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Research Paper

Mycotoxicological and palynological profiles of commercial brands of dried bee pollen

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Abstract

Pollen is used in the human diet as a food supplement because of its high nutritional value; however, this product is prone to fungal contamination that could potentially generate toxins that are harmful to human health. This study aimed to verify the floral diversity of commercial brands of bee pollen and their mycotoxicological safety for human consumption. A total of 27 bee pollen samples were analyzed; these samples represented commercial brands, either showing an inspection seal or not, marketed in the State of Rio de Janeiro. The analyzed parameters included floral diversity through palynological analysis, water activity, fungal counts, identification and toxigenic profiles. The palynological analysis identified nine plant families, of which the Asteraceae was predominant. Analysis of hygienic quality based on fungal load showed that 92% of samples were reproved according to the commercial, sanitary, and food safety quality indicators. *Aspergillus*, *Cladosporium* and *Penicillium* were the most common genera. Toxigenic evaluation showed that 25% of the *A. flavus* strains produced aflatoxins. The high rate of contamination of products bearing an inspection seal emphasizes the need to monitor the entire procedure of bee pollen production, as well as to revise the current legislation to ensure safe commercialization of this product.

Key words: bee products, fungi, mycotoxins, pollen loads, public health.

Introduction

Honeybees (*Apis mellifera*) forage pollen grains of specific floral species, resulting in highly agglutinated pollen loads attached to their hind legs. The bees initiate the preparation of food for the beehive by adding nectar and salivary substances to the pollen. Part of these pollen loads is collected by the beekeeper before the bees enter the beehive and is treated as bee pollen (Lengler, 2002) known as pollinic "mix." The color of the pollen grains is highly variable and reflects the diversity of the botanical species visited by the bees.

The use of bee pollen is common in the human diet. Apitherapy is the field of apicultural science that involves the treatment of diseases (Olegário *et al.*, 2008) either through the significant content in substances of high biological value as proteins, carbohydrates, minerals, and vitamins (Abreu, 1992; Johnson and Nicolson, 2001; Carpes, 2008) or high levels of polyphenolic substances, primarily flavonoids with powerful antioxidant (Campos *et al.*, 2003) and antimicrobial activity (Basim *et al.*, 2006; García *et al.*, 2001). Ishikawa *et al.* (2008) and Kafadar *et al.* (2012) demonstrated that pollen cures certain diseases. This profile has placed pollen in the list of top consumer items

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known for its functional and nutritional properties that offer health benefits (Kroyer and Hegedus, 2001; González *et al.*, 2005).

Food supplements such as pollen are rich in proteins, lipids, and sugar and are thus also susceptible to rapid degradation, which in turn increases the risk for food poisoning due to fungal growth. In general, fungal contamination of food involves the presence of mycotoxins, which are products of fungal secondary metabolism (Fao, 1990). Mycotoxins are carcinogenic, mutagenic, teratogenic, nephrotoxigenic, and immunosuppressive in humans and other animals, and are responsible for global food losses amounting to billions of dollars (Kuiper-Goodman and Scott, 1996).

Dehydration is a common step in bee pollen processing that ensures quality; it prevents the growth of fungi and bacteria and improves its safety and shelf life (Brasil, 2001a). This procedure is feasible when contamination factors associated with the place of origin and hive management do not lead to high levels of contamination, which are conditions that are not always met (Barreto *et al.*, 2006).

In Brazil, the consumer demand for bee pollen is high, which contributes to an increase in apicultural production and improves of the quality of life of farmers. On the other hand, the incidence of food contamination has increased in the fields to such a degree that Brazil is currently recognized as the highest consumer of agrochemicals. Because pollen is highly predisposed to environmental pollutants (Kalbande *et al.*, 2008), it represents a major barrier in the apicultural chain development at the national marketing level.

In the quest to improve the quality of bee pollen production, the present study aimed to verify the floral diversity and microbiological safety of commercial brands of bee pollen for human consumption.

Materials and Methods

A total of 27 samples of dried bee pollen marketed in major commercial establishments in the State of Rio de Janeiro were acquired. Two brands were from the State of São Paulo, three brands from Minas Gerais, one brand from Paraná, and the remainder were from the State of Rio de Janeiro. Most brands featured an inspection seal; 10 of these showed a State seal, whereas 12 showed a Federal seal. Five samples were collected as informal products (products marketed without any kind of inspection).

Pollen analysis of the samples were performed in the Laboratory of Morphology and Viral Morphogenesis at the Oswaldo Cruz Institute, Fiocruz (Rio de Janeiro/Brazil). Two grams of dried pollen were homogenized in 70% alcohol, subjected to ultrasonic bath for 5 min, and centrifuged. These samples were washed in water and water-glycerin, mounted on slides with gelatin-glycerin in duplicate, sealed with paraffin, and used for qualitative evaluation.

Mycological and mycotoxicological analyses were performed at the Núcleo de Pesquisas Micológicas e Micotoxicológicas of the Universidade Federal Rural do Rio de Janeiro. Initially, water activity (A_w) was determined using the AquaLab® equipment (CX 2 model, Decagon Devices Inc., USA). Samples showing water activity of < 0.60 were considered stable and classified as dehydrated (Labuza, 1980).

Mycobiota determination was performed by counting the number of filamentous fungi in colony-forming units occurring per gram of food (cfu g^{-1}) after serial dilution plating (Pitt and Hocking, 1997). For each sample, 1 mL aliquots of each of the four dilutions were inoculated (in triplicate) in three culture media: dicloran bengal rose chloramphenicol (DBRC) agar, which is a general purpose medium used to grow fungi isolated from foods (Pitt and Hocking, 1997); dichloran 18% glycerol agar (DG18), a selective medium for xerophilic species such as the genera *Aspergillus* and *Penicillium* (Pitt and Hocking, 1997); and a peptone base agar and containing 0.1% pentachloronitrobenzene (PCNB), a selective medium for the genus *Fusarium* (NELSON *et al.*, 1983). The plates were incubated at 25 °C for 5 to 7 days under controlled temperature. All plates were observed daily, and those containing between 10 and 100 cfu g^{-1} were selected for counting. The limit of 100 cfu g^{-1} used in the determination of hygienic quality in honey (Brasil, 1997) and royal jelly (Brasil, 2001a) was adopted to assess the degree of contamination of the samples. This value was based on the fact that these products are consumed *in natura* and in small portions. The resolution RDC No. 12 revoked the ordinance on microbiological criteria (Brasil, 2001b), which are considered mandatory parameters for the evaluation of bee pollen.

Fungal colonies of *Aspergillus* and *Penicillium* were subcultivated in slanted tubes containing malt extract agar (MEA), whereas *Fusarium* colonies were cultivated in banana leaf agar (BLA). The identification of *Aspergillus* sp. was conducted according to Klich (2002), that for *Penicillium* sp. was based on Pitt (1988), and that for *Fusarium* sp. was according to Nelson *et al.* (1983). To characterize the toxigenic profile, strains of *Aspergillus* sections Flavi and Nigri were analyzed according to Geisen (1996) and Bragulat *et al.* (2001), respectively.

Shelf life evaluation was only performed on samples bearing an inspection seal using the sell-by date and date of purchase information.

Results

Most of the samples of the pollen products that showed an inspection seal did not present any changes in color and odor, and no fungi were observed at the time of their receipt in the laboratory. Only one sample (without an inspection seal) showed caramelized pollen with an intense brown color.

Analysis of the floral sources of the samples revealed 12 pollen types belonging to nine plant families (Table 1); six types belonged to the predominant class of pollen. The Asteraceae type was the predominant class (26%), which was observed in 13 out of the 27 samples. On the other hand, the Amaranthaceae, Apiaceae, and Solanaceae types were observed as isolated pollen. In terms of floral diversity, 65% of the products showed only one floral source (in the class of predominant pollen), whereas the remaining presented a composition originating from two to four pollen types (in the class of accessory pollen). Samples from the State of São Paulo were mainly of the *Cecropia* (embaúba) and *Cocos nucifera* (coqueiro) pollen types, those from Minas Gerais consisted of *Brassica* and *Mimosa*, whereas those from Rio de Janeiro were of *Eucalyptus*, *Brassica*, *Mimosa*, *Cocos nucifera* and the pollen types of *Myrcia* and Asteraceae, and Paraná from *Mimosa* and the Asteraceae type. Close to 50% of the samples were derived from crops, and most of the pollen types were represented in small amounts, comprising eight types of accessory pollen and nine types of isolated pollen.

The fungal contamination load in the culture media was variable, at a level of 5.0×10^2 cfu g^{-1} in the DBRC media, 2.0×10^2 cfu g^{-1} in the DG18 media, and 1.0×10^2 cfu g^{-1} in the PCNB media. The values obtained in the DBRC and DG18 media did not meet the legal standards described in the ordinance SVS/MS No. 451 (Brasil, 1997). The brands that failed represented 89% of the samples with a state seal (SIE), 92% with a federal seal (SIF), and 100% of the informal products (without an inspection seal). The inspection seals showed a low variation in fungal contamination.

The water activity of the bee pollen samples was low, with a median value of 0.404 and a mean of 0.393 (0.056; the minimum and maximum values were 0.287 and 0.489, respectively. Although the development of most microorganisms responsible for food spoilage is inhibited by $A_w < 0.90$, the high fungal contamination observed in the bee pollen samples indicated that contamination occurs even in low humidity conditions.

Because most of the products included in the study had a shelf life of 2 years, contamination was verified for all brands (Table 2), including one sample with a shelf life of 3 years.

A high diversity of the mycobiota was observed in the bee pollen samples. Seventy-seven strains belonging to 10 filamentous genera were isolated. *Aspergillus* was the predominant (85%) isolated fungal species, followed by *Cladosporium* (63%), *Penicillium* (41%), *Alternaria* (19%), *Wallemia* and *Eurotium* (11%), *Mucor* (7%), *Curvularia*, *Paecilomyces* and *Fusarium* (4%).

Among the *Aspergillus* isolates, the highest relative density was observed in *A. niger* aggregates (39%), followed by *A. flavus* (21%), *A. fumigatus* (18%), *A. versicolor* (11%); *A. ochraceus*, *A. carbonarius*, *A. terreus* and *A. oryzae* (3%) were also detected. For the genus *Penicillium*, *P. citrinum* and *P. citreonigrum* were the predominant species (50% and 33%, respectively); *P. glabrum* and *P. oxalicum* (8%) were also detected. *Fusarium camptoceras* was the only representative of the genus *Fusarium*.

The toxigenic profiles were negative for *A. niger* aggregates, *A. ochraceus*, and *A. carbonarius* strains, which are potentially producers of ochratoxin A. Twenty-five percent of the *A. flavus* strains were positive for the production

Table 1 - Frequency of pollen types present in dehydrated apiarian pollen according to sample content and plant family.

Pollen types	Total frequency[1]	Frequency of pollen types[2]		
		Predominantly	Accessory	Isolated
Asteraceae	26% (13)	23%	46%	31%
Amaranthaceae -*Amaranthus*	4% (2)	0%	0%	100%
Apiaceae	4% (2)	0%	0%	100%
Caesalpiniaceae -*Caesalpinia* type	4% (2)	0%	100%	0%
Mimosaceae - *Mimosa caesalpiniaefolia*	4% (2)	0%	50%	50%
Mimosa scabrella	6% (3)	33%	33%	33%
Palmae - *Cocos nucifera*	14% (7)	26%	57%	17%
Cecropiaceae - *Cecropia*	4% (2)	0%	50%	50%
Brassicaceae - *Brassica*	14% (7)	43%	43%	14%
Myrtaceae - *Myrcia* type	10% (5)	20%	20%	60%
Eucalyptus sp.	8% (4)	75%	0%	25%
Solanaceae - *Solanum*	2% (1)	0%	0%	100%

[1]Number of samples.
[2]Frequency calculated as a function of each plant family.

Table 2 - Relationship between shelf life and fungal load of the dried bee pollen samples marketed in the State of Rio de Janeiro.

Shelf life (years)	DRBC cfu g^{-1} (minimum and maximum)
1	6×10^1 - 8×10^4
2	1×10^1 - 2×10^4
3	5×10^2 - 5×10^3

of aflatoxins B_1 and B_2 in *in vitro* conditions (qualitative data).

Discussion

The diversity of the Brazilian flora has triggered the extensive production of hetero-floral honey and pollen (Freitas *et al.*, 2013). Despite this fact, commercial bee pollen manufactured in Rio de Janeiro mainly originated from monocultures, similar to coconut and *Eucalyptus* cultivation. The occurrence of brands produced from wildlife pollen types, Asteraceae, Mimosaceae and Brassicaceae is highlighted next to the figures of hetero-floral pollen. The floral diversity decreased in each brand due to the presence of floral species, which are effective suppliers of bee pollen, and varied according to the region surrounding the apiary and climatic conditions during blooming.

Microbiological analysis showed that the marketed products are not within the established legal standards (Brasil, 1997, 2001b) of a maximum of 100 cfu g^{-1} of fungi and yeasts. Among all studied samples, 93% showed unsatisfactory conditions or were unsuitable for direct human consumption due to the presence of high fungal activity and possibly mycotoxigenic action (Cast, 2003). Other studies on bee pollen in the market of Bahia, Brasília-DF, Minas Gerais, Paraná, Rio Grande do Sul, Santa Catarina, São Paulo and Sergipe have also detected high microbiological contamination ($> 1{,}3 \times 10^4$ cfu g^{-1}) in at least 12% of the analyzed products (Barreto, 2004).

The findings of this study also suggest that Federal and State surveillance measures might have also been ineffective in reducing product contamination, with informal products showing higher levels of contamination. The high fungal and toxigenic load detected in the samples raises questions on how contamination occurs during its production and pollen collection (Barreto *et al.*, 2006). It is also possible that contamination may be present during storage.

Because tropical conditions could affect the quality of food *in natura*, there is a predisposition for pollen samples to present greater mycobiota diversity (Table 3).

Aspergillus is the predominant genus isolated in previous studies listed in Table 4. *Aspergillus parasiticus* is one of the main producers of aflatoxins (González *et al.*, 2005). Its presence has not been detected in Brazilian samples.

Cladosporium is the second most common genus isolated from commercial pollen products. Modro *et al.* (2009) reported the *Cladosporium* sp. collection by bees at an apiary characterized by high air relative humidity and low availability of food resources (pollen and nectar). The nutritional composition of the fungi pellets presented high protein value, ethereal extract and organic matter and could be used as a resource for the brood.

Penicillium is the third most common genus isolated from commercial pollen products, although previous findings showed variable results. González *et al.* (2005) reported that it is the most prevalent genus, whereas Villalobos *et al.* (2010) described it as the fifth most isolated genus. *Fusarium* was rarely detected in the present study, which was similar to the results of González *et al.* (2005) and Villalobos *et al.* (2010)

The toxigenic results of the present study support those of González *et al.* (2005), who analyzed samples from Spain and Argentina, except those involving OTA production by the *A. ochraceus* and *A. niger* aggregates strains, which was not detected in our samples. Pollen stimulates the production of OTA under *A. ochraceus* growth (Medina *et al.*, 2004; Villalobos *et al.*, 2010), which was not verified in our samples. The mycotoxin production depends on a series of factors such as humidity, temperature, oxygen availability, fungi growth time, substrate composition, quantity of fungi inoculum, as well as the interaction/competition among fungi strains (Cast, 2003) which could explain this variability on results.

Contamination of bee pollen compromises its consumption based on its risk to human health. Despite the severity of this matter, the Brazilian legislation for bee pollen was not defined based on physicochemical limits nor does it contemplate the limits for microbiological contamination or water activity. The National Health Surveillance Agency (Anvisa) regulations do not even treat this product as a food item.

Table 3 - Fungi isolated from commercial pollen samples according to the country of origin.

Country of origin	Isolated mycobiota	Authors
Brasil	*Aspergillus*, *Cladosporium*, *Penicillium*, *Alternaria*, *Wallemia*, *Eurotium*, *Mucor*, *Curvularia*, *Paecilomyces*, *Fusarium*	This study
Argentina and Spain	*Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*	González *et al.* (2005)
Mexico	*Aspergillus*, *Alternaria*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*	Villalobos *et al.* (2010)

Table 4 - *Aspergillus* species isolated from commercial pollen samples originating from various countries.

Country of origin	*Aspergillus* species	Authors
Brasil	*A. niger* aggregates, *A. flavus*, *A. versicolor*, *A. ochraceus*, *A. oryzae*, *A. terreus*, *A. carbonarius*	This study
Argentina and Spain	*A. niger* aggregates, *A. flavus*, *A. ochraceus*, *A. parasiticus*	González *et al.* (2005)
Mexico	*A. flavus*	Villalobos *et al.* (2010)

Therefore, the Brazilian legislation that identifies the item as a component of "other apiculture products" (Normative Instruction 3, from January 19, 2001), which includes bee pollen (in addition to propolis, royal jelly, apitoxin, and beeswax) requires revision to address the growth of apiculture production each year. The main function of the standard is to establish the parameters and technical requirements for food safety of products and processes, certification of the industry organization, protection of their products against any technical restrictions, and compliance to market demands (Camargo, 2008). Despite the small number of samples of the present study, it is clear that if the terms of legislation for this food are weak, the production of low-quality products can arise.

It should also be highlighted that the decline in the quality of the product could be due to the negligence of beekeepers in adopting of hygienic standards and proper handling of the product in one or more stages of production, which may result in products that could be harmful to human health (Vasconcelos, 2009). The timing of pollen harvest might also be a critical factor because these items are not collected daily because of the distance between apiaries and processing sites. Storage of collected pollen prior to processing and packaging also exposes the raw materials to moisture in the air. Pollen with high levels of lipids such as those of several Asteraceae species thus serve as optimal substrates for fungal growth compared to pollen with lower lipid content (Cecropiaceae). Another phase that requires caution is dehydration; this step should be performed in a facility with controlled temperature and immediately after collection. Toxigenic fungi present in pollen can grow and produce mycotoxins if the period between harvest and dehydration is too long. Furthermore, these toxins remain in the bee pollen even after heat exposure during drying.

Cast (2003) and Rodriguez *et al.* (2008) have suggested that the entire process of bee pollen production should be reviewed, from collection to storage, to minimize its risks to human health. Martins *et al.* (2013) have suggested that pollen be identified as a food or supplement, and that the allowable limits of contaminants be established. The improvement in management techniques is another important factor of efficient and safe beekeeping (Fachini *et al.*, 2010). Thus, producers must promote changes based on the findings of published reports.

Conclusions

There is currently a high demand for bee pollen in Brazil, which has resulted in its increased production and in turn requires systematization of the beekeeping business. There is also a serious problem relating to the hygienic-sanitary quality of marketed dried bee pollen in the State of Rio de Janeiro. The present study has detected high fungal contaminations, significantly higher than the established microbiological standards for foods. Fungi produce mycotoxins that cause mycotoxicosis in humans. Microbiological analyses represent an important tool in screening the hygiene conditions of the environment and the production process of food. The same guidelines should be implemented for pollen. There is a need to revise the legislation for this product and increase the surveillance on the quality of pollen for human consumption.

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