

CHEMICAL COMPOSITION AND MICROBIOLOGICAL QUALITY OF BEE POLLEN

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ABSTRACT - Besides honey and propolis, bee products already well consolidated, pollen also has relevant economic, nutritional and functional value. As the quality of the final product is directly related to the region where it is collected and processed, this work has evaluated the nutritional and microbiological quality of two dehydrated bee pollens from São Paulo state and the other produced by beekeepers from northern of Mato Grosso state, but with no inspection. Physical-chemical and microbiological quality, phenolic composition and mineral profile analysis were performed. It was observed that both pollens presented satisfactory quality regarding protein contents ($24.8 \pm 2.4 \text{ g } 100 \text{ g}^{-1}$), total sugars ($36.2 \pm 1.1 \text{ g } 100 \text{ g}^{-1}$), lipids ($4.0 \pm 0.3 \text{ g } 100 \text{ g}^{-1}$), ashes ($2.6 \pm 0.05 \text{ g } 100 \text{ g}^{-1}$), free acidity ($238.7 \pm 4.5 \text{ mEq Kg}^{-1}$) and pH (4.8 ± 0.03), however regarding the humidity levels ($6.6 \pm 2.2 \text{ g } 100 \text{ g}^{-1}$) both samples were not in accordance with recommended by law ($<4 \text{ g } 100 \text{ g}^{-1}$). The bioactive profile has shown a significant amount of phenolic compounds ($37.3 \pm 1.1 \text{ mg GAE g}^{-1}$) and flavonoids ($41.8 \pm 2.5 \text{ mg QE g}^{-1}$), besides potential antioxidants around, approximately, 50 and 80%. Microbiological analyzes have revealed low bacterial contamination ($\leq 3.6 \text{ MPN g}^{-1}$), molds and yeasts (variation between 1.2×10^3 and $4 \times 10^1 \text{ CFU g}^{-1}$), according to values stipulated by the current legislation, being observed the absence of *Salmonella* sp. and *Escherichia coli*. The bee pollens investigated have nutritional quality and safety for consumption. However, pollen from São Paulo state showed greater antioxidant potential, probably due to its higher content of phenolics when compared with pollen from Mato Grosso state.

Keywords: beekeeping of Mato Grosso, nutritional composition, microbiological evaluation.

COMPOSIÇÃO QUÍMICA E QUALIDADE MICROBIOLÓGICA DE PÓLEN APÍCOLA

RESUMO - Além do mel e própolis, produtos apícolas já bastante consolidados, o pólen também apresenta valor econômico, nutricional e funcional relevante. Como a qualidade do produto final está diretamente relacionada com a região onde é coletado e processado, este trabalho avaliou a qualidade nutricional e microbiológica de dois pólenes apícolas desidratados, um inspecionado, oriundo de São Paulo e o outro produzido por apicultores do norte de Mato Grosso, porém sem inspeção. Foram realizadas análises de qualidade físico-química e microbiológica, composição de fenólicos e perfil de minerais. Foram observados que ambos os pólenes apresentaram qualidade satisfatória quanto aos teores de proteína ($24.8 \pm 2.4 \text{ g } 100 \text{ g}^{-1}$), açúcares totais ($36.2 \pm 1.1 \text{ g } 100 \text{ g}^{-1}$), lipídios ($4.0 \pm 0.3 \text{ g } 100 \text{ g}^{-1}$), cinzas ($2.6 \pm 0.05 \text{ g } 100 \text{ g}^{-1}$), acidez livre ($238.7 \pm 4.5 \text{ mEq Kg}^{-1}$) e pH (4.8 ± 0.03), porém quanto aos níveis de umidade ($6.6 \pm 2.2 \text{ g } 100 \text{ g}^{-1}$) ambas as amostras não estavam em conformidade com o preconizado pela legislação ($<4 \text{ g } 100 \text{ g}^{-1}$). O perfil bioativo apresentou relevante quantidade de compostos fenólicos ($37.3 \pm 1.1 \text{ mg GAE g}^{-1}$) e flavonoides ($41.8 \pm 2.5 \text{ mg QE g}^{-1}$), além de atividades antioxidantes entre, aproximadamente, 50 e 80%. As análises microbiológicas revelaram baixa contaminação por bactérias ($\leq 3.6 \text{ NMP g}^{-1}$), bolores e leveduras (variando entre 1.2×10^3 e $4 \times 10^1 \text{ UFC g}^{-1}$), segundo valores estipulados pela legislação vigente, sendo observada a ausência de *Salmonella* sp. e *Escherichia coli*. Os pólenes apícolas investigados apresentaram qualidade nutricional e segurança para consumo. No entanto, o pólen do estado de São Paulo apresentou maior potencial antioxidante, provavelmente devido ao seu maior teor de fenólicos quando comparado com o pólen do estado de Mato Grosso.

Palavras-chave: apicultura de Mato Grosso, composição nutricional, avaliação microbiológica.

INTRODUCTION

In addition to the floral nectar that bees use in their food, pollen is also collected mainly because it is a highly protein food for bees. Unlike floral pollen, bee pollen is defined as the result of pollen agglutination of flowers by worker bees using nectar and its salivary substances, which is collected at the entrance of the beehive (BRASIL, 2001b; BARTH, 2004). Some studies have reported the nutritional quality and therapeutic effects

of bee pollen on human health, such as antimicrobial activity (BASIM et al., 2006), antifungal (OZCAN, 2004), antioxidant (MORAIS et al., 2011), anti-inflammatory (MARUYAMA et al., 2010), anticancer (FURUSAWA et al., 1995), among others.

Bee pollen presents great variation in its chemical composition (ALMEIDA-MURADIAN et al., 2005; NOGUEIRA et al., 2012), being observed the presence of more than 200 substances in its composition

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(KOMOSINSKA-VASSEV et al., 2015). These variations are indicative that the product has great influence of the place where the hives are present, due to the climate, the flora and the seasons of the year in which the pollen is collected from the collection boxes (MELO et al., 2009; ARRUDA et al., 2013), being relevant the investigation of the quality of bee pollen produced in different places.

The production of bee pollen has been gaining attention in most countries, as it is an activity with positive economic, social and ecological impacts. According to Valdés (2014) the countries that produce bee pollen most are Australia, Argentina, Brazil, China, Spain and Vietnam. Currently, all Brazilian states produce bee pollen, and the states of Santa Catarina and Bahia are the largest producers (BARRETO et al., 2006; NEVES et al., 2009). The northern of Mato Grosso state is characterized as a transition area between two ecosystems: Brazilian, cerrado and amazon (IBGE, 2004), so the diversity of the flora is a relevant factor in the composition of honeys, pollen or propolis produced in the region. However, no studies have been found that have characterized and evaluated the quality of bee pollen produced in northern Mato Grosso.

In Brazil, pollen can be commercialized *in natura* and dehydrated, the second one conditioned to provide a maximum of 4% moisture, which prevents the proliferation of microorganisms, particularly pathogenic bacteria. Although it is a product with a low water activity, post-harvest care of pollen grains is necessary to keep its quality, both microbiological and nutritional, mainly because it is a food rich in proteins and carbohydrates (FEÁS et al., 2012; ARRUDA et al., 2013; SATTLE et al., 2015).

Food quality standardization and inspection agencies such as the Ministry of Agriculture, Livestock and Food Supply (Ministério da Agricultura, Pecuária e Abastecimento, MAPA) and the National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária, ANVISA), as well as the state and municipal agencies, certify that the products are safe for consumption. Thus this study has evaluated the nutritional and microbiological quality of bee pollen without inspection, produced and marketed by beekeepers in the northern state of Mato Grosso.

MATERIAL AND METHODS

Sample collection and preparation

Two pollen samples were acquired: one from the Sinop (MT) retail market, produced in Franca (SP) with an inspection seal of the state of São Paulo, sold in polyethylene terephthalate (PET) packages. The second one was collected and benefited by beekeepers of Santa Carmen City (MT), being produced in a handmade way and sold with no inspection seal, in polypropylene (PP) packages.

Once the samples were received at the Food Science and Technology Laboratory of the Federal University of Mato Grosso, Sinop *Campus*, one part was separated into sterile packages for the microbiological

analyzes and the remainder kept in the initial package, both stored in a freezer ($-18 \pm 2^\circ\text{C}$).

Nutritional and bioactive composition

The determination of the nutritional composition followed methods established by Instituto Adolfo Lutz (2008) and Almeida-Muradian et al. (2012). Moisture content analysis was performed by infrared under-radiation drying at 85°C (GEHAKA, MODEL IV - 2000); pH through pH meter (TECNOPON, mPA 210) and water activity per dew point (AquaLab Series 4TE).

The lipid content was determined by Soxhlet extraction using petroleum ether as the solvent; protein by the Kjeldahl method, using the conversion factor 6.25 of nitrogen in protein; ash by incineration of organic matter at 550°C (calcination). Total sugars were determined from the Lane-Eynon method and free acidity by titulometry (or titration) using 0.1 N NaOH as titrant. The levels of heavy metals (Pb and Cd) and essential metals (Cu, Ni, Zn, Cr and Mn) were also measured by atomic absorption spectrophotometry, and the data expressed in mg Kg^{-1} (HSEU, 2004).

The total phenolic and flavonoid compounds in pollen were measured by spectrophotometry in ethanolic extracts of pollen, using gallic acid and quercetin respectively as reference standards and readings at 760 and 425 nm, the results were expressed in mg equivalents of gallic acid (mg EGA g^{-1}) and mg of quercetin (mg EQ g^{-1}) (NEVES et al., 2009). The antioxidant potential was evaluated by sequestration of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, with reading at 517 nm in a UV-VIS spectrophotometer (Biospectro, SP-220), the results expressed as % of inhibition of the free radical using the formula:

$$\text{Antioxidant potential (\%)} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

The identification and quantification of phenolic acids and flavonoids in pollens were conducted by High Performance Liquid Chromatography (HPLC), according to the methodology described by Barbari'c et al. (2011), with changes. Varian Modular Analytical HPLC System was employed, equipped with UV detector to 290 nm, chromatography column C18 reverse phase Agela (4.6 mm x 250 mm, 5 μm particle diameter) and two mobile phases (A and B): water/methanol/acetic acid (93:5:2) (A) and water/methanol/acetic acid (3:95:2) (B). The elution was carried out at a flow rate of 1 mL min^{-1} , using the following gradient expressed in time (min.) by percentage of B (t min^{-1} , % B): (0, 20), (20, 40), (30, 52), (50, 60), (70, 80), (80, 20). The same extracts were used for the analysis of total phenols and an aliquot of 20 μL was injected into the chromatographic system. Standard curves were performed for the identification and quantification of phenolic acids and flavonoids using the following reference substances: gallic acid, caffeic acid, ρ -coumaric acid, ferulic acid, quercetin, kaempferol and apigenin.

Microbiological analyzes

In order to evaluate the microbiological quality of the samples, analyzes of total coliforms, coliforms at 45°C were performed, *Escherichia coli* using multiple tubes technique (KORNACKI and JOHNSON, 2001) and *Salmonella* sp. by biochemical tests: LIA (Lysine Iron Agar), TSI (Triple Sugar Iron), urease, citrate, indole, malonate, MV (Methyl Red) and VP (Voges-Proskauer) (APHA, 2001). The results were expressed in most probable number per gram of sample (MPN g⁻¹). In addition, the standard counts of molds and yeasts on potato dextrose agar (BEUCHAT and COUSIN, 2001), the results being expressed as the colony forming units per gram of sample (CFU g⁻¹).

Statistical analyzes

A completely randomized design was used; the contents of the analyzed variables were presented by means of three replicates and their respective standard

deviations. The parameters were compared with those recommended by the Brazilian legislation for the quality of dehydrated bee pollen (BRASIL, 2001b). It was also carried out a simple correlation analysis between variables using t-test at the 5% level of significance using the Statistica 6.0 software from StatSoft (Tulsa, USA).

RESULTS AND DISCUSSION

Table 1 shows the chemical composition of the analyzed pollens and the standards of the current legislation for the product. In the analyzed pollens, moisture content was higher than that allowed by the legislation for dehydrated bee pollen, with the highest pollen content in São Paulo (BRASIL, 2001b). However, the water activity (Aw) was below 0.52, a value stipulated by AOAC (2010) as a limitation for the food deterioration reactions, thus, the two pollen samples, even with high humidity, Aw was low, which makes the product stable for the action of microorganisms.

TABLE 1 - Physical-chemical and bioactive characterization of bee pollen produced in Mato Grosso State and São Paulo State ± standard deviations.

Parameters evaluated	Mato Grosso State	São Paulo State	Legislation standard ¹
Moisture (g 100 g ⁻¹)	5.46 ± 0.25	7.86 ± 0.15	<4
Water activity	0.418 ± 0.002	0.418 ± 0.003	VNS*
Lipids (g 100 g ⁻¹)	3.74 ± 0.17	4.26 ± 0.51	>1.8
Crude protein (g 100 g ⁻¹)	18.64 ± 1.49	31.05 ± 3.31	>8
Ashes (g 100 g ⁻¹)	2.29 ± 0.06	2.97 ± 0.05	<4
Total sugars (g 100 g ⁻¹)	41.54 ± 1.46	30.96 ± 0.83	14.5 a 55
pH	4.82 ± 0.03	4.82 ± 0.03	4 a 6
Free acidity (mEq Kg ⁻¹)	210.66 ± 3.81	266.72 ± 5.28	<300
Phenolic (mg EGA g ⁻¹)	34.52 ± 1.47	40.02 ± 0.79	VNS*
Flavonoids (mg EQ g ⁻¹)	41.49 ± 3.79	42.7 ± 1.14	VNS*
Potential antioxidant (%)	46.25 ± 9.43	82.7 ± 0.36	VNS*

*VNS: Value Not Specified, ¹Brasil (2001b).

Barreto et al. (2005) evaluated bee pollens from seven Brazilian states and the Distrito Federal, and observed moisture levels within the established by law in half of the locations (Paraná, Rio Grande do Sul, São Paulo and the Distrito Federal), revealing that control of humidity is an aggravating factor for the quality control of the product and, therefore, for the expansion of the production chain of the bee pollen. Possibly the high moisture contents of the samples are related to failures in the product dehydration process, since, according to Bastos et al. (2003), in a survey carried out in the states of São Paulo and Minas Gerais on the quality of dehydrated bee pollen, it was found out that most beekeepers used unsatisfactory dehydration methods such as hair dryers, gas ovens or microwaves. In addition, the permeability of water vapor in the packages should be considered because, in the samples tested, the one produced and processed in Mato Grosso uses a packing classification 05 (polypropylene - PP), while that of São Paulo is classification 01 (polyethylene terephthalate - PET). Bessa et al. (2015) evaluated different plastic packaging materials regarding the permeability to water vapor and

verified that PET packaging presented the highest permeability.

From the simple correlation analysis between variables it was possible to observe that total sugars and crude protein had an inverse relationship ($r=-0.9354$). This behavior was also observed in 36 pollen samples from southern Brazil (CARPES et al., 2009). In addition, Kostić et al. (2015) also performed correlation analysis and observed a value of $r=-0.98$ between carbohydrates and protein. Water activity and pH of both samples were similar, whereas higher ash contents and free acidity were found in the bee pollen of São Paulo (Table 1). These observed variations are possibly related to edaphoclimatic factors or to the botanical origin of the product, important aspects for the characteristic of apicultural products already mentioned by several other authors (BARRETO et al., 2005; CARPES et al., 2008; NEVES et al., 2009; MARTINS et al., 2011; KOSTIĆ et al., 2015).

However, the nutritional composition of the pollen investigated is in accordance with the Brazilian legislation (BRASIL, 2001b) and, similar to the levels reported in other studies, that is, variation of protein levels

from 8.3 to 25.1 to $\text{g } 100 \text{ g}^{-1}$, sugars from 26 to 41.7 to $\text{g } 100 \text{ g}^{-1}$, ashes from 0.5 to 3.1 to $\text{g } 100 \text{ g}^{-1}$ and lipids from 2.3 to 8.2 $\text{g } 100 \text{ g}^{-1}$ (ALMEIDA-MURADIAN et al., 2005; NOGUEIRA et al., 2012; ARRUDA et al., 2013 and DE-MELO et al., 2016). Regarding the antioxidant potential, it was observed that São Paulo pollen was higher than that of pollen from Mato Grosso, reaching almost double. According to the correlation analysis it was possible to observe that the phenolic compounds showed a positive correlation with the antioxidant activity ($r=0.8715$).

Neves et al. (2009) found antioxidant potential variation from 60 to 93 in bee pollen collected in some cities of Alagoas, Bahia, Sergipe and Minas Gerais, and correlated the high antioxidant potential with the significant concentration of total phenolic compounds, except for apicultural pollen of Maceió (AL) and Saúde (BA), indicating that other substances, besides the phenolic ones, also captured the DPPH radicals. The content of phenolic compounds observed was higher in São Paulo pollen, while total flavonoids were similar. The levels of phenolic compounds found in the investigated bee pollen corroborate with a wide variation described in the scientific literature, from 12.9 to 48.90 mg EAG g^{-1} . However, the levels of flavonoids found in this study were higher than those observed by the authors (mean of 5.8 $\text{mg catechin equivalents g}^{-1}$ e 8.92 $\text{mg quercetin equivalents g}^{-1}$) (CARPES et al., 2008; CARPES et al., 2009; FEÁS et al., 2012). This wide variation in the levels of these bioactive compounds demonstrates the need to evaluate the chemical composition of apicultural pollen produced in different Brazilian regions, since they may present differences in potential use as a dietary supplement.

Figures 1 and 2 show the chromatograms obtained by HPLC analysis of São Paulo and Mato Grosso samples, respectively. Mato Grosso pollen showed levels of 0.87 ± 0.01 and $4.15 \pm 0.45 \text{ mg g}^{-1}$, respectively, for gallic and ferrulic acids. While pollen from São Paulo showed significant amounts of p-coumaric acid (3.10 ± 0.22), quercetin (0.81 ± 0.11), apigenin (2.21 ± 0.04) and kaempferol ($3.55 \pm 0.11 \text{ mg g}^{-1}$).

De-Melo (2015) evaluating bee pollen produced in Brazil by *Apis mellifera* bees also found average contents of $0.15 \text{ mg } 100 \text{ g}^{-1}$ of caffeic acid and variation of 0.47 to $6.68 \text{ mg } 100 \text{ g}^{-1}$ of p-coumaric acid; 0.71 to $1.04 \text{ mg } 100 \text{ g}^{-1}$ ferrulic acid; 1.86 to $67.91 \text{ mg } 100 \text{ g}^{-1}$ of quercetin and 5.5 to $44.97 \text{ mg } 100 \text{ g}^{-1}$ of kaempferol. Almeida et al. (2017) reported in the same product, levels of p-coumaric acid, ferrulic acid, quercetin and kaempferol of respectively 0.24 ; 0.01 ; 0.32 and 0.68 mg g^{-1} . However, Cheng et al. (2013) identified other flavonoids in this bee product, such as resveratrol, galangin, vanillic acid, hesperetin, protocatechic acid, as well as those already observed in this and other works.

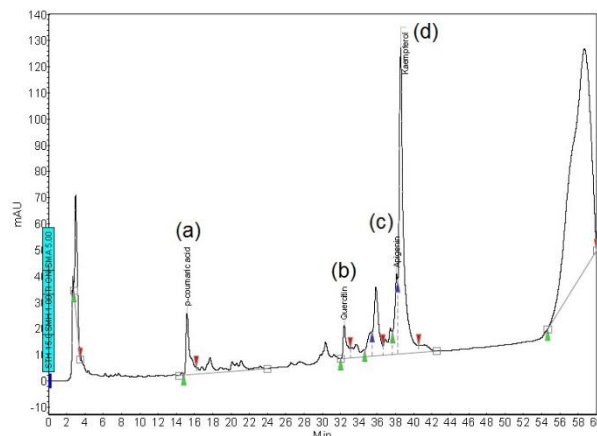


FIGURE 1 - Chromatogram of pollen produced and processed in São Paulo. (a) p-coumaric acid, (b) quercetin, (c) apigenin and (d) kaempferol.

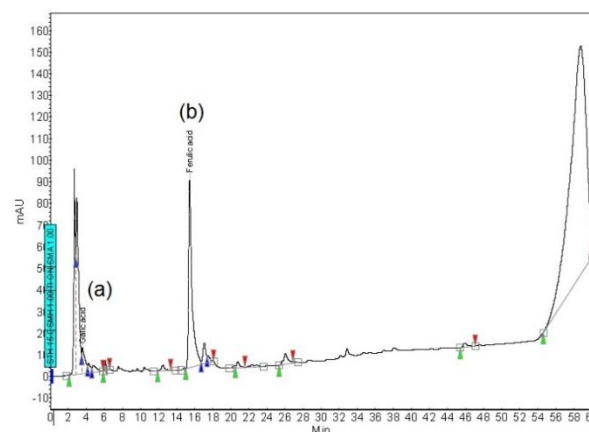


FIGURE 2 - Chromatogram of pollen produced and industrialized (artisanal) in Mato Grosso. (a) gallic acid; (b) ferrulic acid.

The variations in the chemical composition of the investigated pollens may be related to the variation of its botanical origin, since Mato Grosso and São Paulo floras are different, where the first one involves the biome of transition between Amazonia and Cerrado, while in the second one there is the predominance of Atlantic Forest. Since the location and edaphoclimatic factors influence in the chemistry composition of the apicultural products (BARRETO et al., 2005; CARPES et al., 2008; NEVES et al., 2009; MARTINS et al., 2011; KOSTIĆ et al., 2015). Regarding the quantities of essential and toxic metals investigated, São Paulo bee pollen showed higher amounts of Cu and Zn than Mato Grosso. However, Cr, Ni and Mn did not vary between the two samples tested. None of the samples detected the heavy metals Pb and Cd (Table 2).

TABLE 2 - Average values of metals in bee pollen from Mato Grosso and São Paulo (mg kg⁻¹) ± standard deviation.

Bee pollen	Pb	Cd	Cr	Cu	Ni	Zn	Mn
Mato Grosso	ND*	ND	3.85 ± 3.56	1.22 ± 0.01	1.22 ± 0.62	23.41 ± 2.34	18.52 ± 0.83
São Paulo	ND	ND	3.10 ± 1.25	8.27 ± 1.36	1.65 ± 0.53	35.22 ± 3.33	19.67 ± 0.81

*ND: not detected.

Institutions such as the National Research Council (1989), the World Health Organization and the Food and Agriculture Organization of the United Nations (FAO, 2004) recommend for adults the daily intake of 7.0 mg kg⁻¹ of Zn, Mn and 11 for Ni. Therefore, 50 g daily of the analyzed pollen would meet the need of an adult of Zn, Mn and Ni in 21%, 27% and 0.2%, respectively. While the maximum levels of Cu and Cr allowed in foods are 10 and 70 mg Kg⁻¹, then the levels of these minerals found in the analyzed pollen are safe, i.e. they are below the limit prescribed by law (BRASIL, 1998; MERCOSUL, 2011).

Other authors evaluated the levels of essential metals to the human organism and found variations from 6.8 to 13.1 mg kg⁻¹ of Cu, 28 to 76.4 mg Kg⁻¹ of Mn, 29 to 53.6 mg Kg⁻¹ of Zn, and toxic metal values between 0.13 to 0.45 of Pb, 0.007 to 0.051 of Cd, 0.19 to 0.39 of Cr and 0.63 to 1.75 mg kg⁻¹ of Ni (CARPES et al., 2009; MORGANO et al., 2010; 2012; SATTLER et al., 2016). The variations in pollen physicochemical composition is related to edaphoclimatic factors such as soil, flowering, time of year, among others.

Regarding the microbiological quality of the evaluated products, it was observed that for molds and yeasts, the Mato Grosso sample had higher counts than those of the São Paulo sample, being found, respectively, 1.2x10³ and 4x10¹ CFU g⁻¹. Regarding total coliform counts, 45°C and *Escherichia coli* was less than 3 MPN g⁻¹ in 83.3% of the samples tested, being found in 16.7% of the samples 3.6 MPN g⁻¹ for coliforms at 35°C in the São Paulo sample. In addition, the absence of *Salmonella* sp. in the products investigated.

Almeida-Muradian et al. (2012) evaluated bee pollen from Ribeira do Pombal (BA) microregion and found values between 1.5x10² and 1.48x10⁴ CFU g⁻¹ for molds and yeasts, while De-Melo et al. (2015) observed counts of <10 at 7.67x10³ CFU g⁻¹ in samples from nine Brazilian states. Therefore, both apiculture pollens investigated are safe for consumption, since they are in accordance with that recommended by RDC 12/2001 of ANVISA, which establishes the microbiological parameters for food products, recommends a maximum tolerance of 1x10³ MPN g⁻¹ for thermotolerant coliforms and absence of *Salmonella* sp. (BRASIL, 2001a).

The low bacterial count may be related to the Aw lows observed in the evaluated pollens (0.42), since this intrinsic parameter is a limiting factor for the development of microorganisms. De-Melo et al. (2015) evaluating the microbiological quality of pollen from nine Brazilian states found counts of <10 to 2.8x10³ MPN g⁻¹ of total coliforms. Through the biochemical tests performed, none of the analyzed samples showed confirmation for *Salmonella* sp. Féas et al. (2012) evaluating the quality of organic bee pollen also observed the absence of

Salmonella and *E. coli* in all the samples evaluated. Thus, confirming the microbiological quality of the pollens evaluated.

CONCLUSIONS

The bee pollens investigated have nutritional quality and safety for consumption, being indicated mainly as a dietary supplement due to their physical-chemical and microbiological characteristics. However, pollen from São Paulo state showed greater antioxidant potential, probably due to its higher content of phenolics when compared with pollen from Mato Grosso state.

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