Influence of handling procedure on the microbiological quality of propolis
ABSTRACT

The objective of this study was to evaluate the impact of two handling techniques of propolis, from raw material collection to the final product elaboration, in its microbiological quality. The microbiological parameters analyzed included the counting of aerobic mesophilic and psychrotrophic bacteria, molds and yeasts, total coliforms and *Escherichia coli*, enterobacteria, coagulase-positive *Staphylococcus*; and the quantification of sulfite-reducing *Clostridium* spores, and *Salmonella* spp. The collection, packaging, handling, and the storage of propolis considerably influence the microbial counts and therefore the propolis commercial quality, health, and safety. The adoption of good techniques for the handling of propolis is fundamental to maintain the microbiological quality of the product.

**Keywords:** Propolis, *Apis Mellifera*, Bioindicators, Microorganisms, Handling Technique.
INTRODUCTION

Propolis is a resinous product, harvested by bees from the buds and exudates of plants. It undergoes enzymatic transformations in the salivary glands of bees and the addition of fatty acids (SFORCIN et al., 2009). Sforcin et al. (2009) defined propolis as a lipophilic material of variable colors such as green, red, and different shades of brown. Several studies have commented on the biological activities of propolis and its therapeutic applications. Also, they showed that it is essential to investigate the relationship between the chemical composition and biological activity of propolis, in order to be possible to correlate the type of propolis with its therapeutic application (LUSTOSA et al., 2008). The earlier studies with some types of propolis have reported its antibacterial, antifungal, anti-inflammatory, and antitumor activities (WANG et al., 2015; DE-LIMA et al., 2016; EBEID et al., 2016; MAZIA et al., 2016).

Although propolis is accepted to have a health benefit, it needs to be chemically standardized to ensure its quality, effectiveness, and safety. During the collection process, microorganisms can contaminate propolis reducing its commercial value. Thus, it is necessary to adopt Good Manufacturing Practices (GMP), which should cover all aspects of handling, from raw material collection to the final product elaboration (FÉAS et al., 2014). Also, the location of an apiary should be carefully chosen because in environments where animal wastes, fertilizers, or contaminated water are used for irrigation, intestinal pathogens such as Salmonella spp., Escherichia coli, and Shigella spp. can be found as a contaminant (GERMANO; GERMANO, 2011).

The sources of microorganism contamination are generally associated with the processes of the production and the preparation of products, as microorganisms are found in air, water, soil, dust, plants, utensils, intestinal tract of humans, and animals, and sometimes on the skin of animals (FORSYTHE, 2013; DE-MELO et al., 2016). The control of microorganisms requires a high level of hygiene of the manipulators and utensils, as well the processing sites of products (PUCCIARELLI et al., 2014; BÁRBARA et al., 2015).

In Brazil, the quality of propolis is regulated by Normative Instruction n. 3, dated January 19, 2001, which establishes that hygienic practices for the preparation of propolis must be in accordance with the provisions of Administrative Rule 368, of 04/09/97 – “Technical Regulation on the Hygienic-Sanitary Conditions and Good Practices of Elaboration for Establishments Food Processing/Industrialization – Ministry of Agriculture and Supply, Brazil” (BRAZIL, 2001). Besides the lack of precision for regulating the microbiological parameters of propolis, the processes in which the effect of manipulation on the microbiological safety of crude propolis could be evaluated have not been well established. Considering this, the objective of this study was to evaluate the microbiological quality of propolis in natura using two handling techniques during its collection.
METHODS

The experiment was conducted in three apiaries in the Bay of Iguape, Brazil (12°45'S, 38°53'W). The analyses were performed with the propolis samples produced by *Apis mellifera* bees. The samples were collected monthly from August 2013 to January 2014 from three apiaries (one sample per apiary per month per handling), totaling 36 samples, of which 18 samples were handled using stainless steel knives and placed in non-toxic plastic containers (traditional handling). Afterward, visible impurities (wood, leaves, remains of bees, and insects) were cleaned in the laboratory. After weighing and labeling, the samples were identified as traditional handling. Concomitantly, 18 samples were collected following GMP adapted from Fonseca *et al.* (2006), usually used to harvest honey and pollen (GMP handling). For this, the propolis samples were removed using autoclaved stainless-steel knives, packed in aseptic collectors, and transported in temperature-insulated boxes. Upon the arrival of samples in the laboratory, they were weighed and selected in a laminar flow chamber. This form of handling was identified as GMP handling.

Microbiological determinations

The following microbiological parameters were evaluated: counts of aerobic mesophilic and psychrotrophic bacteria, molds and yeast, total coliforms and *Escherichia coli*, entero-bacteria, coagulase-positive *Staphylococcus*, quantification of sulfite-reducing *Clostridium* spores, and *Salmonella* spp. All assays were performed in triplicate.

Preparation of samples

The samples were prepared following the method of the American Public Health Association (DOWNES; ITO, 2001). A 25-g aliquot of each sample was taken for the preparation of the first dilution (10^-1) in 225 mL of 0.1% buffered peptone water, and the preparation of subsequent decimal dilutions (10^-2 to 10^-6) were performed in tubes containing 9 mL of the same diluent.

Quantification of total aerobic bacteria and psychrotrophic bacteria

From each dilution, 1-mL aliquots were transferred to Petri dishes, inoculated in depth in Standard Agar for Counting (PCA, HiMedia®), and incubated at 37 ± 1 °C for 48 h for counting aerobic mesophilic bacteria. For counting psychrotrophic bacteria, inoculation occurred on the surface, and the plates were incubated at 7 °C for 10 days.
Enumeration of yeast and molds

From each dilution, 0.1 mL aliquots were withdrawn into Petri plates containing Sabouraud Agar medium (HiMedia®), added with 0.02% chloramphenicol, and incubated in an incubator (BOD) at 25 °C for 5 days.

Enumeration of total coliforms and Escherichia coli

For counts of total coliforms and \textit{E. coli}, the CEC-CI SimPlate method (AOAC® Official Method 2005) (BioControl® System, Bellevue, WA, USA) was used according to the manufacturer’s instructions. The medium supplied was hydrated in 100 mL of sterile water, and then a 1-mL aliquot of the sample was added to 9 mL of the hydrated medium. The samples were homogenized in a vortex mixer and distributed by scattering on 84-well plates in circular motions such that the 84 wells were covered and contained no air bubbles. The plates were incubated at 35 °C for 24 to 48 h. After incubation, total coliform counts were determined from the color change of the culture medium. The plates were read at 365 nm in a spectrofluorimeter against the uninoculated culture medium used as a blank. The colony count was determined by absorbance using the SimPlate Conversion Table.

Quantification of Enterobacteriaceae

For counting Enterobacteriaceae, the TPC-CI SimPlate method (AOAC® Official Method 2005) (BioControl® System) was used according to the manufacturer’s instructions, similar to the quantification of total coliforms and \textit{E. coli}. The plates were incubated at 35 °C for 24 to 48 h. After incubation, the colony counts were determined by the absorbance of the wells using SimPlate conversion table.

Determination of sulfite-reducing Clostridium spores

Sulfite-reducing \textit{Clostridium} spores were detected and enumerated according to ISO 15213:2003. In sterile test tubes, 1 mL, 5 mL, and 10 mL of the initial sample suspension (in triplicate) were added. The tubes were submitted to thermal treatment in a water bath at 80 °C for 15 min for the inactivation of the samples. Subsequently, the samples were distributed in Petri plates and then topped with iron sulfite agar medium. The plates were sealed with Parafilm in anaerobic jars and incubated at 37 °C for 24 to 48 h.
Quantification of coagulase-positive Staphylococcus

Serial dilutions of the sample were inoculated in Baird-Parker Broth with Egg Yolk Tellurite and Sulfadimidine Solution (HiMedia®, Mumbai, Índia) during 24 h (37 °C). Black halo colonies were inoculated and incubated at 37 °C for 24 h for subsequent enzyme coagulase screening.

Detection of Salmonella spp.

The detection of Salmonella spp. in the samples was performed using an immunodiffusion technique (AOAC® Official Method 989.13), following the manufacturer’s recommendations. The results were visually interpreted by observing the development of a band that is a characteristic pattern of cell immobilization.

Statistical analysis

All statistical treatments were performed using the R statistical software, version 3.2.0 (The R Foundation for Statistical Computing, Vienna, Austria), available on the Internet (https://www.r-project.org/). The packages used were ggplot2 (WICKHAM, 2009), FSA (OGLE, 2018), rcompanion (MANGIAFICO, 2018), agricolae (MENDIBURU, 2016) and MASS (VENABLES; RIPLEY, 2002). All tests were performed at a significance level of 5%.

RESULTS AND DISCUSSION

Results

In general, the microbiological analysis of propolis showed the presence of the quantification of total aerobic bacteria and psychrotrophic bacteria, moulds and yeasts, total coliforms and Enterobacteria, Coagulase-positive Staphylococcus and Escherichia coli microorganisms.

The relative standard deviation (%DPR) of each assay (counts of microorganism performed in triplicate, CFU/g) was <3.0% in all analyses, except in a sample that presented a value of 4.4%, thus indicating an acceptable dispersion of results. Regarding the overall values of counts of microorganisms, the order of the dimension varied, that is, between zero (absence of colony unit formed, CFU) and values greater than 1 million. For data treatment was necessary to establish, as dependent variable in this study, the dimensional level of microorganism’s count level associated to the decimal logarithm of the counting data (considering the value zero when colony countdowns were lower than 10). The statistical analyzes carried out in this study focused on this transformed variable allowed to reduce distribution...
asymmetry and to establish satisfactory GLM models. Considering this situation, generalized linear models (GLM) were used to adjust the dependent variable (dimensional order of the number of microorganism counts) to the factors (also, considering the interaction terms) studied (independent variables): apiary, time period and handling technique. The objective was to identify which factors were significant to justify the transformed values of the counts of all evaluated microorganisms. Table 1 presents the overall results from the adjusted GLM models, considering the quasi-Poisson and Poisson hurdle distributions, as well the slope, intercept and adjusted \( R^2 \) from the linear representation between model predicted values and raw data.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Adjusted model</th>
<th>Linear relation between predicted and raw data</th>
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<tbody>
<tr>
<td></td>
<td>Null deviation</td>
<td>Residual deviation</td>
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<tr>
<td>Quasi-Poisson distribution</td>
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<tr>
<td>Aerobic mesophilic</td>
<td>34.2</td>
<td>3.67</td>
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<tr>
<td>Psychrotrophs</td>
<td>38.4</td>
<td>3.90</td>
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<tr>
<td>Molds and yeasts</td>
<td>25.5</td>
<td>5.96</td>
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<tr>
<td>Total coliforms</td>
<td>60.3</td>
<td>45.6</td>
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<tr>
<td>Enterobacteria</td>
<td>13.7</td>
<td>3.67</td>
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<td>Poisson hurdle distribution</td>
<td>179.2</td>
<td>90.1</td>
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</table>

The distribution that best fit the data from the microorganisms Aerobic mesophilic, Psychrotrophs, Molds and yeasts, Total coliforms and Enterobacteriacea, was the quasi-Poisson distribution. After evaluating the significance of each independent term, through analysis of variance of the models with and without the term under test using the Chi-square test and using the significance of the coefficients of the regression model through the partial Wald tests, the best model was obtained considering only the handling technique and time period variables (p <0.001). Globally, the fitted models showed to represent 61% to 90% of the raw data variability, with exception of the model adjusted for the Total coliforms, which represented only 24% of the variability of the presented data. This result could be related to the influence of environmental factors, which were not controlled. For the first models, the linear relation between predicted values by the model and raw data (dimensional level of microorganism counts) showed values of slope between 0.60 and 0.90 (theoretical value should be 1), intercept between 0.5 and 1.6 (theoretical value should be zero) and adjusted \( R^2 \), within 0.60 and 0.89. The best fitting results were achieved for the Aerobic mesophilic and Psychrotrophs microorganisms. The overall results showed that, in the present study,
the handling technique and time period variables are to be examined to explain most of the results variability.

The only data treatment exception was for the Coagulase-positive *Staphylococcus* microorganisms, where the GMP with Poisson hurdle model was used, due to the high number of samples exempt from this contamination (data with 44% of zeros, meaning absence of growth of bacterial colonies). The best selected model contained the handling variable and the handling-time period and time period-apiary interactions (p <0.05). A linear relation was obtained, between the predicted values by the model and raw data (dimensional order of the number of microorganism counts) with linear tendency, but with a great variability in the represented data. The linear relation presented an adjusted R$^2$ of 0.77, slope of $0.72 \pm 0.04$ and intercept, $0.5 \pm 0.1$. Even with the high variability presented in the data, the model allowed to show that part of the variation was explained by the selected factors and factor interactions.

To visualize and conclude on the existing variability in the data matrix three figures were presented. Figure 1 shows the graphs of the transformed variable (dimensional level of microorganism counts) for each the analyzed microorganisms performed considering the variables: handling (1-traditional procedure and 2 – GMP procedure), time period (related to the propolis collection in the 1st, 2nd, 3rd, 4th, 5th and 6th sampling month), and apiary (three apiaries located in three different locations). The figure shows that there is evident difference in the contamination levels between the traditional and GMP handlings. Traditional handling resulted in the samples with high levels of microorganisms, while GMP handling demonstrated that it is more efficient in reducing contaminations. On average, mesophilic and psychrotrophic aerobic bacteria were the groups of contaminants with higher counts found in the propolis of Bay of Iguape in both treatments, followed by molds, and yeasts, enterobacteria, total coliforms, and coagulase-positive *Staphylococcus* microorganisms.
Figure 1. Mean dimensional level of microorganism counts in propolis samples (with associated error bars) in function of the two handling procedures (traditional and GMP handlings): AMeso - Aerobic mesophilic; APSic - Psychrotrophs; MoYe - Molds and yeasts; ColIT - Total coliforms; EnteB - Enterobacteria; Staph - Coagulase-positive Staphylococcus; and, EsheC - Escherichia coli.
The Kruskal–Wallis test, a non-parametric test, showed that the mean results of the transformed variable of microorganisms’ counts presented significant differences between the two processing techniques for each analyzed microorganism. (p-value < 0.001). Figure 2 shows the general averages (with associated errors) obtained in each handling process, allowing to verify that the highest levels of contamination were in the traditional sample handling. The E. coli count showed a considerable decrease in the samples collected using GMP handling, where good practices were applied since only one bacterial count was found in one of the evaluated apiaries and at a low level. The presence of E. coli was observed in 73.3% of the samples collected using traditional handling and only in 4.8% of the samples collected using GMP handling. Also, in this study, all the samples were negative for sulphite-reducing Clostridium spores and Salmonella spp (<10 CFU/g¹, as zero value for mean dimensional level of microorganism counts).

Figure 2. Mean dimensional level of microorganism counts in propolis samples (with associated error bars) for the two handling procedures (traditional and GMP handlings): AMeso - Aerobic mesophilic; APsic - Psychrotrophs; MoYe - Molds and yeasts; ColIT - Total coliforms; EnteB - Enterobacteria; Staph - Coagulase-positive Staphylococcus; and, EsheC - Escherichia coli.

In Figure 3, the data variability of the variable time period can be observed for the two handling procedures. The Kruskal–Wallis test showed significant differences within each handling procedure in the time periods (p-value < 0.007) for all the microorganisms tested, with exception of the E. coli microorganism for the GMP procedure (p-value = 0.068). In order to determine which groups were different from others, a post-hoc Dunn test was conducted. The results of the post-hoc analysis for the means comparison within each handling procedure are shown in the figure (different letters represent significant differences; in italic for the GMP procedure and in bold for the traditional procedure). Overall, there were significant differences in the in the dimensional levels of microorganism counts present in the propolis samples between the months of harvest, but without evidence of any tendency considering the relation of letters...
obtained between the different groups (months of sample collection). These significant variations found within each handling procedure were considered to be random, that is, they could not be explained by the two hygiene and utensils manipulations procedures in propolis production.

**Figure 3.** Mean dimensional level of microorganism counts in propolis samples (with associated error bars) in function of sampling time periods for the two handling procedures (traditional and GMP handlings): AMeso - Aerobic mesophilic; APsic - Psychrotrophs; MoYe - Molds and yeasts; ColiT - Total coliforms; EnteB - Enterobacteria; Staph - Coagulase-positive *Staphylococcus*; and, EsheC - *Escherichia coli*. The letters display format represents statistical analysis using Dunn post-hoc test. Groups sharing the same letter are not significantly different.
DISCUSSION

The results indicate the influence of the handling and the time of collection on the counts of microorganisms. This confirms that the contamination occurs during the harvesting and processing of propolis. Within each manipulation, the influence of the time period (sampling in different months) was also significant, but it was not possible to obtain an explanation for the presented variability, considering that it was also affected by different external conditions. Considering that the propolis samples were processed in natura and were cleaned for visible contaminations after collection (contaminations associated with environmental factors such as vegetation, soil, insects, and climatic conditions), it is difficult to clearly demark the effect of the collection time. In addition, it should be considered that regardless of the analyzed time periods, the results were directly influenced by the handling during the product processing.

The microorganisms from the groups of mesophilic and psychrotrophic aerobic bacteria include deteriorating organisms that reduce the quality of products and may or may not be pathogenic. Psychrotrophs are able to grow at refrigeration temperatures and cause deterioration of products by forming a fungal mycelium, surface silt, or changes in taste and/or color (FORSYTHE, 2013, DE-MELO et al., 2016).

The high counts of yeast and mold are often related to the environmental exposure and improper storage of products. Gomes et al. (2010) reported that molds and yeasts are among the major microbial contaminants in honey, along with spore-forming bacteria. The authors indicated that several sources exist for microbial contamination of honey, including pollen, the digestive tract of bees, nectar, dust, and soil. These primary sources are more difficult to control, whereas the quality of equipment and facilities can be controlled by adopting GMP (GOMES et al., 2010). In this study, for mesophilic and psychotropic aerobic bacteria, a regular asymmetry was observed, with a reduction of 53.3% and 50.8%, respectively, among the treatments used during the collection. In the mold and yeast counts, a reduction of up to 73.9% in the maximum limits after the application of GMP handling was observed.

Although propolis does not offer an environment conducive to the development of Staphylococcus spp., it may be a carrier for other bee products (cross-contamination). According to Nowakiewicz et al. (2016), this group of microorganisms is a potential threat to public health, as they produce toxins harmful to the health of several animal species, develop antimicrobial resistance, and can grow in adverse environmental conditions. Coagulase-positive Staphylococcus spp. were present in 86.7% of the samples collected without proper hygiene and sanitary conditions. Only 9.6% of these microorganisms were observed when sample collection was done following adequate manipulation techniques.

Microorganisms indicative of fecal contamination may be from the secondary contamination caused by unclean equipment and utensils or during transportation. The E. coli is present...
in high concentrations in the feces of mammals and can be found in soil and water, serving as an indicative parameter of fecal contamination. This bacterium can be distinguished from the other total coliforms because of the absence of urease production and the presence of β-glucuronidase production (EDEN, 2014).

The Enterobacteriaceae family is vast and encompasses microorganisms that inhabit soil and the intestinal tract of animals. This family includes approximately 20 genera, including *E. coli*, all members of the coliform group and the foodborne pathogens *Salmonella* spp, *Shigella* spp, and *Yersinia* spp. According to Eden (2014), these microorganisms are more efficient to be used as indicators of GMP because they present greater resistance to the environment than coliforms. *Salmonella* spp, *Shigella* spp, and *Yersinia* spp have the ability to ferment glucose, while coliforms ferment lactose.

All samples were negative for sulphide-reducing clostridia spores and *Salmonella* sp. This result was extremely satisfactory from the microbiological point of view because the members of the genus *Clostridium* are producers of toxins that are harmful to animals and damage food products (FORSYTHE, 2013). Toxins are small soluble molecules produced by many pathogens and can harm to animals. Among the microorganisms that cause such damages are *Clostridium botulinum*, *C. perfringens*, *Staphylococcus aureus*, *Bacillus cereus*, and *Aspergillus flavus* (FORSYTHE, 2013). *S. aureus* and *E. coli*, develop during unhygienic conditions of the manipulators and can cause a severe threat to the consumers (TIDJANI *et al.*, 2013). This is an important result because these microorganisms are potentially pathogenic and of interest to public health.

Since there are few studies on the microbiological quality of propolis and due to the lack of legislation regulating microbiological parameters of propolis with precision, such studies are fundamental to standardize propolis by region. The lack of standardization in the existing norms for the microbiological parameters can be related to the absence of diagnosis and the limitations and unhygienic conditions of the handling.

These results are based on the importance of GMP in the collection and processing of propolis, as it occurs with other bee products, mainly honey and pollen. Dias, Pereira, and Estevinho (2012), used a similar approach when investigating the microbiological quality of Portuguese propolis and emphasized the importance of evaluating these parameters in propolis *in natura*, since propolis has a great therapeutic value.

Sforcin *et al.* (2009) showed that, in Brazil, the propolis harvest occurs throughout the year and the seasonal variations in the composition of propolis. In addition, the authors showed a synergistic performance between the chemical compounds. Therefore, the results obtained, we can verify that the microbiological quality of propolis is strongly influenced by
primary and secondary contaminating factors and can be controlled by GMP, such as that is applied for honey and other bee products (GRIGORYAN, 2016).

According to De-Melo et al. (2016), the presence of some types of phenolic compounds, water content, and pH may affect bacterial growth and presence. This aspect is very important because a large amount of microbial load may influence the physical, chemical, and biological characteristics of propolis, losing its commercial value and decreasing the content of bioactive compounds (ESTEVINHO et al., 2012), in addition to the risk of contamination of other products by bees. Because propolis is a widely used product, since reducing the microbial load in propolis is essential to minimize any chemical alteration in order to assure its quality and safety for its possible applications as possible damages caused by propolis when used as an antimicrobial agent (DE-LIMA et al., 2016), as a functional food (FREIRES; ALENCAR; ROSALEN 2016), and a natural food preservative (DUMAN; OZPOLAT 2015).

CONCLUSION

Despite bee products have the image of being natural, healthy and clean, this work showed that there are microorganism sources of contamination in propolis production, which they are produced in an environment polluted by different sources of contamination. Thus, it is of utmost importance for beekeepers should identify and control to localise and exclude the different contamination sources for obtaining a product with highest quality, health, and safety. This work highlighted the importance of adopting GMP during the propolis’ process of collecting and processing considering that collection, packaging, handling, and the storage of propolis considerably influence the microbail counts. These factors influence the commercial quality, health, and safety, emphasizing the importance of adopting GMP during the process of collecting and processing of propolis.

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