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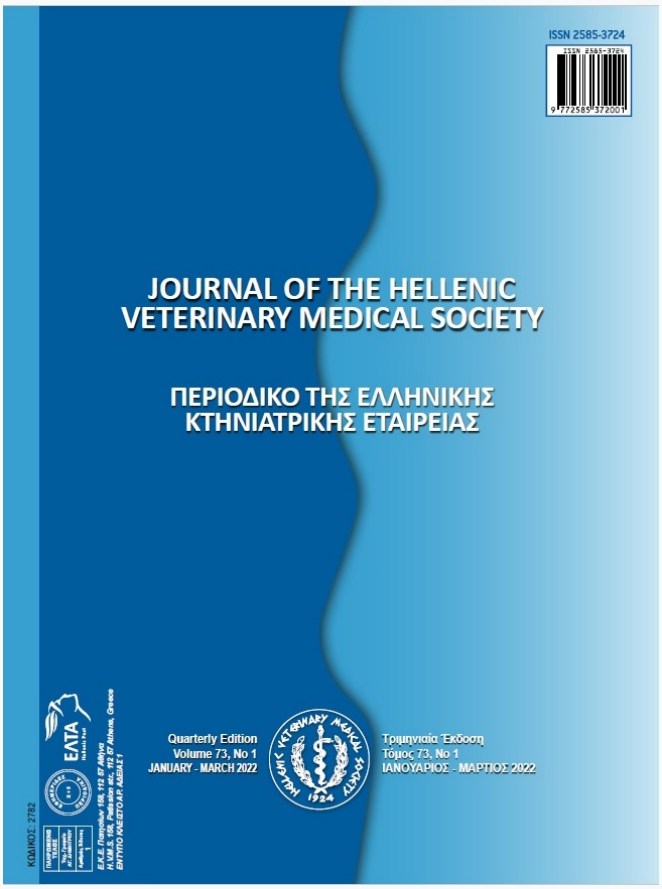

Microbiological characterization of fresh bee pollens from the Aegean region of Turkey

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Microbiological characterization of bee pollen from the Aegean region of Turkey

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ABSTRACT: This study aims to characterize the microbiological safety of Turkish bee pollen for the first time. Twenty-one bee pollen samples were purchased from local beekeepers between September 2020 and October 2020 in Muğla, İzmir, Kütahya and Afyon provinces in the Aegean region of Turkey. The samples were analyzed for total aerobic bacteria, total coliform bacteria, total psychotropic microorganisms, total lactic acid bacteria, *Staphylococcus aureus*, sulfite-reducing clostridia spores, yeast and mold. Total ochratoxin and aflatoxin levels were measured using a lateral flow kit in samples contaminated with mycotoxigenic molds. The counts of the microorganisms ranged from 3.70 to 5.42 log CFU g^{-1} for the aerobic mesophilic, 3.0 to 5.40 log CFU g^{-1} for psychrotrophs, 3.85 to 5.78 log CFU g^{-1} for lactic acid bacteria (LAB), 3.0 to 5.45 log CFU g^{-1} for yeasts-molds, and <10 to 5.0 log CFU g^{-1} for total coliforms in the samples. Besides, *S. aureus* and sulfite-reducing clostridia were not detected among the samples. The predominant mold genera in samples were *Aspergillus* sp. *Penicillium* sp. and *Alternaria* sp. Five samples contaminated with mycotoxigenic molds had aflatoxins in the range of 2.96-9.71 μg/kg. According to the study results, comprehensive prevention, control and surveillance strategies need to be established to reduce bacteria and mycotoxin contaminations in bee pollen. Legal regulation of bee pollen as a food supplement and legal limit of mycotoxins in bee pollen should be defined.

***Keywords*:** Bacteria; Bee pollen; Mold; Mycotoxins; Yeast.

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INTRODUCTION

Bee pollen is a pellet of pollen collected by worker honeybees. The bees store the pollen in the broods to be used as the primary food source for the development of brood and young bees (Carroll et al., 2017). It has wide biological activities known and used since antiquity due to having bioactive contents including amino acids, lipids, carbohydrates, mineral salts, vitamins, phenolic compounds, and flavonoids (Komosinska-Vassev et al., 2015). There has been a growing interest in better understanding its biological properties, and numerous studies reported that the pollen exhibits a variety of beneficial therapeutic properties including antioxidant (Kocot et al., 2018; Tutun et al., 2021), antimicrobial (Erkmen and Ozcan, 2008), antiinflammatory (Eteraf-Oskouei et al., 2020) and wound healing (Olczyk et al., 2016).

Although most of the studies conducted on bee pollen have focused on its chemical composition, chemical residues, botanical origin, and other biological activities, there have been limited studies on its safety risk related to microbiological hazard (Komosinska-Vassev et al., 2015; Mauriello et al., 2017; Puvača, 2018). Its water content and nutritionally important components make pollen an ideal environment for the growth of various bacteria, mold, and yeast (Estevinho et al., 2012). Bee pollen may contain some pathogens including viruses, parasites and bacteria. Also, manipulations by a beekeeper in bee pollen production and storage can lead to a significant increase in the contamination levels of bee pollen by pathogenic microorganisms (Mauriello et al., 2017; de Sousa Pereira et al., 2019; Lika et al., 2021). Toxigenic molds in the pollens can produce one or more mycotoxins that are toxic to vertebrates and other animal groups in low concentrations (Kostic et al., 2019; Tutun and Kahraman, 2020). Thus, the presence of microorganisms in bee pollen can both favor the spoilage of the bee pollen and cause diseases in humans. Its microbial quality in terms of the health and safety of consumers is important and should be monitored properly. This study aimed to investigate the microbiological characterization of honeybee pollens from the Aegean region of Turkey.

MATERIAL AND METHODS

Sampling of bee pollen

Twenty-one pollen samples were purchased from local beekeepers and warehouses linked to these apiaries between September 2020 and October 2020 in four provinces (Muğla, Kütahya, Afyon and İzmir) belong to the Aegean Region of Turkey (Table 1). All the samples were sent to the laboratory in sterile glass vials and stored at 4°C, until testing. They were analyzed within 48 h of sampling in the Laboratory of the Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University.

Physicochemical analysis

The water activity (a_w) of the pollen samples was determined using a hygrometer (Testo 650, Germany) and the a_w value was recorded at 20±0.2 ºC. Moisture content in pollen samples was measured using a moisture analyzer (AND MX-500, AD Company). The pH value of each sample was measured using a pH meter (WTW Lab-pH Meter InoLab® pH 7110) at 25ºC ± 2ºC after 10 g of each sample was dissolved in 90 ml deionized water.

Microbial analysis

Microbiological determinations were evaluated as described previously by De-Melo et al. (2015). The samples were analyzed for total aerobic bacteria, total coliform bacteria, total psychotropic microorganism, total lactic acid bacteria, *Staphylococcus aureus*, sulfite-reducing clostridia spores, yeast and mold.

Table1. Sampling regions

Sample code	Number of samples	Province	District	Number of Apiary
1-5	5	Muğla	Döğüşbelen	A-E
6-7	2	Muğla	Seydikemer	F-G
8-11	4	Muğla	Dalaman	H-K
12-13	2	Muğla	Menteşe	L-M
14-15	2	Muğla	Datça	N-O
16	1	Muğla	Milas	P
17	1	İzmir	Seferihisar	Q
18, 19	2	Kütahya	Kadınhanı	R, S
20, 21	2	Afyon	İscehisar	T, U

Sample preparation

A 10 g sample from each bee pollen was placed aseptically into sterile stomacher bags (VWR, Belgium), diluted in 90 mL of sterile saline peptone water (0.85%±0.1%, w/v) and homogenized in a stomacher (Stomacher 400, Seward, London, UK) for 2 min at room temperature. Then, serial dilutions were made in 9 mL of sterile saline peptone water (1:10, v/v). The appropriate dilutions were plated by spread plate technique (0.1 mL) in duplicate and counted after incubation. The colonies were expressed as log colony forming units g^{-1} (log CFU g^{-1}).

Microbial enumeration

Count of total aerobic microflora was performed within 24-36 h after the spread plate inoculation of 0.1 mL of each dilution on plate count agar (PCA, Merck, 5463) and incubation at 30 °C. To count the total psychotropic microorganism, 0.1 mL of each dilution were inoculated on PCA and incubated at 7±0.5 °C for 10 days. To determine the total lactic acid bacteria (LAB) count, 0.1 mL of each aliquot was inoculated onto de Man, Rogosa and Sharpe agar (MRS, Merck 110660) and incubated under anaerobic conditions using Anaerocult® A (Merck 113829) with anaerobe jar at 30°C for 48-72 h. Enumeration of total coliforms was carried out by spreading on plates of violet red bile glucose agar (VRBG, Merck 110275). Plates were overlaid with VRBG agar. Then, solidified plates were incubated at 37°C for 24-48 h. For the determination of *S. aureus*, 0.1 ml aliquots of the dilutions were transferred to Baird-Parker agar (BP, Merck, 105406) enriched with egg yolk and incubated for 24 h at 37±2°C. The occurrence of sulfite-reducing clostridia spores was determined according the procedure as defined by ISO 15213:2003 (2003). The dilutions (10, 5 and 1 mL) were placed in a water bath at 80°C for 10 min, inoculated in petri dishes containing sulfite iron agar (SIA, Merck, 110864) and incubated at 37ºC for 48 h under anaerobic conditions. The serial dilutions of pollen homogenates were inoculated into dichloran rose Bengal chloramphenicol agar (DRBC, Merck 1.00466) and incubated at 25±1 °C for 5-7 days. At the end of the incubation period, yeast and mold colonies were counted.

Isolation and identification of yeast and mold

Single colonies of different yeast grown on DRBC agar were sub-cultured on Sabouraud's dextrose agar (SDA, Merck, 105438) supplemented with chloramphenicol (Oxoid, SR0078E) and incubated in aerobic conditions at 37 °C for 24-48 h. Isolated yeasts were defined at the genus level by colony morphology, Gram staining, germ tube test and urease tests (Seeliger, 1956; Quinn et al., 2011; Khadka et al., 2016). In the germ test, *Candida albicans* ATCC 90028 was used as positive control and *C. tropicalis* ATCC 13803 was used as a negative control. The yeast isolates were sub-cultured to CHROMagar Candida (CHROMagar, Paris, France) and incubated at 37°C for 24-48 h under aerobic conditions. At the end of the period, the growth ability and colony colors of the yeast isolates on CHROMagar Candida were evaluated according to the manufacturer's instructions. *C. albicans*, *C. tropicalis*, *C. krusei*, *C. kefyr* or *C. glabrata* and *Candida* sp. appear as green, metallic blue, purple fuzzy, mauve-brown and white to mauve colored colonies, respectively.

Single colonies of different mold grown on DRBC agar were duplicate sub-cultured on SDA supplemented with chloramphenicol and incubated at 25°C and 37°C for 4-7 days. Slides were prepared by the sticky tape method and stained with lactophenol cotton blue solution for microscopic examination of mold colonies (Quinn et al., 1999). The morphological characteristics of isolated colonies were identified based on macroscopic (colony appearance) and microscopic (hyphae, conidia, conidiophores and arrangement of spores) examination at genera level (Quinn et al., 1999; Pitt and Hocking 2009).

Mycotoxin analyses

Total aflatoxin and ochratoxin levels were analyzed in the bee pollen samples contaminated with *Aspergillus* sp. and/or *Penicillium* sp. The concentrations of total aflatoxin and ochratoxin in the samples (n=5) contaminated with *Aspergillus* sp. and/or *Penicillium* sp. were quantified by Symmetric Total Green 0-30 Lateral Flow kit (ProGnosis Biotech, Larissa, Greece) and Symmetric Ochratoxin Lateral Flow kit (ProGnosis Biotech, Larissa, Greece) according to the manufacturer's instructions, respectively.

Statistical analysis

Analyses were performed in duplicate. The Pearson correlation coefficient was calculated with SPSS 21 (SPSS Inc., IBM Corporation, Armonk, New York, USA). A p-value less than 0.05 is statistically significant.

RESULTS

The values of moisture, a_w and pH of bee pollens

ranged from 12.92% to 20.99%, 0.59 to 0.78 and 3.78 to 4.71, respectively (Table 2). While the samples from Menteşe had lower water content and the a_w values, the samples from Kütahya province had higher than the others.

The microbial communities of bee pollens are shown in Table 2. The counts of microorganisms ranged from 3.70 to 5.42 log CFU g^{-1} for the aerobic mesophilic bacteria, 3.0 to 5.40 log CFU g^{-1} for psychrotrophs, 3.85 to 5.78 log CFU g^{-1} for LAB, 3.0 to 5.45 log CFU g^{-1} for yeasts and molds, and <10 to 5.0 log CFU g^{-1} for total coliforms. *S. aureus* and sulfite-reducing clostridia were not detected among the samples. No correlation was shown between counts of bacteria including aerobic mesophilic bacteria, psychrotrophs and total coliform and the a_w and %RH values for bee pollen (Table 3). A positive correlation was observed between LAB counts and a_w ($r=0.507$; $p<0.05$) and %RH values ($r=0.551$; $p<0.01$) for bee pollens. Also, there is a significant positive correlation between yeast-mold communities and a_w ($r=0.464$; $p<0.05$) and %RH values ($r=0.529$; $p<0.05$) of bee pollen. These results indicate that a_w and %RH had a positive effect on the yeast-mold and LAB growth.

Totally 21 fungal isolates representing 4 fungal species were detected in all bee pollen samples (Table 4). Fourteen yeasts were isolated in pure form on SDA. White-cream color, smooth, pure growth yeast colonies were observed on SDA. Gram-positive, 3-4 µm in diameter, oval-round, budding yeast cells were seen in these colonies by microscopic examination. All yeast isolates were urease negative and no germ tube formation was detected in any of the isolates. Colonies of yeast isolates in CHROMagar Candida were white to mauve in color and the isolates were interpreted as *Candida* sp. The *Candida* sp. was the most frequently found with 14 isolates. *Penicillium* sp. (5 isolates from Seydikemer, Dalaman and Kütahya regions) was the second most prevalent fungal genera. *Aspergillus* sp. and *Alternaria* sp.were the least fungal contaminant found in bee pollen.

Total aflatoxin and ochratoxin production were analyzed in 5 samples contaminated with *Aspergillus* sp. and *Penicillium* sp. There was a good accuracy with recovery 95% and 96%, and the detection limit were 1.20 and 1.50 µg $kg^{-1}$for aflatoxin and ochratoxin, respectively.

Total aflatoxin levels ranged from 2.96 to 9.71 µg kg^{-1} (Table 5). The samples from the Dalaman region had the highest total aflatoxin level (9.71 µg kg^{-1}). Ochratoxin was not detected in the samples**.**

Table 2. Microbial and physicochemical evaluation of 21 bee pollen samples

Regions	n	Aerobic mesophilic	Psychotrophs	LAB	Yeast -mold	Total coliforms	*S. aureus*	Sulfite-reducing Clostridia	a_w	pH	%RH
Döğüşbelen	5	4.20 -5.42	4.08-4.71	5.18-5.46	3.00-4.04	3.48-3.78	<10	<10	0.70-0.72	4.18-4.36	18.05-18.72
Seydikemer	2	4.30-4.88	4.11-4.46	4.60-5.04	3.48-4.65	3.50-3.80	<10	<10	0.66-0.68	4.27-4.36	16.26-16.48
Dalaman	4	3.95-4.65	3.00-4.60	3.85-5.78	3.00-5.45	3.48-4.65	<10	<10	0.67-0.75	4.03-4.44	16.49-20.99
Menteşe	2	3.95-3.98	3.00-4.30	4.00	3.00	<10	<10	<10	0.59-0.60	4.57-4.61	12.92-13.50
Datça	2	4.30-4.90	5.23-5.40	5.00-5.20	3.00	3.00-4.60	<10	<10	0.69-0.72	4.09-4.45	17.20-19.03
Milas	1	4.30	3.30	5.48	5.48	<10	<10	<10	0.69	4.28	20.90
Seferihisar	1	3.78	3.48	4.00	5.00	<10	<10	<10	0.76	3.78	20.55
Kütahya	2	3.81-4.30	4.00-4.60	4.00-5.30	4.40-4.48	<10	<10	<10	0.68-0.78	3.93-4.45	16.94-24.74
Afyon	2	3.70-3.72	3.00-4.70	5.00	3.00-5.00	3.40-5.00	<10	<10	0.71-0.72	4.24-4.71	17.66-19.95

Note: microbial communities express as log CFU g^{-1}

Table 3. Correlation between the microbial counts, moisture content and a_w in bee pollen

	a_w	%RH
Aerobic mesophilic	0.119	0.136
Psychotrophs	0.212	0.137
LAB	0.507*	0.551**
Yeast-mold	0.464*	0.529*
Total coliforms	-0.99	-0.710
a_w	1.000	0.916**
%RH	0.916**	1.000

* $p<0.05$; **$p<0.01$

Table 4. Fungal species isolated from bee pollen

Areas	Sample No	*Candida* sp.	*Aspergillus* sp.	*Penicillium* sp.	*Alternaria* sp.
Döğüşbelen	1-5	+	-	-	-
Seydikemer	6	+	-	+	-
Seydikemer	7	+	-	-	-
Dalaman	8	+	-	+	-
Dalaman	9	+	-	+	+
Dalaman	10	+	+	+	-
Dalaman	11	-	-	-	-
Menteşe	12-13	-	-	-	-
Datça	14-15	-	-	-	-
Milas	16	-	-	-	-
Seferihisar	17	-	-	-	-
Kütahya	18	+	-	-	-
Kütahya	19	+	-	+	-
Afyon	20-21	+	-	-	-

Table 5. Total aflatoxin and ochratoxin levels of the pollen samples

Sample Region	**Total Aflatoxin (µg/kg)**	**Ochratoxin (µg/kg)**
Seydikemer	2.96	*
Dalaman	8.04	*
Dalaman	9.71	*
Dalaman	5.03	*
Kütahya	4.92	*

*: Not detected.

DISCUSSION

Since bee pollen moisture value plays an important role in organoleptic properties and extension of their shelf life, it is one of the important parameters for assessing quality control of this product (Anjos et al., 2019). The higher a_w values give the opportunity to the proliferation of microorganisms including pathogenic bacteria grown at a_w>0.85 and fungi grown well at a_w>0.6 (Mathlouthi, 2001; Rahman, 2010). In the current study, the average a_w, pH and moisture values of the bee pollen were 0.69, 4.30, and 18.05%, respectively. The $a_w$values of all samples were greater than 0.6, which enables microorganisms to grow in stored bee pollen (Rahman, 2010). The pH average and water content of the samples in the current study were similar to those of bee pollens reported in the literature (Estevinho et al., 2012; Petrovic et al., 2014; Arslan and Durmaz, 2019; Beev et al., 2020; Dinkov, 2020) and the Brazilian legislation (pH, 4.0-6.0). In the current study, although the a_w and %RH values of the bee pollens had a positive effect on the growth of LAB and yeast-mold, they did not show a positive effect on the growth of the bacteria including aerobic mesophilic bacteria, psychrotrophs and total coliform. The reason for LAB and yeast-mold growth may be high a_w and %RH in pollen samples. Many literatures also confirm these results (Gonzalez et al., 2005; Hani et al., 2012; Nuvoloni et al., 2021)

The studies on the microbiological safety of fresh bee pollen samples showed a vast majority of microbial diversity according to inadequate hygienic manipulations during harvesting, processing and storage conditions (Nogueira et al., 2012; De-melo et al., 2015; Beev et al., 2020). In the present survey, some samples had not perfect microbial quality characteristics and similar observations were reported by other researchers (Belhadj et al., 2014; Shevtsova et al., 2014; Beev et al., 2020). There was a variation in the microbiological quality among those collected from a different apiary in the same province. In a sample collected in Döğüşbelen province the higher aerobic mesophilic count (5.42 log CFU g^{-1}) was observed, for psychrotrophs the highest value was observed in pollen sample from Datça (5.40 log CFU g^{-1}). The sample from Milas province had the highest counts of LAB and yeast-mold counts (5.48 and 5.48 log CFU g^{-1}, respectively). The highest value of total coliform was observed in one sample from Afyon province (5.0 log CFU g^{-1}). As a member of the *Enterobacteriaceae*

family, the presence of coliforms that cause various food-borne diseases in humans is linked to fecal contamination of food (Jay et al., 2005). It is well known that *S. aureus* can produce thermostable enterotoxin that causes food poisoning (Argudin et al., 2010) and *Clostridium botulinum* is responsible for serious neuroparalytic illness due to produce neurotoxin (Vasquez, 2009). All fresh bee pollen samples were negative for *S. aureus* and sulfite-reducing clostridia. This is a promising result considering the importance of these microorganisms are potentially pathogenic and have significant effects on public health.

In the present study, the most prevalent fungi genus in bee pollen samples was *Candida* sp., followed by *Penicillium* sp.*, Aspergillus* sp. and *Alternaria* sp., which is similar to those of previous studies on bee pollen (Kostic et al., 2017; Sinkevičienė et al. 2019; Beev et al., 2020). In terms of the yeast-mold count, most of the samples (Except 3) are comply with the European Union regulations which allow for up to 50.000 CFU $g^{-1}$of yeast and mold in bee pollen samples. There is no regulation on microbiological quality standards of bee pollen in Turkey.

Mycotoxins belong to the category of toxic secondary metabolites produced by *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. and cause health hazards to people and animals. The mycotoxin-producing fungi contamination of foods may result in the production of mycotoxins such as aflatoxins and ochratoxins (Gilberth et al., 2000; Jay et al., 2005). We analyzed the total aflatoxin and ochratoxin in the samples contaminated with *Aspergillus* sp. and/or *Penicillium* sp. and detected the only aflatoxin in the range of 2.96-9.71 μg kg^{-1} in all tested samples (average 6.13 μg kg^{-1}). According to Regulation of European Commission (No: 165/2010) and Turkish Food Codex (No: 26/2008), the maximum levels of total aflatoxin is from 2.0 μg kg^{-1} to 15.0 μg kg^{-1} depending on the type of foods, such as maize, wheat, and milk. However, there is no information about the maximum levels for mycotoxins in bee pollen in these regulations. Campos et al. (2008) proposed that maximum permissible concentration (MPC) in pollen should be set at 2.0 μg kg^{-1} for aflatoxin B1 and 4.2 μg kg^{-1} for total aflatoxins. On the other hand, since bee pollen is consumed less than foods with the tolerable limit for mycotoxins, these proposed MPCs may not be realistic. In addition, according to the coexistence of mycotoxins in bee pollen, the MPC should be reconsidered, as some combined mycotoxins have a more detrimental effect on human health (Smith et al., 2016; Kostic et al., 2019). Determining the MPC of mycotoxins especially aflatoxins and ochratoxins in bee pollens will be of great importance.

Bee pollen has ideal nutritional and chemical composition for bacterial colonization and growth. Improper movements while beekeepers collect and process bee pollen may result in microorganism contamination (Mauriello et al., 2017). Several factors, such as humidity, temperature, drying process, and storage conditions can affect the growth of different types of microorganisms in bee pollen (Kostic et al., 2017). In the current study, the reason for the high microorganism load in a few samples could be the results of contact of the bee pollen with microorganisms by beekeepers' manipulation mistakes. Aegean region, situated in the western part of Turkey, has a Mediterranean climate that provides a favorable condition for microbial growth especially fungi. Also, the high moisture and temperature in these regions could contribute to the microbial growth in the samples (Arruda et al., 2017; Tarazona et al., 2019).

Monitoring of the microbiological quality of raw bee pollen allows evaluating of processing and storage conditions (Rocha, 2013; De Melo et al., 2015). Microbiological contamination of bee pollen can happen during the steps of production, due to inadequate hygienic practices, the lack of proper handling at the harvesting, transportation and storage steps (Nogueira et al. 2012). It is known that each apiary has different collection and processing practices. Therefore, the variation in the counts of microorganisms observed in samples is acceptable considering different apiary manipulation.

CONCLUSION

This is the first study in Turkey to describe the microbiological quality of bee pollen from the Aegean region. The findings suggest that microbiological criteria should be established for bee pollen regulated by national authorities. Adoption of proper harvesting practice and post-harvest processing techniques can help to prevent possible microbial contamination of Turkish bee pollen by equipment or handling. Also, quality control and monitoring systems are necessary for maintaining the safety of bee pollen for humans.

CONFLICT OF INTEREST

None declared by the authors.

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