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Microbial flora in organic honey samples of Africanized honeybees from Parana River islands

Flora microbiana em amostras de mel orgânico de abelhas africanizadas provenientes das ilhas do rio Paraná

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Abstract

The purpose of this study was to verify and compare the main contamination sources and the hygienic/sanitary conditions of organic honey samples of *Apis mellifera* from Parana River islands. Thirty-three (33) samples were analyzed between January 2005 and August 2006. Eleven (11) samples were collected by beekeepers and twenty-two (22) samples were collected and processed in accordance with ideal personal hygiene norms and good manufacturing practices. The samples underwent microbiological analysis in search of coliforms at 35 °C and 45 °C, as well as fungi enumeration analysis. As for fungi counting, the samples harvested by beekeepers showed values above the maximum established by Resolution n° 15/94 of Common Market Group - Mercosul. The results showed that secondary contamination sources are responsible for the reduction of organic honey quality.

Keywords: microbiological analysis; good manufacturing practice; honey contamination; fungi; *Apis mellifera*.

Resumo

Este estudo teve como objetivo verificar e comparar as principais fontes de contaminação e as condições higiênico-sanitárias de amostras de mel orgânico de *Apis mellifera* proveniente das Ilhas do rio Paraná. Foram analisadas 33 amostras, de janeiro de 2005 a agosto de 2006, sendo que, 11 foram coletadas por apicultores e 22 foram colhidas e processadas de acordo com as normas de higiene pessoal e práticas corretas de manipulação de alimentos. As amostras foram analisadas para a presença de coliformes a 35 °C, 45 °C e enumeração de bolores e leveduras. Todas as amostras apresentaram ausência total de coliformes a 35 °C e 45 °C ($44,5 \pm 1$ °C). Para contagem de bolores e leveduras, as amostras colhidas pelos apicultores apresentaram valores acima do máximo estabelecido pela Resolução Mercosul Grupo de Mercado Comum, N° 15/94. Os resultados indicam que as fontes de contaminação secundárias são as responsáveis pela redução da qualidade do mel orgânico.

Palavras-chave: análise microbiológica; boas práticas de fabricação; contaminação do mel; fungos; *Apis mellifera*.

1 Introduction

Honey, a natural product derived from the nectar of flowers and other sources, represents an important source of energy, being widely consumed. There is a high interest in the number and type of microbes in honey, since it is used as food or as an ingredient in the food, pharmaceutical and cosmetic industries, as well as in alternative medicine.

The *Apis mellifera* honey has a peculiar microbiological pattern due to its physicochemical composition, with a high degree of resistance to microorganisms proliferation. Its microbial flora consists of common microorganisms, present in the sporulated state, such as the *Bacillus* genus bacteria and other incidental or accidental, the genus *Penicillium*, *Mucor* and *Saccharomyces*, which can negatively influence its final quality as they multiply in honey exposed to action of adverse external factors, such as handling conditions, spores contamination, high temperature storage and high relative humidity (RODRIGUES; MARCHINI; CARVALHO, 1988; SNOWDON; CLIVER, 1996).

The primary sources of microorganisms are: pollen, bee's digestive system, dust, air and flowers (GILLIAM, 1971; SNOWDON; CLIVER, 1996). According to Gilliam; Moffett, Kauffeld (1983), the nectar and pollen contain many microorganisms. Tysset and Rousseau (1981) reported that the secondary sources of microorganisms are found in men, equipment, containers, wind, dust, insects, animals and water; this is probably the same for other foods. According to Ezenwa (2006), the presence of these microorganisms in honey is considered a contaminant in the programs of HACCP (Hazard Analysis and Critical Control Point).

In general, the presence of yeasts in honey is due to neglect and lack of good sanitary procedures during harvesting, handling and processing. As an example, we could mention the resting of honey frames on the ground, poorly washed centrifuges, brass centrifuges, very dark honeycombs and prolonged storage of honey in the frames, bringing up microorganisms to the product

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(RODRIGUES; MARCHINI; CARVALHO, 1988; SNOWDON, 1999). Spores, present in the air (SNOWDON; CLIVER, 1996), are either incorporated into the honey by the bees themselves during the collection and preparation of nectar and pollen, or by the poor care during handling in open places (SNOWDON, 1999).

Although these microorganisms cannot grow in natura honey – except for a few yeasts and fungi, they may survive and be transmitted to a new product in which the honey is used as an ingredient, thus deteriorating the product (SNOWDON, 1999).

These microorganisms may also show up in the production of food, such as alterations, enzymes production, antibiotics and mycotoxins, increase of vitamins, amino acids promoting metabolic conversion and inhibition through microbial competition (GOERZEN, 1991; MARTINS; MARTINS; BERNARDO, 2003).

This research was carried out in order to verify and compare the main contamination sources and hygienic conditions of organic honey samples of *Apis mellifera* from Parana River Islands.

2 Materials and methods

The tests were performed at the Laboratory of Food Microbiology at the Universidade Tecnológica Federal do Paraná (UTFPr), Campo Mourão (PR) campus.

2.1 Material

Collection of organic honey samples:

Thirty-three samples of organic honey were analyzed and compared, from January 2005 to August 2006; eleven were collected from beekeepers without any instruction regarding hygienic care and twenty-two were obtained following personal hygiene techniques and proper handling practices from three apiaries located in 'Floresta' and 'Laranjeira' islands along the riverfront of Parana River. The region climate, according to the Köppen classification, is humid temperate with hot summers "Cfa" (MAAK, 1968).

2.2 Methods

Implementation of Good Manufacturing Practices for obtaining organic honey.

Good Manufacturing Practices (GMP) are a set of procedures and corrective actions that seek to eliminate or reduce risks to acceptable levels and to ensure compliance with the basic requirements established for safe food preparation.

Different corrective actions were adopted to improve the process quality following the instructions laid down by the National Sanitary Surveillance Agency (BRASIL, 1997): hygiene in harvesting and honeycombs transportation, adequacy of the honey house facilities, personal hygiene, workplace cleanliness and implementation of Hygiene Standard Operating Procedure (PPHO), which includes the cleaning and sanitization of equipment and utensils used in honey handling.

Microbiological analysis

Samples were analyzed for the presence of coliforms at 35 °C and 45 °C and quantification of yeasts and molds (see Figure 1)

following the international standards method of Compendium of Methods for the Microbiology Examination of Foods (AMERICAN..., 1992).

Twenty-five grams of honey were diluted in 225 mL of 0.1% Buffered Peptoned Water (BPW). Preparations of subsequent decimal dilutions were performed in tubes containing 9 mL of the diluent up to 1/1000.

The multiple tube method, carried out in two stages, was used to determine the Most Probable Number (MPN) of coliforms at 35 °C to 45 °C. First, the sample was inoculated in Lauryl Sulfate Tryptose (LST) Broth, which inhibits the accompanying microbiota, at the same time, in a bacteria enrichment medium of the coliforms group or Brilliant Green Bile Lactose Broth (BGBL). Bacteria from this group cause turbidity in the medium with gas formation, detected in Durham tubes after 48 hours of incubation at 35 ± 2 °C. The second stage was performed by inoculating the lauryl-positive broths into *Escherichia coli* (EC) selective broths. If turbidity occurs in the EC broth after the incubation at 44.5 °C for 24 hours, then it is positive for coliforms at 45 °C. Dilutions were inoculated in three series, each containing three tubes of 2% BGBL with Durham tube, incubated at 35-37 °C for 24-48 hours. The presence of gas in the Durham tube was regarded as positive evidence. Conventional tables were used to calculate the MPN.

One mL aliquots of each dilution were grown in depth, in triplicate, to count yeasts and molds using potato dextrose agar (PDA) acidified with tartaric acid to 3.5%. After the medium solidification, the plates were incubated at 25 °C for five days, and the colonies were quantified.



Figure 1. Yeasts and Molds Counting (UFC.g⁻¹) in samples of organic honeys.

Statistical analysis

The design was completely randomized with three replications for each parameter, and the data were submitted to variance analysis and *F* test ($p > 0.05$).

3 Results and discussion

Table 1 shows the means, minimum and maximum values of coliforms at 35 °C and at 45 °C, and molds and yeasts in organic honey samples from the Paraná River islands. Table 2 shows *F* values with their respective probability (*P*) and variation coefficient (VC%) of the analyzed samples.

3.1 Coliforms at 35 °C and 45 °C

All analyzed samples presented results < 3.0 MPN.g⁻¹ for the quantification of coliforms at 35 °C and at 45 °C, showing the safety regarding the presence of enteric pathogens. These results can be explained by the honey physicochemical composition, determining whether an organism will be able to develop or not (SILVA, 2000; SEREIA, 2005). Sodré et al. (2007) found similar values (< 3.0 CFU.g⁻¹) when analyzing 58 honey samples in the Sates of Ceara (20) and Piaui (38).

By knowing the food characteristics, it is possible to predict the microbial flora that may grow in it, since its development is influenced by several external factors, such as temperature and relative humidity; and by intrinsic factors, such as water activity, pH, redox potential and food composition; besides its physical and sanitary conditions (BANWART, 1981; MUNDO et al., 2004). The maximum moisture content in mature honey must be 20% at the most (MARCHINI; SODRÉ; MORETI, 2004), any smaller figure is considered insufficient to promote the enteric pathogens growth

and indicates contamination, since these microorganisms need higher humidity values to grow. Snowden and Cliver (1996) had already reported a population of coliforms at 45 °C ranging from 10 to 10².CFU.g⁻¹. Nevertheless, these authors did not mention the moisture content of the studied samples.

3.2 Fungi

A maximum of 3.8×10^1 and a minimum of $< 10^1$ CFU.g⁻¹ values were obtained for quantification of moulds and yeasts in honey harvested in ideal hygienic conditions and a maximum of 1.1×10^3 and a minimum of 1.9×10^2 CFU.g⁻¹ for honey samples harvested without the GMP regulations.

According to the Mercosul (1994) # 15/94 GMC Resolution, which approved the Technical Regulation for Determining Honey Identity and Quality, adopted by Ordinance # 367, September 4, 1997, honey must contain a maximum of 100 CFU.g⁻¹. All honey samples harvested in disagreement with the GMP regulation showed results higher than those permitted by the Brazilian legislation, that is, 100 CFU.g⁻¹ (Table 1). These results corroborate with Souza (2008), who, when analyzing honey samples from stingless bees (Apidae: Meliponinae), noticed that 52.2% of samples showed moulds and yeasts counting above the maximum permitted by the Brazilian legislation. These studies show the importance of implementing the Standardized Operating Procedures (POPs) linked to the GMPs regarding both personal hygiene and food contact surfaces hygiene.

Oliveira et al. (2005) observed differences in the microbiological quality when analyzing *Melipona compressipes fasciculata* honey samples harvested by different methods (producer and aseptic methods). According to them, the installation of honey houses in untidy environments, as well as environmental variables, could

Table 1. Means, minimum and maximum values of coliforms at 35 °C and at 45 °C, molds and yeasts in *Apis mellifera* honey collected from the Paraná River islands, with and without the use of Good Manufacturing Practices (GMP).

Accomplished analysis	Honey samples without GMP (n = 11)		Honey samples with GMP (n = 22)		*Standard values
	Minimum	Maximum	Minimum	Maximum	
Coliforms at 35 °C (MPN.g ⁻¹)	< 3	< 3	< 3	< 3	-
Coliforms at 45 °C (MPN.g ⁻¹)	< 3	< 3	< 3	< 3	-
molds and yeasts (CFU.g ⁻¹)	1.9×10^2	1.1×10^3	< 10 ¹	3.8×10^1	1×10^2
Mean of molds and yeasts (CFU.g ⁻¹)	5.3×10^2		1.1×10^1		1×10^2

*Mercosul GMC res. 15/94.

Table 2. Values of *F* with the probability (*P*), variation coefficient (VC%), coliforms means at 35 °C and 45 °C, yeasts and molds of microbiological testing of *Apis mellifera* organic honey samples, collected from the islands of Paraná River, with and without implementation of Good Manufacturing Practices (GMP).

Variation source	Microorganisms		
	35 °C (MPN.g ⁻¹)	45 °C (MPN.g ⁻¹)	Molds and yeasts (CFU.g ⁻¹)
<i>F</i> Values	ns	ns	9.09 (<i>P</i> = 0.0088)
CV (%)			88.68
Means without GMP	<3 a	<3 a	5.3×10^2 a
With GMP	<3 a	<3 a	1.1×10^1 b

MPN = Most probable number; CFU = Colony forming unit; ns = Non significative; Different small letters in the same column differs significantly ($p < 0.05$).

be responsible for the presence of moulds and yeasts in quantities above the ones permitted by the standards. The presence of osmophilic yeasts is a risk for food, since they can grow in acidic conditions and in high concentrations of sucrose. The fermentation process involves the hydrolysis of sugars to produce alcohol and carbon dioxide. The alcohol can be converted into acetic acid in the presence of oxygen (WHITE JUNIOR, 1978; CRANE, 1979).

Analyzing the results regarding the significant increase in the content of molds and yeasts in honey not processed according to the GMP regulation, it can be inferred that this contamination is linked to secondary sources of contamination, for instance, incorrect handling, equipment, facilities and environmental conditions during the harvesting, processing, packaging and final product storage (Table 2).

Comparisons of *Apis mellifera* honey with honey from native Brazilian social bees, the stingless bees, have shown that honey responds differently to strains of bacteria, and in general, the stingless bees honey has higher bacteriostatic and bactericidal properties than those of *Apis* (CORTOPASSI-LAURINO; GELLI, 1991). Fonseca, Sodré and Carvalho (2006) suggested that, although these honeys have antimicrobial activity, it is important to implement the GMP associated with harvesting and conservation techniques to prolong the shelf life of these products. According to Sereia (2005), the organic honey from the islands of the triple border (the States of Parana, Sao Paulo and Mato Grosso do Sul) usually presents higher moisture, low viscosity and osmolarity. These features lead us to infer that the lower internal friction of the molecules determines the higher degree of fluidity with less resistance to oxygen transmission and dissolution currents. It also lowers antimicrobial action, favoring the growth of higher forms of aerobic and micro-aerobic osmophilic yeasts.

The personal behavior of food handlers should be the beekeepers responsibility, as well as other employees' routines involved in the harvesting process of organic honey. Honey suppliers that use the GMPs implementation increase the product quality. Further studies ought to be carried out to identify fungi genus and/or species in honey samples, characterizing them either as spoiling or toxigenic - with risks to the human health.

4 Conclusions

The results observed in this study are above the parameters set by official bodies and accepted by the scientific community.

The secondary contamination sources are responsible for reducing the organic honey quality.

The microbiological quality of organic honey is highly dependent on the implementation of correct techniques regarding the cleaning and sanitization of equipment and utensils, the care during honey harvesting and transportation; as well as on the environmental conditions where the processing stages are carried out.

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