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NOTES AND COMMENTS

Microbiological and chemical characterization of bee pollen throughout the production process in the Southwest of Buenos Aires Province (Argentina)

Leticia A. Fernández[a]*, Juliana Susca Tromba[a], Adriana M. Alippi[b], Fernando M. López[c], Mónica Pérez[d] and Liliana M. Gallez[a]

[a]*Laboratorio de Estudios Apícolas (LabEA-CIC), Departamento de Agronomía, Universidad Nacional de Sur, Bahía Blanca, Argentina;* [b]*Centro de Investigaciones de Fitopatología, CIDEFI (CIC/UNLP)-Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina;* [c]*Departamento de Agronomía, Universidad Nacional de Sur, Bahía Blanca, Argentina;* [d]*Departamento de Química, Universidad Nacional del Sur, Bahía Blanca, Argentina*

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In order to evaluate if the different stages of bee pollen production could lead to changes in the microbiota, counts of filamentous fungi and yeast, culturable heterotrophic mesophilic bacteria, aerobic spore-forming bacteria, sulfite-reducing clostridia, enterobacteria, total and thermotolerant coliforms, and the study of human pathogenic bacteria were performed. Also, the chemical characterization was carried out. We report the analysis of 36 bee pollen samples which were obtained from different sampling points throughout the production process (collecting, freezing, drying, and cleaning) in the Southwest of Buenos Aires province, Argentina. In bee pollen samples, *Escherichia coli, Salmonella* sp., coagulase-positive *Staphylococcus,* and *Clostridium perfringens* were not detected. A total of 2.90 x 10^3 colony forming units of *Bacillus cereus* group were counted. Culturable heterotrophic mesophilic bacteria as well as yeasts showed the highest values and drying and freezing stages did not reduce either. Although bee pollen samples were manipulated following appropriate practices under good hygienic conditions they presented counts of filamentous fungi and yeasts higher than the Argentine Food Code tolerance while they did fit the European Codes requirements. Thus, this data offers scientific support to suggest a revision of our Code in order to establish an official method for carbohydrate content analysis and to allow higher limits for filamentous fungi and yeasts for bee pollen destined to human consumption.

Keywords: bee pollen; beekeeping; yeasts; filamentous fungi; culturable heterotrophic mesophilic bacteria

Nowadays, large quantities of bee pollen (BP), either processed or unprocessed, are sold worldwide for human and animal consumption (Belhadj, Harzallah, Dahamna, & Khennouf, 2014). BP can be characterized as a functional food which means that it resembles traditional foods with demonstrated physiological benefits: improving health and reducing disease risk (Soares de Arruda et al., 2017).

There are several publications from different countries on microbiological characteristics of BP ready to be sold in the retail market (Belhadj et al., 2014; De-Melo, Estevinho, & Almeida-Muradian, 2015). However, there is still a lack of information on the microbiological quality of BP at different stages of the production process.

We aimed to characterize the microbiological quality and the chemical composition of BP samples which were obtained from different sampling points of the process (collecting, freezing, drying, and cleaning) in the Southwest of Buenos Aires province, Argentina. We hypothesized that the different stages of BP production could lead to changes in the microbiota. This information would allow to know the microbiological traits of hygienically processed BP and to identify the critical control points to achieve a quality product.

BP production process designed by Cooperativa de Trabajo Apícola Pampero Limitada (CAP) (Bahía Blanca, Argentina) involved four stages separated in time and space: collecting, freezing (-10 °C), drying in an electric oven with forced air circulation at 40 °C during 48 h, and cleaning in a special machine designed by CAP. The study was done with 36 BP samples from the production line of three beekeepers from the Southwest of Buenos Aires province. Nine samples were obtained from pollen traps, while 27 from the stages of freezing, drying and cleaning between February and June 2016. All samples were aseptically collected in sterile 100 ml vials and were stored refrigerated (4 °C).

Ten grams of each BP sample were homogenized in 90 ml of peptone water. Decimal serial dilutions were performed and counts of filamentous fungi (FF) and yeast, culturable heterotrophic mesophilic bacteria (CHMB), aerobic spore-forming bacteria (ASFB), sulfite-reducing clostridia (SRC), *Enterobacteriaceae*, total coliforms at 37 °C, and thermotolerant coliforms at 45 °C were evaluated as previously described by Fernández, Ghilardi, Hoffmann, Busso, and Gallez (2017). The following human pathogenic bacteria were studied in 25 g of each BP sample: *Salmonella* sp., *Shigella* sp., coagulase-positive *Staphylococcus, Clostridium perfringens,* and *Escherichia coli*

*Corresponding author. E-mail: lafernan@uns.edu.ar

Table 1. Microbiological determinations in 36 bee pollen samples obtained from the production process in the Southwest of Buenos Aires province (Argentina).

Production process: sampling point	Culturable heterotrophic mesophilic bacteria	Filamentous fungi	Yeasts	Aerobic spore-forming bacteria	Total Coliforms
Collection	4.18 ± 0.41[a]	3.34 ± 0.26	4.63 ± 0.58	2.88 ± 0.00	1.26 ± 1.16
Freezing	4.78 ± 0.12	4.05 ± 0.41	5.03 ± 0.13	3.19 ± 0.06	1.17 ± 0.27
Drying	4.90 ± 0.81	4.11 ± 0.33	5.08 ± 0.36	3.92 ± 1.48	1.55 ± 0.72
Cleaning	4.56 ± 0.13	3.82 ± 0.37	4.78 ± 0.51	3.00 ± 0.00	0.58 ± 0.15

Colony forming units (CFU) values were $\log_{10}$-transformed prior to statistical analysis.
[a]Results are mean of three replicates from each beekeeper (3) ± its standard deviation. ANOVA revealed that there were no significant statistically differences among means ($p \leq 0.05$).

(Fernández et al., 2017). Isolation and identification of *Bacillus cereus sensu lato* (*B. cereus* group) was done according to López and Alippi (2007).

Chemical analyses such as moisture, pH, total carbohydrate (Dubois et al. 1956), and nitrogen content (Campos et al., 2008) as well as ash content were performed in triplicate and results were reported based on air-dried samples (except for pH).

Infostat software (Di Rienzo et al., 2013) was used for statistical analyses. The data from counts on FF, yeasts, CHMB, ASFB, and SRC between the three beekeepers at the different stages of the BP production process were analyzed statistically with the one-way analysis of variance. When a significant F-value was detected, means were compared with LSD test ($p < 0.05$). T-tests were used to compare microbiological counts between BP collection and cleaning stage.

The comparison of MY, CHMB, total coliforms, and ASFB counts for the three beekeepers at the different stages are shown in Table 1. Statistical analysis indicated that none of the microbiological groups showed significant differences among the stages of BP production. SRC and *Enterobacteriaceae* were not detected in any of the 36 BP samples. Regarding total coliforms, counts ranged between 3 to 3.90 x 10^3 MPN g^{-1} of BP at all of the studied sampling points. Thermotolerant coliforms were detected only in samples obtained from collecting and drying stages of a single beekeeper, MPN g^{-1} of BP 1.0 x 10^3 and 45, respectively. Even when no differences in microbial counts were found at the different stages of BP processing (Table 1), all BP samples from a single beekeeper showed the highest counts of microorganisms including coagulase-negative *Staphylococci* and *B. cereus s. l.* as well as the presence of thermotolerant coliforms. Consequently, our results confirmed and reinforced the idea that proper BP handling and sanitation practices would allow to improve the microbiological quality of BP.

Pathogenic bacteria, except *Bacillus cereus s.l.*, were not detected in any of the BP samples throughout the production process. Counts of *Bacillus cereus s.l.* revealed no statistical significant differences in BP samples at the different sampling points. Bacteria belonging to this group are well known for their potential to cause two types of food poisoning syndromes when the levels reach up to 10^5 CFU g^{-1} of food (Lucking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013). It is important to mention that none of the BP samples from the different sampling points reached this value. However, infants, older persons, women who are pregnant and anyone with a compromised immune system are especially susceptible to food-borne illness (Lucking et al., 2013). These people should pay attention when *Bacillus cereus s.l.* have been detected in food.

The effect of processing conditions on the hygienic status of BP samples showed that the stages of drying as well as freezing did not reduce the amounts of the CHMB, FF, and yeasts (Figure 1). These observations were in agreement with those reported by other researchers (Puig-Peña, del Risco-Ríos, Pazos Álvarez-Rivera, Leyva, & García Neninger, 2012; Estevinho, Rodrigues, Pereira, & Feás, 2012) who stated that freezing and drying stages allow the multiplication of microorganisms. Also, the comparison between the initial and the last stage of BP production demonstrated that there were no statistical differences in the counts of CHMB and yeasts. Nevertheless, a logarithmic increase of 0.723 was observed in the number of FF between BP traps and cleaning. Thus, other treatments should be considered in order to prevent microorganism multiplication in BP such as gamma irradiation (Hosny, Sabbah, & El-Bazza, 2018; Meeus et al., 2014).

Moisture, pH, ash, protein, and total carbohydrates of BP samples from the final stage of processing (cleaning) were analyzed. The average values were: moisture 6.08 ± 0.63% (maximum value 5.38 and minimum value 7.52); pH 4.28 ± 0.09 (4.14 and 4.54); ash 2.34 ± 0.05% (2.2 and 2.46); nitrogen 4.88 ± 0.14% (4.66 and 4.99); total carbohydrates 31.76 ± 0.94% (30.48 and 33.68). Protein content showed an average of 27.38 ± 0.78% and ranged from 26.14 to 27.98%. There were no statistically significant differences among BP samples of the three studied beekeepers. Our results were in agreement with literature values reported by several authors even when the samples were collected in different latitudes of South America (De Melo et al., 2016; Soares de Arruda et al., 2017). However, the regulations establish between 45 and 55% for carbohydrate contents. In this study, this chemical parameter was below that minimum. It is possible that this discrepancy was partly due

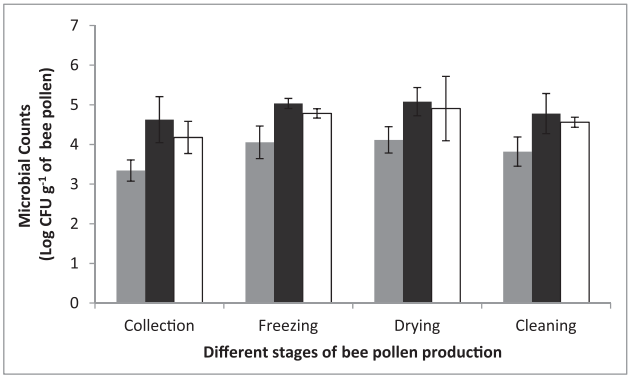

Figure 1. Microbiological counts of filamentous fungi (grey bars), yeasts (black bars) and culturable heterotrophic mesophilic bacteria (white bars) during the different stages of bee pollen production in the Southwest of Buenos Aires province (Argentina). Data are presented as the mean value of three independent counts ± standard deviation.

to differences in the methods used for analysis. In our determinations, total carbohydrate content was analysed by phenol-sulphuric acid test (DuBois et al., 1956), since the regulation does not specify which technique is appropriate.

Taking into account Argentine Food Code, FF and yeasts were over the maximum established limit (100 CFU g^{-1} of BP). Several works on BP from different regions of the world showed that counts of FF and yeast exceed 1.0 x 10^4 colonies g^{-1} of pollen (Estevinho et al., 2012; Belhadj et al., 2014; De Melo et al., 2015; Puig-Peña et al., 2012). Campos et al. (2008) described pollen standards of countries such as Brazil, Bulgaria, Poland, and Switzerland where BP is considered to be a food. They proposed microbiological criteria for regulations very different from those of the Argentine Food Code. In fact, European legislation established a maximum of 5.0 x 10^4 CFU g^{-1} of FF and yeast.

To the best of our knowledge, this is the first work conducted in Argentina on a comprehensive microbiological evaluation of BP throughout the production process. Our results demonstrated that the stages of freezing and drying are the critical control points in bee pollen production. Bee pollen samples manipulated under good hygienic conditions presented counts of FF and yeasts higher than the Argentine Food Code tolerance while they did fit the European Codes requirements. Thus, our data offers scientific support to suggest a revision of our Code in order to establish an official method for carbohydrate content analysis and to allow higher limits for FF and yeasts for BP destined to human consumption.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Leticia A. Fernández http://orcid.org/0000-0003-3785-8003
Juliana Susca Tromba http://orcid.org/0000-0001-8018-1400
Adriana M. Alippi http://orcid.org/0000-0003-4749-1567
Mónica Pérez http://orcid.org/0000-0002-3443-4048

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