance, temperature. Most of molds proliferate at a temperature range between 25 and 30 °C (Daou et al., 2021), so the low total mycotoxins' concentration found in fresh bee pollen could be due to the storage conditions that were chosen for this processing type (−20 °C). On the other hand, bee bread and dry bee pollen were stored at room temperature, as recommended by the manufacturers' instructions, probably favoring in this way the growth of molds and the production of mycotoxins. Analyzing separately the samples subjected to the same storage and temperature conditions, it was possible to observe a positive correlation between moisture percentage and mycotoxins content, as it was also previously reported by Petrovic et al. (2014).

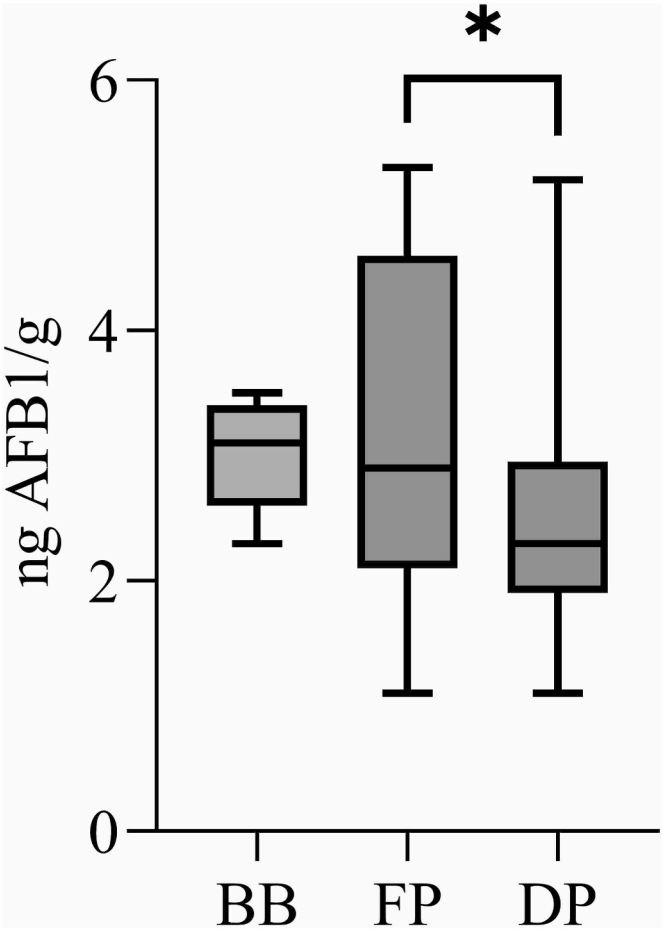

Fig. 2. aGraphical one-way ANOVA results for AFB1 in bee bread, fresh pollen, and dry pollen.

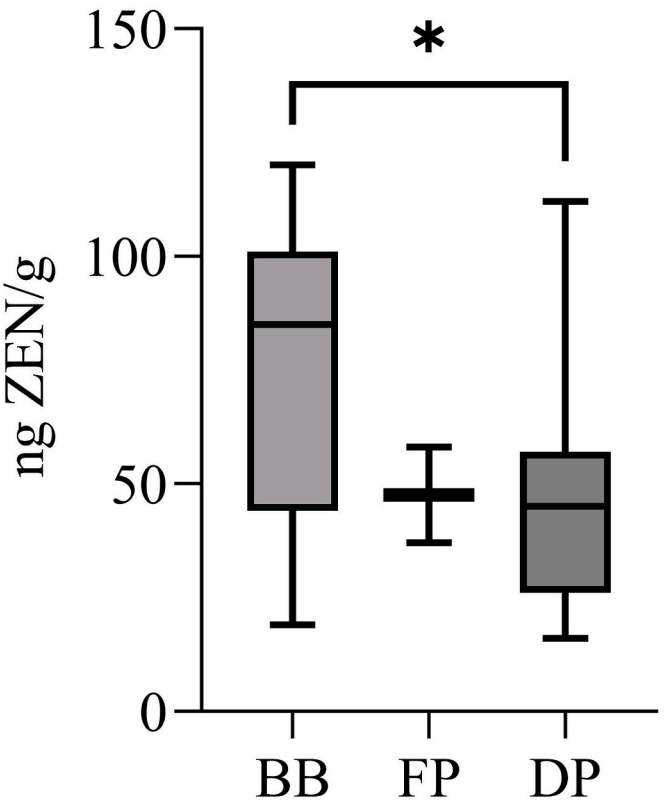

Fig. 2b. Graphical one-way ANOVA results for ZEN in bee bread, fresh pollen, and dry pollen.

3.3. Risk assessment mycotoxins

The risk associated with the consumption of bee pollen due to its content of mycotoxins was separately calculated for the three different types of processing considered in this study: dry bee pollen, fresh bee pollen and bee bread (Table S2, S3 and S4 of Supplementary material). For each subgroup the average content of each mycotoxin was calculated excluding eventual extreme values.

The single and cumulative risk assessment was then carried out considering the consumption parameters obtained from the EFSA Comprehensive European food consumption database, and the carcinogenic potential of the analyzed mycotoxins.

3.3.1. Single mycotoxins

The risk assessment related to the presence of single non-carcinogenic mycotoxins (DON and ZEN) in the analyzed samples showed very similar results in dry/fresh bee pollen and bee bread.

For the three types of differently processed samples, the HQ_{NOAEL} calculated for DON exceeded the safe limit in about 28% of the analyzed cases. The interested countries and population groups are the ones who consume higher amounts of bee-derived formulations in comparison with others. Particularly at potential risk are adolescents from Cyprus; adults, elderly and very elderly from France; other children from Hungary and Italy; elderly and very elderly from Italy; toddlers and other children from Latvia; adolescents from Netherlands; toddlers from Portugal and Slovenia. In 61% of the mentioned cases, the exposition to dangerous levels of DON regards females since they consume higher amounts of bee derived supplements, according to the conducted surveys. Examples of both chronic and acute consumption of bee-based supplements were found among the cases overstepping the safe limit of exposition to DON.

On the other hand, ZEN concentration was averagely not worrying in any of the analyzed cases and types of processing, considering both

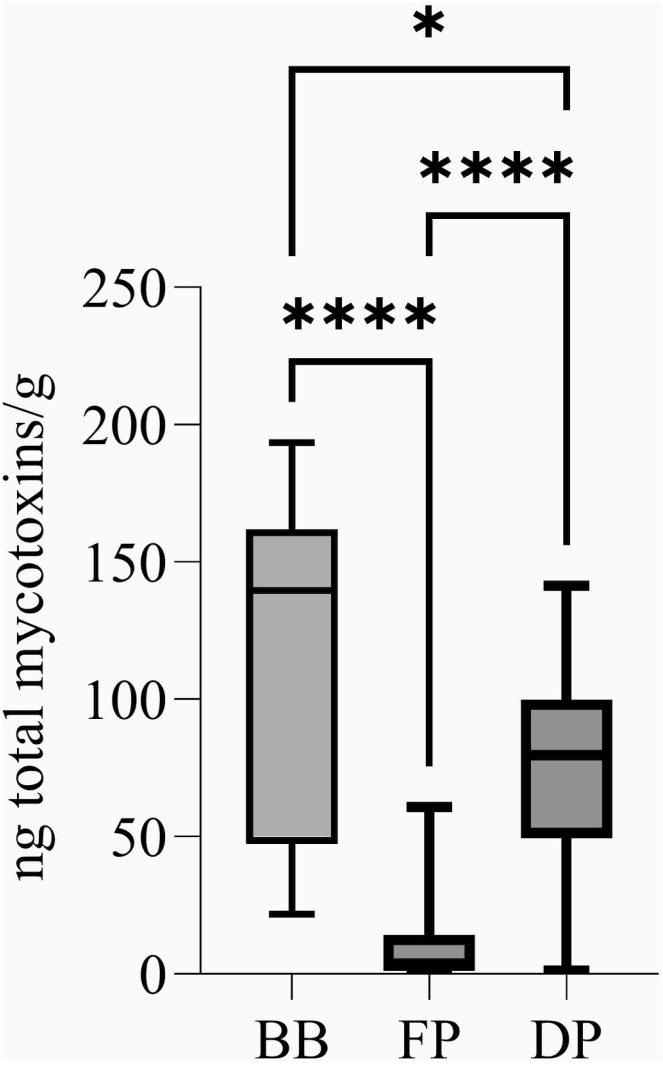

Fig. 3. Graphical one-way ANOVA results for total mycotoxins in bee bread, fresh pollen, and dry pollen.

HQ_{TDI} and HQ_{NOAEL}.

Regarding the analyzed carcinogenic mycotoxins, in fresh/dry bee pollen and bee bread, the MOE calculated for AFB1 resulted of high public health concern in 84% of the considered cases. Due to the generally high concentration of AFB1 in the analyzed samples, the only cases of low public health concern were those characterized by a negligible consumption of bee-based supplements (0.01 g/kg · bw per day).

In bee bread samples, no MOE level could be calculated for OTA, since it was never detected in this matrix, while for fresh and dry bee pollen, 7% of the analyzed cases could be considered of high public health concern. Among the considered groups of consumers, the ones more exposed to OTA are adolescents from Cyprus, elderly from France, other children from Hungary, and toddlers from Slovenia. Once again, females represent the great majority (80%) of the categories at risk, generally because of episodes of acute bee-based supplements consumption.

3.3.2. Cumulative risk assessment of co-occurring mycotoxins

Cumulative risk assessment was carried out for mycotoxins possessing comparable toxic properties (DON and ZEN, AFB1 and OTA).

Regarding DON and ZEN, the HI_{NOAEL} exceeded the safe limit in around 28% of cases in bee bread and fresh/dry bee pollen, with the highest contribution being given by DON.

In fact, the countries, and the population groups more at potential risk for the combined exposition to DON and ZEN, due to their consumption of bee-based supplements, were the same that were individuated for DON. The only new introduction among the categories at risk was represented by the very elderly from Portugal when consuming bee bread, because of its high average content of ZEN which consistently contributed to the cumulative HI_{NOAEL}.

The MOE_T for AFB1 and OTA could not be calculated for bee bread (since OTA was not detected in it), while the content of these two carcinogenic mycotoxins in dry and fresh bee pollen could be considered of high public health concern in none of the analyzed cases.

4. Conclusions

AFB1 had the highest incidence rate (98.75%), followed by DON (66.25%), ZEN (55%) and OTA (28.75%). T-2 mycotoxin was not detected in any of the samples. Co-occurrence of the carcinogenic mycotoxins AFB1 and OTA was found in 28.75% of the samples, while the co-occurrence of non-carcinogenic mycotoxins DON and ZEN was detected in 33.75%. From the multiple comparisons carried out among samples coming from different climatic areas and sub-areas, it was not possible to observe any significant difference in terms of single and total mycotoxins concentration. No significant differences in terms of single and total mycotoxins' content were found by comparing organic and conventional production. The comparison between mono and multifloral samples revealed that the total mycotoxins' concentration was significantly higher in the analyzed multifloral samples than in monofloral ones. In the three types of processing, a positive correlation between moisture content and total mycotoxin concentration was found. For the three types of differently processed samples, the HQ_{NOAEL} calculated for DON exceeded the safe limit in about 28% of the analyzed cases. In fresh/dry bee pollen and bee bread, the MOE calculated for AFB1 resulted of high public health concern in 84% of the considered cases. Regarding cumulative risk of co-occurring DON and ZEN, the HI_{NOAEL} exceeded the safe limit in around 28% of cases in bee bread and fresh/dry bee pollen, with the highest contribution being given by DON. The MOET for AFB1 and OTA could not be calculated for bee bread (since OTA was not detected in it), while the content of these two carcinogenic mycotoxins in dry and fresh bee pollen could be considered of high public health concern in none of the analyzed cases.

Disclaimer

Samples collection and data analysis were performed following the principle of statistical ethics. Samples were chosen in order to obtain the greatest amount and variety of samples possible, based on the availability of bee pollen in herbal stores, grocery stores and online shops. The authors had absolutely no intention of making a selection that could undermine specific countries or companies. More samples than the ones included in this study were selected to be purchased and analyzed, but most of them were never delivered.

CRediT authorship contribution statement

Maria Antonietta Carrera: Investigation, Formal analysis, Validation, Writing – original draft. **Esther Miguel:** Investigation. **Amadeo R. Fernández-Alba:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing. **María Dolores Hernando:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.109816.

Appendix A. Equations for risk assessment

$$PDI = Occurrence * Consumption/bw * 1000 \quad \text{(A1)}$$

$$HQ_{TDI} = PDI/TDI \quad \text{(A2)}$$

$$HQ_{NOAEL} = PDI/NOAEL \times UF \quad \text{(A3)}$$

$$MoE = BMDL_{10}/PDI \quad \text{(A4)}$$

$$MoE_T = \frac{1}{\left(\frac{1}{MOE_{AFB1}} + \frac{1}{MOE_{OTA}}\right)} \quad \text{(A5)}$$

$$HI = HQ_{TDI,ZEN} + HQ_{TDI,DON} \quad \text{(A6)}$$

$$HI = HQ_{NOAEL,ZEN} + HQ_{NOAEL,DON} \quad \text{(A7)}$$

bw = body weight (kg) reported by volunteers.

$BMDL_{10}$ = Benchmark Dose Lower Confidence Limit ($\mu g \cdot kg^{-1} \cdot bw \cdot day^{-1}$)
Consumption = reported consumption (g) of food on the previous day
HI = Hazard Index
HQ = Hazard Quotient
MoE = Margin of Exposure
NOAEL = No Observed Adverse Effect Level ($\mu g \cdot kg^{-1} \cdot bw \cdot day^{-1}$)
Occurrence = mycotoxin content ($\mu g \cdot kg^{-1}$) determined in food analysis
PDI = Probable Daily Intake ($\mu g \cdot kg^{-1} \cdot bw \cdot day^{-1}$)
TDI = Tolerable Daily Intake ($\mu g \cdot kg^{-1} \cdot bw \cdot day^{-1}$)
UF = Uncertainty Factor

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