

Apis mellifera* honey sanitary quality related to its form of collection*Qualidade sanitária do mel de *Apis mellifera* em relação a forma de coleta**

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ABSTRACT

The Beekeeping is a source of income for the population of the Amazon region in Brazil, standing out for its diverse vegetation that provides food and water for bees almost all year round. However, the lack of technical assistance in honey production can compromise its production and cause food poisoning. Thus, the objective of article was to evaluate and compare the hygienic-sanitary conditions of honeys, collecting in aseptic conditions and in a traditional way by the beekeeper. The numbers of mesophilic bacteria, coliforms at 35°C and 45 °C, moulds and yeasts were determined and the presence of *Clostridium* sulfite-reducers (CSR). The presence of CSR was detected in a traditionally collected sample. A total of nine (42.85%) samples collected aseptically presented contamination by fungi, above that permitted by law and four (26.6%) samples traditionally collected had high counts of mesophilic bacteria. There was a difference in yeast contamination

when comparing the different collection methods, aseptically and traditionally collection. The sanitary hygienic quality of honey was considered one of the problems in the region studied due to the non-use of Good Manufacturing Practices (GMP) in bee creation. Evidencing the need for technical support for beekeepers to ensure health security for honey consumers.

Keywords: Africanized Hybrid Bee, *Clostridium* Sulfite-Reducers, Good Manufacturing Practices.

RESUMO

A Apicultura é uma fonte de renda para a população da região amazônica no Brasil, se destacando por sua vegetação diversa que fornece alimento e água para as abelhas quase o ano todo. Porém, a falta de assistência técnica na produção do mel pode comprometer sua produção e causar aos consumidores graves intoxicações alimentares. Assim, o objetivo deste artigo foi avaliar e comparar as condições higiênico-sanitárias de meis, colhidos em condições assépticas e de forma tradicional pelo apicultor. Foram determinados os números de bactérias mesófilas, coliformes a 35°C e 45 °C, bolores e leveduras e a presença de *Clostridium* sulfito-redutores (CSR). A presença de CSR foi detectada em uma amostra coletada de forma tradicional. Um total de nove (42,85%) amostras coletadas assepticamente apresentaram contaminação por fungos, acima do permitido pela legislação brasileira e quatro (26,6%) amostras tradicionalmente coletadas apresentaram contagens elevadas de bactérias mesófilas. Houve diferença na contaminação por leveduras quando foram comparados os dois métodos de coleta, asséptica e tradicional. A qualidade higiênica sanitária do mel foi considerada um dos problemas da região estudada devido à não utilização das Boas Práticas de Fabricação (BPF) na criação de abelhas. Evidenciando assim a necessidade de suporte técnico aos apicultores para garantir segurança sanitária aos consumidores de mel.

Palavras-chave: Abelha Híbrida Africanizada, *Clostridium* Sulfito Redutores, Boas Práticas de Fabricação.

1 INTRODUCTION

The Brazil has great beekeeping potential due to the diversity of flora, wide territorial extension and climatic variability that makes it possible to produce honey throughout the year (Nogueira-Neto, 1972; Holanda et al., 2015). Beekeeping is an activity that stands out in the regions of the North and Northeast, particularly the State of Maranhão, for exploring the potential of native flora providing work and income to beekeepers who are generally medium and small farmers.

Bee food from wild flowers, including mangrove, which bloom in up to eight months during the year, strongly contributes to the state of Maranhão to present strategic conditions in the production of honey (Atlas do Maranhão, 2002; Muniz et al., 2004; Marques et al., 2011; Souza et al., 2016). The creation of Africanized bees has been highlighted in the Amazon region for being a privileged area in Local Productive Arrangements (LPA) of government projects that promote the development of family farming in the exploration of honey (Vasconcelos et al., 2002).

Honey is a complex food produced by honey bees, easy to digest and assimilate, which consists of a source of energy for the balance of biological processes as it contains adequate

proportions of nutrients and bactericidal and aromatic substances (Brasil, 2000; Komatsu et al., 2002).

The quality of honey is directly related to its own physical-chemical characteristics such as sugar and moisture content, as it has a high capacity to absorb moisture from the environment, thus becoming a food favorable to the growth of molds and yeasts (De Camargo et al., 2003). In addition to external factors such as microbiological contamination that can be caused by the lack of hygiene in the extraction, processing and packaging of honey, which thus reduces the shelf life of this product (Souza et al., 2009; Moura et al., 2014).

As this product is consumed in natura by children and adults, it is important to assess the sanitary quality from production to commercialization, even though the honey of certain bees has antimicrobial properties (Caldas et al., 2020).

Thus, Brazilian legislation through the Ministry of Agriculture (MAPA) regulates an acceptable limit of molds, yeasts and coliforms at 35°C and 45°C (Brasil, 2000; Brasil, 2001). However, honey for being rich in carbohydrates facilitates the development of other microorganisms, such as aerobic mesophilic bacteria, among which stands out *Escherichia coli* (Souza, et al. 2009a).

Thus, an important aspect in the beekeeping obstacle in Maranhão is the use of Good Manufacturing Practices (GMP) associated with the maintenance of its hygienic quality, which is correlated with several factors, among them the hygienic habit of bees (Nogueira-Neto, 1997), the conditions of collection (Oliveira et al., 2005), type of bottle for filling and storage conditions of the product (Holanda et al., 2015; Oliveira et al., 2005).

However, the evaluation of the quality of the product becomes essential for it to be marketed, ensuring greater health security for honey consumers. Therefore, the objective of our study was quantify the rate of contaminating microorganisms by comparing two collection methods: aseptic (laboratory) and traditional (performed by beekeepers) in order to identify the critical points associated with traditional management.

2 MATERIAL AND METHODS

2.1 COLLECTIONS OF HONEY SAMPLES

The honey samples from Africanized bees were collected in locations belonging to the Amazon region of Maranhão, in three different cities, A (03° 18 '27 "S; 46° 15 '03" W), B (02° 42 '33 "S; 45° 59 '28 "W) and C (02° 16 '50"; 45° 55 '45 ").

Two different collection methods were employed in this work, in the first (1) the combs were collected directly from the hive, uncapped and the honey was drained and filtered with sieves and stored in 250 mL glass bottles and stored at room temperature.

The entire procedure was performed with previously sterilized material, in order to avoid any contamination by handling. In the second method (2), the samples were collected and extracted in a traditional way by beekeepers and stored in their own marketing packages. In the two methods used, a total of 36 samples were collected with approximately 250 g of honey between January 2011 and August 2013.

2.2 MICROBIOLOGICAL ASSAY

Serial dilutions were used, where 25 g of honey was used in 225 mL of buffered peptonized water (10^{-1}), subsequently 1 mL of this dilution was added in test tubes containing 9 mL of the same diluent (10^{-2}) and so on until 10^{-3} dilution. Microbiological analysis were performed to determine the Most Probable Number (MPN) of Coliforms at 35 °C and 45 °C, Standard counting in Mold and Yeast and Mesophilic Bacteria (CFU) plates, isolation of *Clostridium* sulfite-reducers (CSR) according to the Analytical Methods Manual Officials for Microbiological Analysis for Control of Products of Animal Origin and Water (Brasil, 2003).

2.3 COLIFORMS AT 35 °C AND 45 °C

The determination of coliforms was performed using the multi-tube fermentation technique, a presumptive test performed with lauryl sulfate tryptose broth; from the serial dilutions of the honey samples. From the prepared dilutions, 1 mL of each dilution was inoculated in the lauryl broth series and the tubes incubated in a bacteriological incubator at 35 °C for 48 hours. The lactose fermentation was observed by the formation of gas inside the tubes of Duran or effervescence when the test tubes were shaken gently (Brasil, 2003). For confirmatory analysis of coliforms at 35 °C, the broth medium bright Green Bile (GB) is used, where aliquots of positive growth in lauryl are transferred to the GB medium and the tubes are again incubated in a bacteriological oven at 35 °C for 48 hours. For confirmatory analysis of coliforms at 45 °C, the *Escherichia coli* (EC) broth is used and, from the positive reading of the GB tubes, the inoculum are transferred to EC tubes and these are incubated in a water bath 45 °C for 24 hours. The most probable number table (MPN) was used to interpret the tests (BRASIL, 2003) was used to interpret the coliforms tests (Brasil, 2003).

2.4 STANDARD COUNT ON MOLDS AND YEASTS PLATES

For counting molds and yeasts, 1mL of decimal dilutions were added in Petri dishes and 20 mL of potato dextrose agar (PDA) was poured, the medium being acidified with 10% lactic acid to pH 3.5. The plates was incubated in an oven at 25 ° C for 3 - 5 days.

2.5 STANDARD COUNT ON MESOPHILIC BACTERIA

For the counting of mesophilic bacteria, 1 mL of decimal dilutions were added in petri dishes and 20 mL of standard counting agar (SCA) were added and then incubated in an oven at 35 °C for 48 hours. The CFU numbers were quantified with the help of the colony counter. For the calculation of CFU.g⁻¹ of the samples, the number of identified colonies was multiplied by the inverse of the inoculated dilution, the result being estimated (Brasil, 2003).

2.6 CLOSTRIDIUM SULFITE REDUCERS

For the counting of *Clostridium* Sulfite Reducers (CSR), 1 mL of the serial dilution was seeded in Petri dishes containing 20 mL of Selective Perfringens agar (SPS), after homogenization and solidification of the medium, a second layer was added and incubated in a anaerobiosis with the Gas-Pak® system (Ragazanii, 2008) in an oven at 46 °C, for 48 hours. After this period, they were selected to confirm the growth of black colonies, characteristics of sulfite-reducing Clostridia on SPS agar and for confirmation, Gram stain was performed (Hucker and Conn, 1923).

2.7 STATISTICAL ANALYZES

The results were compared to the acceptable limit recommended by the Ministry of Agriculture, Supply and Livestock (MAPA), through Normative Instruction No. 11, of October 20, 2000, which recommends up to 1.0x10² CFU.g⁻¹ for molds and yeasts and <3.0 MPN.g⁻¹ for coliforms (Brasil, 2000). The means were analyzed using the Chi-squared test at the 5% level, to compare the collection methods using the BioEstat 5.3 software (Ayres *et al.*, 2017).

3 RESULTS AND DISCUSSION

3.1 COUNT OF CONTAMINATING MICROORGANISMS IN HONEY

The detection of microorganisms present in foods above current legislation is a relevant control in the prevention of food infections. The results of the microbiological analysis of *A. mellifera* honeys from the Amazon region can be seen in Table 1.

Table 1 Count of contaminating microorganisms from *Apis mellifera* honey collected in aseptic and traditional ways in different cities in the Amazon region of Maranhão.

Sample	City	Collection Method	Coliforms Group		Molds and Yeasts (CFU.g ⁻¹)	Mesophilic Bacteria (CFU.g ⁻¹)	<i>Clostridium</i> Sulfite Reducers
			35 °C (MPN.g ⁻¹)	45 °C (MPN.g ⁻¹)			
1	A		ND	ND	5.3 x 10 ²	NO	(-)
2			ND	ND	5.0 x 10 ²	NO	(-)
3			ND	ND	NO	6.3 x 10 ²	(-)
4			ND	ND	>300	NO	(-)
5			ND	ND	>300	NO	(-)
6			ND	ND	>300	>300	(-)
7			ND	ND	>300	NO	(-)
8	B	Aseptic	ND	ND	>300	NO	(-)
9			ND	ND	>300	NO	(-)
10			ND	ND	>300	NO	(-)
11			ND	ND	NO	NO	(-)
12			ND	ND	NO	NO	(-)
13			ND	ND	NO	1.9 x 10 ²	(-)
14			ND	ND	NO	NO	(-)
15			ND	ND	NO	NO	(-)
16			ND	ND	NO	NO	(-)
17			ND	ND	NO	NO	(-)
18			ND	ND	NO	NO	(-)
19	ND	ND	NO	NO	(-)		
20	C		ND	ND	NO	NO	(-)
21			ND	ND	NO	NO	(-)
22	A		ND	ND	NO	4.4 x 10 ²	(+)
23	B	Traditional *	ND	ND	NO	NO	(-)
24			ND	ND	NO	NO	(-)
25			ND	ND	NO	NO	(-)
26			ND	ND	NO	NO	(-)
27			ND	ND	NO	NO	(-)
28			ND	ND	NO	NO	(-)
29			ND	ND	NO	>300	(-)
30			ND	ND	>300	NO	(-)
31			ND	ND	NO	>300	(-)
32			ND	ND	NO	1.0 x 10 ²	(-)
33			ND	ND	NO	NO	(-)
34			ND	ND	NO	NO	(-)
35			ND	ND	NO	NO	(-)
36			ND	ND	NO	NO	(-)
Standard			< 3.0	< 3.0	1.0 x 10 ²	x	(-)

Legend: ND = Not detected (< 3.0 MPN.g⁻¹), NO = Not observed (< 30 CFU.g⁻¹), (+) = Presence, (-) = Ausence, CFU = colony-forming unit, MPN = most probable number, Standard = maximum allowed in 25 g of honey (Brasil, 2000).* In city C there was no availability of honey collected traditionally by beekeepers. x = Not required in national regulations (Brasil, 2000; Brasil, 2001).

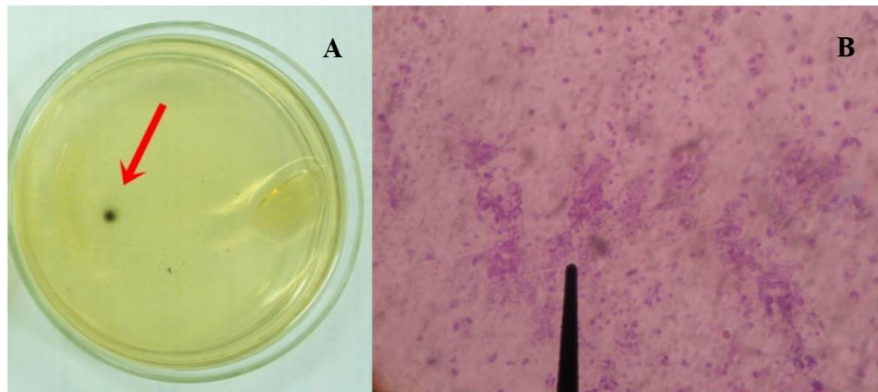
3.2 PRESENCE OF CLOSTRIDIUM IN HONEY

The presence of reducing *Clostridium* sulfite was detected in a sample of honey from the traditional collection (Figure 1.), made by beekeepers and the confirmation was made based on Gram stain, its presence in honey occurred possibly due to cross contamination during their honey

extraction, because the same sample, 22, showed contamination by aerobic mesophilic bacteria (4.4×10^2 CFU.g⁻¹).

These organisms are spore-forming which are naturally present in the soil and pollen and are capable of reducing sulfite to sulfide, which reacts with ammonium citrate and iron III, forming a black precipitate (Brasil, 2003).

Fig.1 Presence of *Clostridium* sulfite reducers agents in *Apis mellifera* honey, collected traditionally in the Amazon region. A: colony isolated on SPS solid medium; B: Gram staining of the gram-positive rods of the colony found; red arrow: black colony formed typical of *Clostridium* sulfite reducers by reducing sulfite to sulfide, which reacts with ammonium citrate and iron III.



According to Finola et al. (2007), evaluating the quality of commercialized bee honey, detected the presence of sulfur-reducing *Clostridium* spores in 70% of the analyzed samples, higher than that found in this study. However, its presence in food functions as an indicator of the presence of *Clostridium* species, such as *C. botulinum*, a microorganism capable of producing toxins that causes the death of many species, mainly human (Tysset et al., 1974; Solomon and Lilly, 2001). Therefore, in this work we can say that the presence of *Clostridium* sp. it was due to cross contamination due to improper handling of honey, thus showing that the presence of this microorganism constitutes a risk to the health of honey consumers.

Honey is a food that has its own primary contamination source from bees inserted into the hive through pollen, which are made up of fungal and bacterial spores (Olaitan et al., 2007). As a secondary source, they can be introduced into honey using the equipment used in handling, extraction, processing and filling (Souza et al. 2009a; Silva et al. 2015). Thus, effective research in the detection of pathogens in honey is a fundamental measure to guarantee the microbiological safety of honey, as this food does not undergo any type of treatment in its processing to control microorganisms before being consumed (Ragazani et al., 2008).

3.3 MICROORGANISMS THAT DETERIORATE HONEY

Coliforms were not found at 35 °C and 45 °C. However Nogueira Neto (1997) describes that some bees can develop unhygienic habits by collecting human, animal and polluted water when the hives are installed close to these sources. Thus, the results presented show the hygienic habit of *A. mellifera* bees.

Coliform are a group of microorganisms present in the human and animal intestines, which do not represent a direct risk, but can serve as indicators of the presence of pathogenic microorganisms such as *Enterobacter* and *Klebsiella* in contaminated foods (Sousa, 2006). In general, these microorganisms are not found in honey (Holanda et al., 2013; Lima et al., 2015; Fernandez et al., 2017), thus not differing from what was found in this study.

A total of ten samples showed values between 5.0×10^2 CFU.g⁻¹ and greater than 300 CFU.g⁻¹ of molds and yeasts, that is, higher than the established by the national legislation of 1.0×10^2 CFU.g⁻¹ (Brasil, 2000). The presence of these microorganisms in honey is considered natural, as they may be present in plant pollen (Keller et al., 2005), however values higher than those established by MAPA may represent risks to consumers where some of these fungi produce mycotoxins (Grabowski and Klein, 2017). Therefore, these samples were considered unfit for consumption.

According to Abreu et al., (2005), the development of molds and yeasts in honey is associated with some physical-chemical parameters, such as humidity and acidity and also with the conditions of storage and storage of the product such as, temperature and relative humidity. Matuella and Torres (2000) found a significant presence of fungi in furniture, where one of the analyzed hives was highlighted, with a higher count of 3.2×10^2 CFU.g⁻¹, as well as Oliveira et al., (2005), who found counts that ranged from 1.5×10^2 CFU.g⁻¹ to 6.5×10^3 CFU.g⁻¹ corroborating the values found in this study.

As for the presence of aerobic mesophilic bacteria, seven samples were found, considered unfit for consumption. This analysis is not currently included in the national legislation, but was used for the purpose of evaluating the hygienic conditions of the product, the collection sites and handling for the presence of coliforms (Brasil, 2000).

The parameter used in honey to indicate hygiene in relation to handling is made from the coliform count (Brasil, 2000) where in this study the growth of these organisms in honey was not detected. What can fail to represent the actual contamination by microorganisms that may be pathogenic to man and that was evidenced by the standard count in mesophilic aerobic bacteria

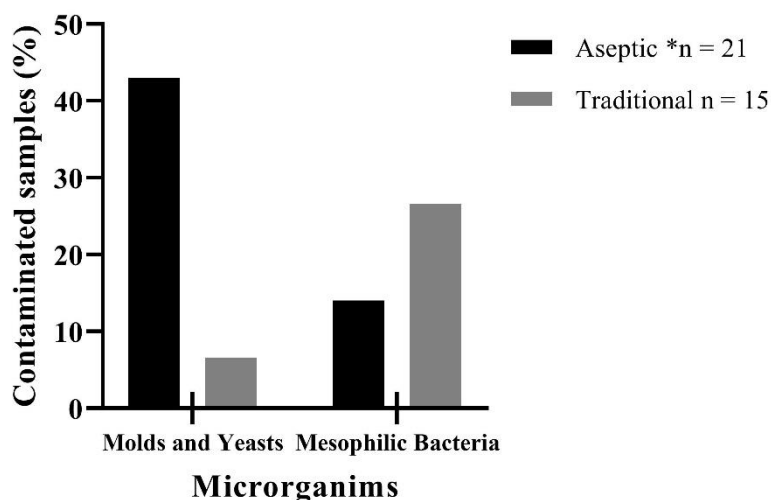
plates in the analyzed honey samples, thus suggesting that it could be used as a standard analysis MAPA for this food.

Most of the foodborne pathogenic bacteria are mesophilic, therefore, a high count of aerobic mesophilic bacteria means conditions favorable to their multiplication, such as handling without the use of good food handling practices (Sousa, 2006; Souza et al., 2009a). Currently, there are no established microbiological standards for mesophilic bacteria in honey, however this count is routinely used in other sugary foods such as chocolate (Anvisa, 1978) and it should also be used in honey to indicate some inadequate procedure from the sanitary point of view (Franco and Landgraf, 2008).

3.4 COMPARISON BETWEEN ASEPTIC AND TRADITIONAL HONEY COLLECTION METHODS

From the honey samples collected in the Amazon region, in which two collection methods were used, the percentage of contaminated samples can be seen in Figure 2.

Fig.2 Percentage of honey samples from *Apis mellifera* contaminated with molds and yeasts and mesophilic aerobic bacteria according to the collection method. * n = number of samples analyzed.



The highest percentage (43%) of samples with values greater than 1.0×10^2 CFU.g⁻¹ of molds and yeasts was identified in the form of aseptic collection. What can be explained because in Amazonian regions where the humidity rate can reach 80%, and high temperatures favor the growth of molds and yeasts in the hive that cause the fermentation of honey and this process can occur more easily in "green" honeys", which are harvested from combs that did not have their alveoli properly operated by the bees (Camargo et al., 2003).

Honey, being a food rich in sugar, favors the development of osmophilic yeasts, which are capable of growing at high concentrations of sugar. These yeasts are often isolated from the hive, from wax, nectar and dead bees (Sousa et al., 2009a).

While 26.66% of the samples collected in the traditional way, using honey extraction facilities by beekeepers, had the presence of mesophilic bacteria, possibly due to poor hygiene in handling the honey or filling in inappropriate reusable PET packaging, thus being considered inappropriate for consumption.

Silva et al., (2010) stated that quantifying mesophilic bacteria in food is useful in assessing quality, because high populations of these bacteria may indicate deficiencies in sanitation or processing failure. Although the use of GMP in all stages of honey collection, extraction and processing can reduce contamination by this microorganisms (Moura et al., 2014).

We demonstrated in this work that the lack of Good Beekeeping Practices (GBP) throughout the production of honey in the Amazon region of Maranhão is an aggravating factor in the quality of honey, because even when collecting honey aseptically this contamination was already present in the hive.

When comparing the ways of collecting *A. mellifera* honey in the Amazon region (Table 2), the Chi-squared test for independence, the χ^2 calculated = 16.84, less than the χ^2 table = 2.65 where the hypothesis that does not exist was accepted difference between the forms of collection used (H0) and the alternative hypothesis (H1) was rejected and thus it was concluded that there is no significant difference at the level of 5%, in the form of collection for molds and yeasts, possibly due to the lack of GMP in relation to the maintenance of apiaries, which consequently affected the microbiological quality of the analyzed honey but did not affect the concentration of aerobic mesophilic bacteria.

According Oliveira et al., (2005) analyzed *Melipona fasciculata* honeys collected aseptically and by the producer found that 65% of the samples collected by the producers had a mold and yeast count above the maximum established by Brazilian standards, while 25% of the samples collected aseptically, there were no significant differences related to the form of collection, similar to that found in this study.

Table 2 Comparison of the results of the counts outside the standards of molds and yeasts and mesophilic bacteria according to the form of collection.

Collection Method	Number of Samples	Number (%) of non-standard samples	
		Molds and Yeasts	Mesophilic Bacteria
Traditional	15	1(6.6)*	4(14.3) ^{ns}
Aseptic	21	9(42.85)*	3(26.6) ^{ns}

* $\chi^2 = 16.84$; $p = 2.65$; ^{ns} $\chi^2 = 0.039$, $p = 0.23$

According to Grosso et al., (2002) the facilities of the apiaries in unhealthy environments, the non-use of GMP of honey during the production chain and the environmental variables can favor the growth of microorganisms that are causal agents of various food poisonings, a fact verified in some apiaries visited during collection, where many hives needed maintenance and cleaning (Grosso et al., 2002; Oliveira et al., 2005).

Moura and collaborators (2014) evaluated the microbiological quality of honey in relation to the level of GMP use by producers and concluded that filamentous fungi and yeasts were found in values higher than allowed by national legislation in up to 90% of apiaries, which did not use gmp and did not have a Honey House. For those who benefited from a better level of GMP and had Honey House, this percentage dropped to 50 %, thus showing that the modification of hygienic habits of beekeepers is an efficient tool for improving the microbiological quality of honey produced by honey bees in the Amazon region of Maranhão.

4 CONCLUSION

In this study, the presence of these microorganisms in high counts even under aseptic collection conditions may be related to environmental conditions during the collection period. Most of the collected samples showed satisfactory hygienic-sanitary quality, therefore these were recommended for human consumption. The non-use of Good Manufacturing Practices during the production of honey in the region constitutes an aggravating factor in the quality of honey produced by honey bees.

It became evident that there is a need for specialized technical support from beekeepers related to the honey production chain for the implementation of Good Practice measures in beekeeping in the Amazon region and that this income-generating activity in the region can be developed through obtaining certifications for marketing as well as ensuring microbiological safety for honey consumers.

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