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ORIGINAL ARTICLE

A diagnosis of the microbiological quality of dehydrated bee-pollen produced in Brazil

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Significance and Impact of the Study: Contamination of bee-pollen can occur during production, collection and processing, but there are few studies of the microbiological quality of this product. Brazil is an important producer of dehydrated bee-pollen, therefore, a diagnosis of the microbiological status is important to ensure the safety of many consumers. *Salmonella* sp., genus *Clostridium*, coagulase-positive *Staphylococcus*, *Escherichia coli* and even some yeast species are micro-organisms of public health concern and their presence must be monitored. Furthermore, the determination of spoilage micro-organisms indicates whether the production and the processing practices carried out by beekeepers and warehouses were adequate.

Keywords
apicultural, *Apis mellifera*, bee-pollen, microbiological quality, pathogens, spoilage micro-organisms.

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Abstract

Bee-pollen is an apicultural product with potential for medical and nutritional applications; therefore, its microbiology quality should be monitored. In this context, the objective of this study was to diagnose the microbiological quality of 45 dehydrated bee-pollen samples collected from November 2011 to December 2013 in nine Brazilian States. All the samples were negative for sulphite-reducing *Clostridium* spores, *Salmonella*, coagulase-positive *Staphylococcus* and *Escherichia coli*, which are micro-organisms of public health concern. Total aerobic mesophilic micro-organism counts ranged from <10 to $1{\cdot}10 \times 10^4$ CFU g^{-1}, with psychrotroph counts ranging from <10 to $1{\cdot}12 \times 10^3$ CFU g^{-1} and total coliforms from <10 to $2{\cdot}80 \times 10^3$ CFU g^{-1}, while the values for yeasts and moulds were between <10 to $7{\cdot}67 \times 10^3$ CFU g^{-1}. According to the literature, the microbiota observed in this study were typical; however, it is important to consider that these micro-organisms may cause spoilage and diminish shelf life, reason by which quality control programs should be implemented.

Introduction

Bee-pollen is the result of the agglutination of different pollen grains collected by bees from flowers, as well as salivary secretions and small amounts of nectar and/or honey (Brazilian Legislation, 2001; Campos *et al.* 2008). The consumption of this food is driven by the demand for natural products, complementary to dietary or therapeutic effects (Barreto *et al.* 2005). In addition, there is a growing concern with the sanitary quality of food. To meet the demand for bee-pollen and to prevent harm to consumer health, permanent controls in the beekeeping chain and the implementation of good production and processing practices by beekeepers and warehouses are essential.

There are few studies of the microbiological quality of dehydrated bee-pollen, and we are aware of only two publications analysing samples collected in Brazil (Barreto *et al.* 2005; Hervatin 2009). There is no specific international legislation for this product and Brazilian legislation establishes limits only for physico-chemical parameters such moisture, protein, lipid, ash, total sugars, free acidity

and pH (Brazilian Legislation, 2001). The lack of regulation of the microbiological quality of bee-pollen may be related to the absence of diagnostics, attesting the need for limitations.

Given the requirement for a diagnosis of the sanitary status of bee-pollen produced in Brazil, this study evaluated the microbiological quality of dehydrated bee-pollen samples from different Brazilian States.

Results and discussion

The choice of microbiological indicators to determine the sanitary quality of the samples was based on previous studies (Campos *et al.* 2008; Estevinho *et al.* 2012; Feás *et al.* 2012; Nogueira *et al.* 2012; Puig-Peña *et al.* 2012). The results are shown in Table 1. The aerobic mesophilic micro-organism counts ranged from <10 to $1{\cdot}10 \times 10^4$ CFU g^{-1}, psychrotrophs from <10 to $1{\cdot}12 \times 10^3$ CFU g^{-1}, yeasts and moulds from <10 to $7{\cdot}67 \times 10^3$ CFU g^{-1} and total coliforms from <10 to $2{\cdot}80 \times 10^3$ CFU g^{-1}. The highest counts were observed for aerobic mesophilic micro-organisms. There was a variation in the microbiological quality between the samples, even among those collected and processed in the same apiary. In a sample collected in Mato Grosso State it was observed the higher aerobic mesophilic count ($1{\cdot}10 \times 10^4$ CFU g^{-1}), for psychrotrophs the highest value was observed in a dehydrated bee-pollen from Paraná State ($1{\cdot}12 \times 10^3$ CFU g^{-1}). Two samples, one from São Paulo State and one from Bahia State, had the highest counts of yeasts and moulds ($7{\cdot}67 \times 10^3$ CFU g^{-1}). The highest value of total coliform was observed in a different sample collected in Bahia State ($2{\cdot}80 \times 10^3$ CFU g^{-1}) (Table 2). In samples from the same State were observed variation in the counts of micro-organisms. It must be considered that each apiary is a unit of production and adopts different collection and processing practices.

The diagnosis of the microbiological quality of a food allows the evaluation of processing conditions, storage and distribution (Rocha 2013). The presence of micro-organisms in dehydrated bee-pollen is related to inadequate hygienic practices during the steps of manipulation during harvesting and processing, and the handling and contamination of floral pollen grains on the plant or by bees (Barreto *et al.* 2005; Hervatin 2009; Nogueira *et al.* 2012). In the production process, a critical moment is during the time that bee-pollen *in natura* remains in the collection traps fixed in the hive entrance because the grains are in contact with air, dust and other dirt. Certain seasons and exposure to high humidity and temperature also favour microbial growth (González *et al.* 2005).

The proliferation of micro-organisms in a food matrix depends on favourable conditions. Estevinho *et al.* (2012) observed a positive correlation between water activity (a_w) and the population of yeasts and moulds. The a_w in dehydrated bee-pollen varies from 0·25 to 0·60 (Bastos *et al.* 2003; Estevinho *et al.* 2012; Nogueira *et al.* 2012; Rocha 2013) and it is a limiting factor for microbial proliferation in dehydrated bee-pollen (Estevinho *et al.* 2012). There are two hypotheses for the presence of viable micro-organisms in samples of dehydrated bee-pollen: that the low temperature used in the dehydration process is insufficient to remove micro-organisms and that contamination of the product occurs during the processing steps after dehydration (Barreto *et al.* 2005; Hervatin 2009).

The aerobic mesophilic micro-organism count is commonly used as a general indicator of food quality. Microbiological diagnostics for dehydrated bee-pollen indicate counts in the range of <10 to 10^4 CFU g^{-1} (Estevinho *et al.* 2012; Feás *et al.* 2012; Hani *et al.* 2012; Nogueira *et al.* 2012), although values above 10^4 CFU g^{-1} have been reported (Puig-Peña *et al.* 2012). Based on the literature, it was expected that the number of total psychrotrophs would be lower due to storage and marketing at room temperature, which we confirmed. Total coliforms are also indicators of contamination during the production stages, and the collection and processing of food, but they are the best indication of the presence of pathogens of enteric origin, such as *Escherichia coli*. Reports of total coliforms in this matrix indicate values <10–10^4 CFU g^{-1} (Hani *et al.* 2012; Rocha 2013). Counts of yeasts and moulds in dehydrated bee-pollen samples

Table 1 Percentage of the dehydrated bee-pollen samples in each range of determination.

CFU g^{-1}/MPN g^{-1}	Aerobic mesophilic, %	Psychotrophs, %	*Staphylococcus aureus*, %	Yeasts and moulds, %	Total coliforms, %	*Escherichia coli*, %
<10	6·67	57·78	100	15·56	33·33	100
10–100	15·56	35·56	–	37·78	15·56	–
101–1000	53·33	4·44	–	31·11	37·78	–
1001–10000	22·22	2·22	–	15·56	13·33	–
Above 10001	2·22	–	–	–		–

Table 2 Counting range of micro-organisms in dehydrated bee-pollen samples according to the Brazilian State

Origin/Brazilian State	Number of samples	Aerobic mesophilic (CFU g^{-1})	Psychotrophs (CFU g^{-1})	Yeasts and moulds (CFU g^{-1})	Total coliforms (MPN g^{-1})
Bahia	14	$2{\cdot}50 \times 10$ to $2{\cdot}13 \times 10^3$	<10 to $5{\cdot}00 \times 10$	$2{\cdot}00 \times 10$ to $7{\cdot}67 \times 10^3$	<10 to $2{\cdot}80 \times 10^3$
São Paulo	13	<10 to $3{\cdot}53 \times 10^3$	<10 to $9{\cdot}80 \times 10$	<10 to $7{\cdot}67 \times 10^3$	<10 to $1{\cdot}67 \times 10^3$
Paraná	4	$1{\cdot}11 \times 10^2$ to $1{\cdot}50 \times 10^3$	<10 to $1{\cdot}12 \times 10^3$	$3{\cdot}70 \times 10$ to $4{\cdot}00 \times 10^3$	$5{\cdot}00 \times 10$ to $1{\cdot}40 \times 10^3$
Rio Grande do Norte	4	<10 to $3{\cdot}50 \times 10^2$	<10	$2{\cdot}40 \times 10$ to $4{\cdot}50 \times 10^2$	<10 to $2{\cdot}20 \times 10$
Rio Grande do Sul	3	$3{\cdot}70 \times 10^2$ to $7{\cdot}42 \times 10^2$	<10 to $1{\cdot}70 \times 10$	$2{\cdot}30 \times 10$ to $2{\cdot}17 \times 10^2$	$6{\cdot}00 \times 10^2$ to $2{\cdot}27 \times 10^3$
Sergipe	2	$6{\cdot}84 \times 10^2$ to $7{\cdot}64 \times 10^2$	<10 to $1{\cdot}82 \times 10^2$	$2{\cdot}72 \times 10^2$ to $2{\cdot}80 \times 10^2$	<10
Santa Catarina	2	$4{\cdot}80 \times 10$ to $6{\cdot}80 \times 10$	<10 to $1{\cdot}30 \times 10$	<10 to $2{\cdot}20 \times 10$	<10 to $6{\cdot}70 \times 10$
Mato Grosso	2	$1{\cdot}94 \times 10^2$ to $1{\cdot}10 \times 10^4$	<10 to $2{\cdot}70 \times 10$	<10 to $8{\cdot}70 \times 10$	$6{\cdot}70 \times 10$ to $3{\cdot}33 \times 10^2$
Mato Grosso do Sul	1	$4{\cdot}80 \times 10^3$	$4{\cdot}70 \times 10$	$6{\cdot}70 \times 10^2$	$2{\cdot}67 \times 10^2$

indicate values between <10–10^4 CFU g^{-1} (Barreto *et al.* 2005; Santos *et al.* 2010; Hani *et al.* 2012; Rocha 2013).

All samples were negative for sulphide-reducing clostridia spores and *Salmonella* sp. and did not show growth of *E. coli* and coagulase-positive *Staphylococcus*. This is an important result because these micro-organisms are potentially pathogenic and of interest to public health. *Clostridium perfringens* gastroenteritis is caused by eating food contaminated with enterotoxin produced by the bacteria and the most common symptoms are diarrhoea and abdominal pain (Monma *et al.* 2015). Botulinum neurotoxin produced by *C. botulinium* can causes a serious neuroparalytic illness (Vasquez 2009). *Salmonella* sp. is the cause of one of the most common diseases transmitted by food, with global distribution. Infection by *Salmonella* sp. can cause gastrointestinal disease and, in more severe cases, septicaemia and meningitis (Chiappini *et al.* 2002). *Escherichia coli*, besides a micro-organism indicator of hygiene during production and processing and possibly the presence of other micro-organisms of enteric origin (Franco and Landgraf 2009), is potentially pathogenic because some strains acquire specific virulence factors responsible for diarrhoea, acute inflammation, haemorrhagic colitis, urinary tract infections, septicaemia and neonatal meningitis (Kaper *et al.* 2004; Müller *et al.* 2007). *Staphylococcus aureus* is a coagulase-positive *Staphylococcus* that produces a thermostable enterotoxin that when ingested causes food poisoning (Franco and Landgraf 2009).

Few countries have microbiological criteria for marketing dehydrated bee-pollen. Researchers at the International Honey Commission (IHC), an international commission whose objectives are to propose quality criteria and an international standard of identity and quality for bee-pollen, recommended limits for total aerobic micro-organisms (<10^5 CFU g^{-1}), yeasts and moulds (<5×10^4 CFU g^{-1}), Enterobacteriaceae (max. 10^2 CFU g^{-1}), *E. coli* (absence in 1 g), *Salmonella* sp. (absence in 10 g) and *Staph. aureus* (absence in 1 g) (Campos *et al.* 2008). Using the limits established by IHC, all the samples analysed were within the standard.

Fourteen species of yeast were identified in 26 samples (57·7%). No growth of these micro-organisms or correspondence with the patterns provided by the commercial system was observed in the other samples. Of the 14 identified species, five belonged to the genus *Candida* (*C. magnoliae, C. parapsilopsis, C. norvegensis, C. krusei, C. stellata*) and three to the genus *Zygosaccharomyces* (*Z. bailii, Z. rouxii, Z. lentus*). The other species were *Rhodotorula mucilaginous*, *Hanseniaspora uvarum*, *Cryptococcus humicola*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Pichia membranifaciens*. The occurrence of each species in the 45 samples tested is shown in Fig. 1.

Among the identified yeast, the genus *Candida* predominates. This genus comprises diverse commensal micro-organisms that inhabit the gastrointestinal tract primarily in humans, but it is also part of the vaginal microflora and can be found in the urethra and the lungs. These yeasts can become pathogenic if there is an imbalance in their relationship with the host, so they are called opportunistic. Although *C. albicans* is considered the most important pathogenic yeast in humans, other species were identified in recent decades in infectious processes, including *C. parapsilopsis*, *C. norvegensis* and *C. krusei* (Giolo and Svidzinski 2010). *Rhodotorula* species are opportunistic pathogens able to colonize and infect susceptible patients. *Rhodotorula mucilaginous* is commonly isolated from foods and beverages but the most cases of infection with *Rhodotorula* spp. are fungemia associated with catheter use (Wirth and Goldani 2012). Although considered a deteriorating agent, *Cryptococcus humicola* has been described as a cryptococcosis agent in immunosuppressed patients, although it is not the most common species associated with this pathology (Harris *et al.* 2011). Opportunistic pathogens cause infection only in special circumstances. Eating the food contaminated with these micro-organisms will not necessarily result in infection. According to Bishop (2012), in individuals with immuno-

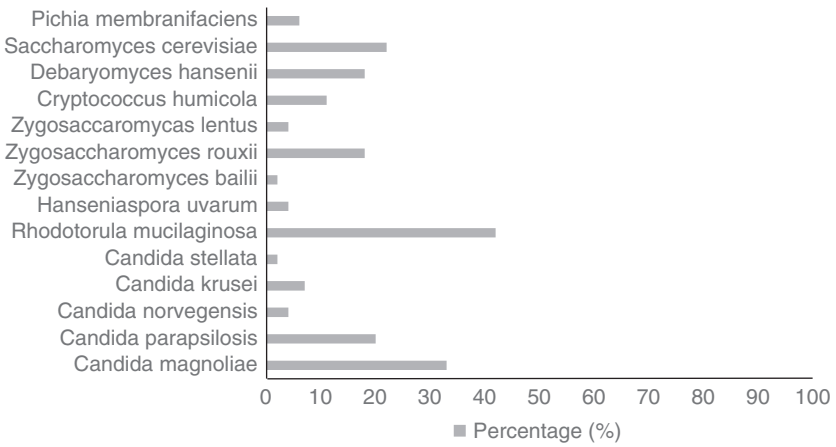

Figure 1 Occurrence of yeasts isolated in 45 dehydrated bee-pollen collected in nine Brazilian States.

suppression can occur a population imbalance of these opportunistic pathogens, resulting in infection. This imbalance can also occur due to stress or changes in intestinal pH.

Zygosaccharomyces rouxii was also found in commercial samples of dehydrated bee-pollen commercialized in Portugal and Spain (Nogueira *et al.* 2012). The *Z. rouxii* is a spoilage micro-organism known for its high tolerance to osmotic stress (Pribylova *et al.* 2007). *Saccharomyces cerevisiae*, *Pichia membranifaciens* and *Hanseniaspora uvarum* deteriorate foods by fermentation (Moreira *et al.* 2011; Nogueira *et al.* 2012). *Debaryomyces hansenii* has been found in different foods of low water activity and products with elevated sugar content. This species is also associated with deterioration processes (Breuer and Harms 2006).

Although, as far as we know, only one study identifying yeast in bee-pollen has been published (Nogueira *et al.* 2012), the presence of these micro-organisms in honey has been extensively reported (Teixeira *et al.* 2003; Carvalho *et al.* 2005). Considering that both are bee products, it is possible that the bee itself is the source of contamination or contamination that occurs because of the wide distribution of these micro-organisms in nature, and consequently, are present on pollen grains. In this study, the presence of yeast in the samples seems unrelated to the season or region where the dehydrated bee-pollen were produced, since these micro-organisms have been identified in products from all nine States and in different seasons.

This is the most comprehensive study of the microbiological quality of dehydrated bee-pollen produced in Brazil, including a large number of samples and analyses. The values for groups of aerobic mesophilic micro-organisms, psychrotrophs, total coliforms and moulds and yeasts are in agreement with the results reported in the literature and are considered acceptable by the IHC. The absence of pathogenic bacteria is a positive result, although the identification of potentially pathogenic yeast is of concern. It is necessary to create health quality criteria for bee-pollen and to require more attention from producers and warehouses to management practices in the field and processing to prevent endangering the health of consumers and to increase the shelf life of the product.

Material and methods

The study was conducted in the Microbiology Laboratory (ML) of the Agrarian Superior School/Polytechnic Institute of Bragança, Portugal. Samples were submitted from beekeepers and warehouses linked to the Brazilian Confederation of Apicultural or State Associations, as well as randomly purchased on the market for investigative purposes. Forty-five bee-pollen samples from *Apis mellifera* bees, dehydrated and ready for consumption, were collected between November 2011 and December 2013 in 22 apiaries in nine Brazilian States (Table 3) and sent to the Food Analysis Laboratory of the Faculty of Pharmaceutical Sciences/University of São Paulo (São Paulo, Brazil). Samples were coded and stored at 0°C. Groups of samples were sent at room temperature to the ML (five groups, shipping time: up to 5 days), then stored at 0°C until analysed.

The packages were opened in a laminar flow cabinet and homogenized to obtain a 10 ± 0·2 g portion for analysis. The portion was placed aseptically into sterile bags and homogenized in a presterilized Stomacher Lab-Blender (Seward type 400, London, UK) for 1 min with 90 ml of sterilized peptone water (HiMedia, Mumbai, India). Serial dilutions were also prepared using peptone water (1 : 10, v/v).

The aerobic mesophilic micro-organism count was performed 48 h after the incorporation of 1 ml of each dilution in plate count agar (PCA) (HiMedia, Mumbai,

Table 3 Sample code, collection date, origin and apiary of the 45 dehydrated bee-pollen samples

Sample code	Collection date	Origin/Brazilian state	Apiary*
1	November/11 to December/11	Rio Grande do Norte	A
2	December/11	Santa Catarina	B
3	12–20/April/12	Sergipe	C
4	21/April to 11/May/12	Sergipe	C
5	19/June/12	Bahia	D
6	21/June/12	Bahia	E
7	23/July/12	Bahia	D
8	01/August/12	Bahia	D
9	12/August/12	Bahia	D
10	September/12	Santa Catarina	F
11	03/September/12	São Paulo	G
12	14/September/12	Paraná	H
13	18/September/12	Bahia	E
14	23/September/12	Bahia	D
15	25/September/12	Rio Grande do Norte	A
16	01/October/12	Rio Grande do Norte	I
17	03/October/12	São Paulo	G
18	05/October/12	Rio Grande do Norte	J
19	06/October/12	Bahia	D
20	15–30/October/12	Rio Grande do Sul	K
21	16/October/12	São Paulo	G
22	25/October to 08/nov/12	São Paulo	G
23	01–20/nov/12	Rio Grande do Sul	K
24	20/November to 04/December/12	São Paulo	G
25	25/November to 10/December/12	Rio Grande do Sul	K
26	28/November/12	Bahia	L
27†	December/12	São Paulo	M
28	December/12	Mato Grosso	N
29	06–13/December/12	São Paulo	G
30	08–15/January/13	São Paulo	G
31	18–25/January/13	São Paulo	G
32	11/February/2013	Bahia	L
33	26/April to 04/May/13	Bahia	O
34	25/April to 01/May/13	Bahia	P
35†	May/13	Paraná	Q
36	June/13	Mato Grosso	R
37	June/13	Bahia	S
38†	June/13	São Paulo	M
39	June/13	Bahia	T
40†	September/13	São Paulo	U
41	05/September/13	Mato Grosso do Sul	V
42†	November/13	Paraná	X
43†	November/13	São Paulo	U
44†	December/13	São Paulo	U
45†	December/13	Paraná	Q

*Equal letters in this column indicate samples collected in the same apiary.

†Samples purchased on the market.

India) and incubation at 30 ± 1°C (Portuguese Standard, 2002a). The count was determined using the formula:

$$\text{CFU g}^{-1} = \sum C/[V * (n_1 + 0{\cdot}1n_2) * d]$$

where: $\sum C$ = sum of colonies counted on all plates (15–150 colonies);

V = volume of inoculum inoculated in each plate;

n_1 = number of plates on which the first dilution was counted;

n_2 = number of plates on which the second dilution was counted;

d = dilution from which the first counts were obtained.

To count the total number of psychrotrophs, 0·1 ml aliquots of the dilutions were inoculated onto the surface of Petri dishes containing solidified PCA and incubated at 6·5 ± 0·5°C for 10 days (Cousin *et al.* 2001). Yeasts and moulds were counted according to ISO 21572-2:2008 (2008), where 0·1 ml of the dilutions was seeded onto the surface of Petri dishes containing Potato Dextrose Agar (HiMedia, Mumbai, India) acidified with tartaric acid and then incubated at 25 ± 1°C for 5 days. The results were expressed as colony forming unit per g of dehydrated bee-pollen (CFU g^{-1}).

Yeasts were identified using an API 20C AUX system (BioMerieux, Hazelwood, MO, USA) with a pattern of strains previously identified by PCR for comparison. Previously, the cultures were isolated in Petri dishes containing Sabouraud agar (HiMedia, Mumbai, India) after incubation for 48 h at 25 ± 2°C. Each strain was inoculated into 2 ml of sodium chloride solution (0·85%, w/v) to achieve turbidity visually equivalent to a 2 McFarland turbidity standard. Then, 100 μl of this preparation was inoculated into tubes containing C medium, and the contents were transferred to the corresponding API 20C AUX yeast panel. Each panel was incubated at 30 ± 2°C for 48 h. Identification was performed according to the manufacturer's instructions (Nogueira *et al.* 2012).

The enumeration of total coliforms and *E. coli* was performed using a SimPlate of BioControl kit (AOAC, 2000). The culture medium was prepared following the manufacturer's instructions. Then, 1 ml of the dilution was inoculated in culture medium and vortexed. The *inocula* were transferred to the kit plates and the plates were incubated at 37 ± 1°C for 24–48 h. Wells where the colour changed from blue to pink after incubation were considered positive for total coliforms, and these were considered positive for *E. coli* if they fluoresced under a UV lamp (Viber Lourma, Merck, Paris, France, TLC Silica gel 60 F_{254}) at a wavelength of 365 nm. The number of wells positive for total coliforms and *E. coli* were converted to the most probable

number per g of dehydrated bee-pollen (MPN g^{-1}) according to the table provided by the manufacturer.

Determination of coagulase-positive *Staphylococcus* was performed as described in Portuguese Standard (2002b). First, 0·1 ml aliquots of the dilutions were inoculated by spreading on Baird-Parker agar (HiMedia, Mumbai, India) enriched with egg yolk and incubated for 24 h at 37 ± 2°C. Typical and atypical colonies were inoculated into tubes containing brain heart infusion broth (VWR, Denver, CO, USA) and incubated for 24 h at 37 ± 2°C. Finally, we proceeded to the coagulase test using rabbit serum (Merck, Darmstadt, Germany). The results were expressed as colony forming units per g of dehydrated bee-pollen (CFU g^{-1}).

The occurrence of sulphite-reducing clostridia spores was determined as described in the ISO 15213:2003 (2003). First, 10, 5 and 1 ml samples of the initial dilution were placed into sterilized test tubes and subjected to heating in a water bath at 80°C for 10 min. The contents of the tubes were poured into Petri dishes and then incorporated into culture Iron Sulphite Agar (HiMedia, Mumbai, India) previously prepared according to the manufacturer's instructions. The incubation occurred at 37 ± 2°C for 48 h under anaerobic conditions. Plates with characteristic black colonies were considered positive. The results were expressed as the presence or absence of sulphite-reducing clostridia spores in 0·01 g of sample.

To determine the occurrence of *Salmonella* sp., we used the 1–2 TEST kit (BioControl) (AOAC, 2000) prepared according to the manufacturer's instructions. A 100 μl initial dilution was added to the kit, followed by incubation for 14–30 h at 37 ± 2°C. The results were defined as positive for *Salmonella* sp. upon the formation of a white band, i.e. U-shaped or meniscus-shaped. The results were expressed as the presence or absence of *Salmonella* sp. in 25 g of dehydrated bee-pollen.

The results were shown as mean and standard deviation of three replicates. The dates were submitted to ANOVA with a F test and, when relevant, a Tukey's HSD test with $\alpha = 0{\cdot}05$. The *Statistical Analysis System* 6.0 program was used for the calculations.

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Conflict of Interest

The authors have no conflict of interests.

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