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Microbiological characterization of dried and frozen pollen in Viracachá-Colombia

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ABSTRACT

Objective. Microbiologically characterize dry and frozen pollen produced in the municipality of Viracachá-Boyacá. **Materials and methods**. Through a quantitative descriptive cross-sectional study, samples from 5 apiaries were taken, each with 10 hives, separating the pollen dry and frozen, determining for each sample: mesophilic aerobes, total coliforms, fecal coliforms, *Staphylococcus aureus*, *Clostridium* sulfite reducer, and fungus. The data obtained were analyzed according to international regulations and compared with research results in other countries. **Results.** Total and fecal coliforms were found in three of the five apiaries evaluated and only in dried pollen samples. Also, in two apiaries when dry pollen was analyzed, *Staphylococcus aureus* was found. The microbiological results of most samples are within the ranges of some international regulations; however, the best results in terms of microbiological quality were determined for frozen pollen. **Conclusions**. The pollen freezing process offers advantages related to maintaining microbiological quality compared to the drying process. It is necessary to evaluate the microbiological quality of both products throughout the storage time.

Keywords: Bee product; *Apis mellifera*; storage; bee pollen; sanitary quality (*Sources: CAB,DeCS*).

RESUMEN

Objetivo. Caracterizar microbiológicamente el polen seco y congelado producido en el municipio de Viracachá-Boyacá. **Materiales y métodos.** A través de un estudio transversal descriptivo cuantitativo se tomaron muestras de 5 apiarios, cada uno con 10 colmenas, separando el polen en seco y congelado, determinando para cada muestra: aerobios mesófilos, coliformes totales, coliformes fecales, *Staphylococcus aureus*, *Clostridium* sulfito reductor y hongos. Los datos obtenidos se analizaron de acuerdo a normatividades internacionales y se compararon con resultados de investigaciones en otros países. **Resultados.** Se encontraron coliformes totales y fecales en tres de los cinco apiarios evaluados y solo en muestras de polen seco. Además, en dos apiarios cuando se analizó polen seco se encontró *Staphylococcus aureus.* Los resultados microbiológicos de la mayoría de las muestras

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se encuentran dentro de los rangos de algunas normatividades internacionales, sin embargo, los mejores resultados en cuanto a calidad microbiológica se determinaron para el polen congelado. **Conclusiones.** El proceso de congelamiento del polen ofrece ventajas relativas al mantenimiento de la calidad microbiológica en comparación con el proceso de secado. Se hace necesario evaluar la calidad microbiológica de ambos productos a través del tiempo de almacenamiento.

Palabras clave: Producto apícola; *Apis mellifera*; almacenamiento; microbiología; polen; calidad sanitaria (*Fuente: CAB, DeCS*).

INTRODUCTION

Beekeeping is a highly important economic sector in several countries due to the wide variety of products it generates, allowing producers to obtain several layers of income from their sale, taking advantage of consumption trends for natural products (1,2). According to their origin, bee products may be classified as: products collected with no transformation (pollen and propolis), collected with transformation (honey and fruit-honey), by-products from secretions (beeswax, royal jelly, apitoxin) and others like (queen bees, nuclei, bee packs, drone larvae, pollination services) (3).

Bee pollen results from the agglutination of several grains of flower pollen collected by bees, mixed with saliva and small quantities of nectar or honey (4,5,6). This product is traditionally used as food for humans, and it has been demonstrated to possess antimicrobial, anti-inflammatory, antitumor antimutagenic, antioxidant, antiallergenic, antiviral, hypolipidemic, hypoglycemic, liver and kidney protective and immune stimulant properties (7,8,9,10,11).

Due to its manipulation during its collection by bees, its collection at the hive, transportation and storage, pollen is exposed to several sources of contamination that could alter its organoleptic characteristics and, depending on the type of micro-organism that may grow in it, place its sanitary quality at risk and lead to the transmission of diseases, whether as a result of the consumption of microorganisms, or due to the intake of toxic substances resulting from its intermediate metabolism, as in the case of fungi (12,13,14,15).

In Colombia, studies related with the production chain are scarce as compared with other farming topics, a situation that leads to a vacuum in quality scientific information which guides public policies geared to improving the productive chain. In consequence, among other facts, in Colombia there is no regulation regarding the microbiological quality of this type of products, and since they are mostly consumed directly, they might turn into a source for the creation or transmission of diseases; hence, its microbiological monitoring must be rigorous in order to offer a product with optimal conditions (4,16).

Hence, the objective of this study was to microbiologically characterize the dried and frozen pollen produced in the municipality of Viracachá- Boyacá.

MATERIALS AND METHODS

Type and location of the study. A cross-sectional, descriptive, quantitative study was conducted in five apiaries located in the municipality of Viracachá- Boyacá, at the Marquez province, in the central-eastern region of the department of Boyacá, at a height of 2,500 m above sea level, with an average temperature of 15ºC and an annual average rainfall of 824 mm (17).

Sampling. Five apiaries were selected, with 10 hives each, with Canadian-type pollen traps. Every apiary is located at a different village in the municipality of Viracachá. Villages differ in altitude by 200 meters. Samples were taken in each one of them, only once per week, in the months of August-September, 2019.

The pollen collected from the collecting trays in every apiary was packed into independent plastic containers and then transported to a storage facility at the Pueblo Viejo village. There, the pollen from every apiary was submitted to two cleaning procedures, first by air (mechanical cleaning) and then manually, with the help of a magnifying glass, to extract extra elements from the pollen. Every sample was then separated: 50% of it was taken to a drying machine at

a temperature between 45-50ºC for 8 hours, while the rest was taken to freezing point, at a temperature of 0ºC for 8 hours.

200-gram samples were taken from every apiary, and they were placed into sterile jars with screw lids, 100 grams of frozen pollen in one and 100 grams of dried pollen in another one, and on the next day they were processed at the veterinary microbiology department at UPTC.

Transportation. The samples were transported on the same day they were collected. The dried pollen was transported in plastic stacks at room temperature. In turn, the frozen pollen was transported in cellars with ice, monitoring for temperatures between 0 and 4ºC.

At the time of arrival at the veterinary microbiology laboratory, the frozen pollen samples were kept at a temperature of 0ºC till the next day, when they were processed. The dried pollen samples were kept at room temperature during the same period of time.

Microbiological Analysis. The samples were opened in a laminar flow cabin and there, 25 grams from each sample were diluted in 225 mL of sterile peptone saline solution and they were homogenized in an orbital homogenizer at 200 rpm for 10 minutes. Then, dilutions were made in 9 mL of peptone sterile solution. To do this, 1 ml was taken from the first dilution and it was added in the tube to complete 10 ml; then, the same procedure was followed until the three dilutions were completed. The colonies formed in plates were counted and expressed as colony forming units/gram (CFU/gr) (5,18,19).

The microbiological analyses were made with 1 mL of the last solution. The microorganisms to be determined were: Aerobic mesophiles (petrifilm plates incubated at 30ºC for 3 days), total coliforms (violet red bile glucose- VRBG agar, these were incubated at 35ºC and black-reddish colonies measuring 0.5 mm or more in diameter were counted 24 hours after the culture), fecal coliforms (petrifilm® plate for coliforms, incubated at a temperature of 30ºC for 3 days), *Staphylococcus aureus* (Baird-Parker agar and incubated at 37ºC for 48 hours)*, sulfite-reducing Clostridium* spores (the dilution was placed in water at 80ºC for 10 minutes and then inoculated in petri boxes containing iron-sulfite agar; they were incubated for 2 days at 37ºC under anaerobic conditions), molds and yeast (Saboureaud agar, and incubated for 5 days at 30ºC) (5,18).

In order to prevent the peptone solution from altering the results, 1 ml of this was inoculated in each one of the agars previously mentioned and the results are shown in table 1.

Statistical Analysis. An analysis of variance (ANOVA) was conducted for the growth values for each one of the microorganisms assessed for each type of pollen (dried and frozen) in each apiary, taking as reference Fisher's least significant difference procedure, with a confidence level of 95% by means of the Statgraphics-Centurion® Windows 10 software; significantly different values are expressed in ($p<0.05$).

Table 1. Results of the microbiological analysis conducted on dried and frozen pollen.

Microbiological Analysis*	Apiary 1		Apiary 2		Apiary 3		Apiary 4		Apiary 5		Control
	D.P.[1]	F.P.[2]	D.P.	F.P.	D.P.	P.C	D.P.	F.P.	D.P.	F.P.	NA
Aerobic Mesophiles x10^3	12	4	10	6	11	3	9	2	12	6	0
Total Coliforms x10^3	2	0	0	0	3	0	1	0	0	0	0
Fecal Coliforms x10^3	1	0	0	0	2	0	1	0	0	0	0
Staphylococcus aureus x10^3	2	0	1	0	0	0	0	0	0	0	0
S-reducing Clostridium x10^3	0	0	0	0	0	0	0	0	0	0	0
Molds and yeasts x10^3	2	0	5	2	4	3	6	0	1	0	0

*Expressed in CFU/gr. [1] D.P: Dried Pollen. [2] F.P: Frozen Pollen. NA: Not Applicable

RESULTS

Table 1 shows the results of the microbiological analyses conducted on the pollen samples and the peptone solution. No microorganisms were found to have grown from the peptone solution, so the results show microbiological characteristics of pollen. The smallest level of microorganisms growth was found in the frozen pollen sample (Table 1).

Figure 1 shows the statistical differences found for the results in each one of the apiaries assessed. Statistically significant differences were found between dried and frozen pollen regarding the growth of microorganisms. No evidence was found on the presence of sulfite-reducing *Clostridium* spores in any of the pollen samples in all the apiaries assessed.

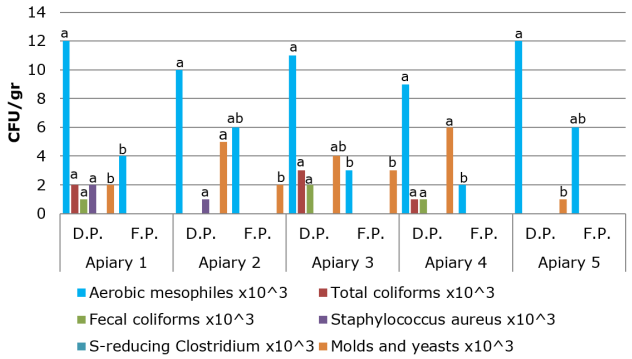

Figure 1. ANOVA for the results of each one of the microbiological analyses. The bars marked with the same letter indicate they belong to the same statistical group.

DISCUSSION

In Colombia, microbiological quality ranges for pollen or other apiculture products for human consumption have not been regulated by national authorities. This situation leads to a serious sanitary problem, due to the likelihood of food poisoning resulting from bee products, not only due to the presence of pathogen microorganisms in food, but also due to the possible production of toxins resulting from their intermediate metabolism, as in the case of some fungi (2,14).

Microbiological contamination of food products may take place at different stages due to the lack of proper handling at the recollection, transportation, processing and packing stages (18). In the case of bee products, these may be polluted from the time of collection and production by the bee, a situation that is mainly influenced by climatic variables (2).

In a study conducted in Colombia, on microorganisms growing in the mature pollen from *Apis mellifera* (after its processing in the beehive) Garcia et al (20) found the growth of microorganisms like: *Streptococcus sp*, *Micrococus sp*, *Pseudomona sp*, *Yersinia sp* and *Arthrobacter sp*, associating them mainly to microorganisms found in the bees' intestines. These microorganisms are not the focus of this study; however, this study shows the correlation between the presence of microorganisms in pollen and the pollen production process itself.

Results show a statistically significant difference between the results for dried and frozen pollen for aerobic mesophiles. In Argentina, national regulations determine some values for microbiological results for pollen; for aerobic mesophiles, the maximum range allowed is $150x10^3$ CFU/gr, which indicates that the two types of pollen assessed comply with this regulation, as well as with regulations from countries like México, Brazil and the European Union, where results should be below $1x10^4$ CFU/gr (1,21,22,23). This shows that, even though both processes comply with international regulations, the best microbiological results were found in frozen pollen (Table 1).

The number of microorganisms (aerobic mesophiles) found reflects the microbiological quality of bee products; high numbers of these reveal contamination of the material at the traps, inadequate cleaning and disinfection of surfaces, insufficient hygiene in pollen preservation, inadequate storage conditions in terms of temperature, or a combination of some of these circumstances (2,24).

Total and fecal coliforms are some of the microorganisms with the highest epidemiological risk due to their capacity to cause alimentary diseases and in consequence they should not be found in food samples for direct human consumption (1). Table 1 shows the growth of total and fecal coliforms in three of the five apiaries assessed, and only in dried pollen samples.

Regulations of the countries previously mentioned require the absence of these microorganisms in pollen samples; hence, these three apiaries do not comply with these regulations. It is worth

highlighting that no coliform growth, whether fecal or total, was found in any frozen pollen sample (1,21,22,23). Besides, it is necessary to clarify that the presence of these microorganisms at the time of collection was not assessed, so it is not possible to affirm when the sample could have been contaminated.

Studies in Portugal have reported the absence of these microorganisms in the total of commercial and organic pollen samples, Estevinho et al (25), Nogueira et al (26) and Fea's et al (27), a situation that differs from the one reported by this study. Contamination by fecal and total coliforms is an indicator of the hygiene conditions of the material, the raw materials, the equipment and instruments used for the collection of the material (2).

In the case of *Staphylococcus aureus*, this should not be found in pollen, according to Mexican and European Union regulations (1,22); however, this microorganism was isolated in dried pollen from two apiaries; besides, in the same two apiaries, the frozen pollen samples did not provide evidence for this agent (Table 1).

The Cuban regulations (28), determine the absence of colonies of sulfite-reducing *Clostridium* due to its pathogenic potential for human beings. All pollen samples, both dried and frozen, show the absence of growth of this microorganism, showing that these samples would comply with the regulations mentioned.

Likewise, a study in Brazil (2) determined the absence of these microorganisms in pollen samples collected for a period of 3 consecutive years in eight different districts from different parts of the country.

The European Union (1) allows for up to 50.000 CFU/gr of mold and yeast in pollen samples, hence, under these standards, all pollen samples would comply with the provisions; on the other hand, the Argentinian (21), Brazilian (23) and Cuban (28) regulations allow a maximum of 100 CFU/gr of these microorganisms, which means that the pollen samples would not comply with the latter standards.

The presence of these microorganisms is mainly associated with environmental conditions in the location of the apiaries, and they serve as a parameter to determine proper or inadequate conditions for their management (2,26).

In several countries, the presence of pollen-polluting microorganisms has been analyzed, (18) in Algeria, the presence of aerobic mesophiles was determined in samples of dried pollen in three apiaries from different municipalities, with values of 620 $x10^3$, $1x10^3$ and $470x10^3$ CFU/gr; these values are high as compared with the ones found in this study for any of the apiaries assessed. Likewise, in the case of total coliforms, they found values of $300x10^3$, 01 $x10^3$ and $19x10^3$ CFU/gr, results which are higher than the ones determined in this study, with the likelihood, therefore, that in Colombia the management conditions of the product are better. When analyzing the product for mold and yeast, the same authors found values of 400, 50 and 230 UFC/g, results which are lower than the ones found in this study for dried or frozen pollen in any apiary.

A similar study conducted in Brazil, including eight states where the quality of dehydrated pollen was assessed, the values for aerobic mesophiles ranged between <10 and 1.10 $x10^4$ CFU/g; molds and yeasts ranged between <10 up to 7.67 $x10^3$ CFU/g, and total coliforms from <10 up to $2.80x10^3$ CFU/g, relating these findings with inadequate hygiene practices during the manipulation, processing and packing process; even including plants contamination, or even, during the time when the pollen is sitting in the traps, as it is exposed to several environmental factors, added to the fact that certain seasons might favor the conditions for the growth of microorganisms (2,4).

On the other hand, also in Brazil, in a stingless bee (*Malipona mandacaia)*, in the regions of João Dourado and Uibaí, Santa Barbara et al (29) did not find microbiological growth of *Salmonella sp*, sulfite-reducing *Clostridium*, fecal coliforms, *Escherichia coli, Staphylococcus aureus*, molds and yeast, which came in sharp contrast with the result of this study, as here, these microorganisms, except for *Salmonella sp* and *Eschericha coli* which were not considered, appeared to grow in the samples collected. Aerobic mesophiles, on the other hand, were considered in this study, finding values of 121.58 CFU/g for the João Dourado region and 375.67 CFU/g for the Uibaí region, results which are lower than the ones determined in this study for any of the two Brazilian municipalities.

Variations among the studies with respect to the latter group of microorganisms may have been due to the differences in antimicrobial

capacity in the pollen of each bee species; for example, Santa Barbara et al (11) determined that the *Apis mellifera* pollen was efficient in the control of Gram positive microorganisms, while its efficiency was lower for Gram negative microorganisms due to factors related with its chemical structure at the cell wall.

In their study, the microbiological characteristics of pollen in three types of stingless bees were determined (*Melipona, Scaptotrigona* and *Frieseomellita*) Santa Barbara et al (19) determined that these samples did not show growth of microorganisms like: *Salmonella sp*, sulfite-reducing *Clostridium* spores, total coliforms, *Escherichia coli* and *Staphylococcus* coagulase positive. They only found a growth pattern in all the samples assessed, for aerobic mesophiles. These results show the disparity between the microbiological results among bee genera, so the management in each of the productive systems at the level of transformation must be adapted to this behavior.

Kačániová et al (30) determined the presence of aflatoxins produced by fungi in samples of frozen pollen, through the ELISA technique, determining that the ZON and T2 mycotoxins appeared in all the pollen samples, showing the great importance of the absence of these microorganisms in food products, which are found in frozen pollen in the three apiaries assessed.

According to Estevinho et al (31), the pollen conservation processes (drying or freezing) show statistically significant differences with respect to their physicochemical composition, together with environmental factors like the collection season and the botanical species present, and this factor may have an influence in the microbiological conditions, since, as previously shown, pollen has an antimicrobial capacity that may be affected by this concentration of nutrients. The same authors mention it is better to consume pollen frozen at a temperature of -20ºC than dried pollen.

The results of this study reveal better microbiological characteristics for frozen pollen as compared with dried pollen for most of the microorganisms assessed. It is necessary that the microbiological quality of these same processes is assessed when the product is submitted to different storage times, as well as the possible changes in its organoleptic characteristics resulting from this process.

Conflicts of interest

The authors declare no conflict of interest.

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