

Evaluation of physic contaminants and contamination with coliforms, molds and yeasts of honey from the Northern Brazil*

Avaliação de contaminantes físicos e contaminação com coliformes, bolores e leveduras de mel do Norte do Brasil

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Abstract

The Mercosur Technical Regulation for honey determines microbiological parameters for total coliforms and molds and/ yeasts, and absence of physical contaminants, of any nature. The purpose of this study was to verify the biological and physical contamin of *Apis mellifera ligustica* and *Melipona fasciculata*, commercialized in the northeastern region of the State of Pará, Brazil. The fourteen samples from informal trade of the municipalities of Bragança, Capanema, Nova Timboteua, São João de Pirabas, Salinas and Tracuateua were analyzed for the Most Probable Number of coliforms at 35°C, Fungus and Yeast counts, besides the detection of dirt by Macro and Microscopy techniques. The results obtained from the microbiological analyzes were compared to the standard required by MERCOSUR, for coliforms. 57.14% of the samples were non-standard and ranged from <3.0 NMP/g to 20 NMP/g. The molds and/ yeast counts ranged from 1×10^1 CFU/g to 8.6×10^2 CFU/g, with a total of 21.42% of the samples being in disagreement with the legislation. From the macroscopic analysis, it was observed that, although 50% of the samples did not present any apparent dirt, microscopically it was possible to observe dirt in 100% of the samples of honey analyzed, which is out of the standard required by the Brazilian legislation. This shows the low sanitary quality of honey commercialized in this region of the state of Pará.

Keywords: honey. Macroscopic evaluation. Microscopy evaluation. Microbiologic quality. *Apis mellifera ligustica*. *Melipona fasciculata*.

Resumo

O Regulamento Técnico do Mercosul para mel, determina parâmetros microbiológicos para coliformes totais e fungos e/leveduras, e ausência de contaminantes físicos, de qualquer natureza. A proposta do presente estudo foi verificar a contaminação biológica e física em méis de abelha das espécies *Apis mellifera ligustica* e *Melipona fasciculata*, comercializados na região Nordeste do Estado do Pará, Brasil. As amostras provenientes do comercio informal dos municípios de, Bragança, Capanema, Nova Timboteua, São João de Pirabas, Salinas e Tracuateua, foram analisadas quanto a determinação do Número Mais Provável de coliformes a 35°C, Contagem de Fungos e/leveduras, além da detecção de sujidades pelas técnicas de Macro e Microscopia. Os resultados obtidos a partir das análises microbiológicas foram comparadas ao padrão exigido pelo MERCOSUL, para coliformes, 57,14% das amostras encontravam-se fora do padrão variaram de <3,0 NMP/g a 20NMP/g. Já a contagem de fungos e/leveduras variou de 1×10^1 UFC/g a $8,6 \times 10^2$ UFC/g, no total de 21,42 % das amostras apresentaram-se em desacordo com a legislação. A partir da análise macroscópica observou-se que, embora 50% das amostras não apresentassem sujidades aparentes, porém microscopicamente foi possível observar a presença sujidades em 100% das amostras de méis analisadas encontrando-se fora do padrão exigido pela legislação Brasileira. Demonstrando a baixa qualidade sanitária do mel comercializado nessa região do estado do Pará.

Palavras-chave: Microbiologia do mel. Macroscópica. Microscopia. *Apis mellifera ligustica* e *Melipona fasciculata*.

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Introduction

Honey quality can be determined by its sensory attributes, chemical, physical and microbiological characteristics (KUNOVÁ et al., 2015). Contaminations generally occur during extraction and handling (ADJLANE et al., 2014; VÁZQUEZ-QUINONES et al., 2018). Pre-harvest contamination can be attributed to pollen, digestive tract of bees, dust, air, soil and nectar (GALLEZ & FERNÁNDEZ, 2009). Post-harvest contamination comes from manipulators, cross-contamination, equipment and utensils, which can be controlled by good manufacturing practices (PUCIARELLI et al., 2014).

Brazilian legislation does not define a microbiological standard for honey in its Technical Regulation of Identity and Honey Quality (BRAZIL, 2000) neither in the Technical Regulation of Microbiological Standards for Food (BRAZIL, 2001). In the latter, it can be only observed standard values for sugary products similar to honey, such as syrup. However, the the Technical Regulation for honey that governs MERCOSUR countries (MERCOSUL, 1994) establishes criteria indicator microorganisms such as coliforms and molds and / yeasts, physical contaminants in addition to pathogens such as *Salmonella* and *Shigella* (THRASYVOULOU et al., 2018).

Honey naturally has a very low microbial load (GALLEZ & FERNÁNDEZ 2009), due to its supersaturated sugar content (KHALIL et al., 2014), low water content (TAORMINA et al., 2001), its natural acidity, low protein quantity and high viscosity. These parameters limit the penetration of atmospheric oxygen, which is particularly stressful for several microorganisms (KUNOVÁ et al., 2015). Another important factor for the low microbial activity of honey is the presence of phenolic compounds and hydrogen peroxide, which are produced by glucose oxidation from the glucose oxidase enzyme action (IURLINA & FRITZ, 2005). In addition, there is glycolic acid that modifies the acidity and pH of honey, turning it into a hostile environment to molds and bacteria growth.

Microorganisms of concern in honey are molds, yeast and spore-forming bacteria. Molds and yeasts are responsible for the honey fermentation when it presents a moisture content above 21% and *Penicillium* spp. and *Mucor* spp. are generally found in these cases (FINOLA et al., 2007). Through the microscopic analysis it is possible to observe the presence of physical contaminants in honey. It should be free of dirt (MERCOSUR, 1994; BRAZIL, 2000), as undesirable foreign matter, apparent dirt, parasites, larvae (FILHO et al., 2012), insects and sand grains (SOUZA & CARNEIRO, 2008), which may come from unsatisfactory sanitary conditions and inappropriate practices of honey extraction and manipulation.

Due to the importance of honey as a food, its consumption and commercialization in the State of Para, it is imperative that the quality of this product is within microbiological standards. There are few studies in the northern region, which analyze the microbiological quality of honey, and especially the physical contamination of this food. In this sense, studies involving the hygienic quality of honey are of utmost importance.

Thus, the objective of this study was to assess the presence of indicator microorganisms in honey commercialized in the state of Pará, Brazil, by determining the most Probable Numbers of

coliforms at 35°C and the molds and yeast counts, as well as the presence of macroscopic and microscopic dirt.

Material and Methods

For carrying out the study, the total of 14 (fourteen) honey samples commercially available from *Apis mellifera ligustica* (M1, M3, M5, M7, M9, M11, M13) and *Melipona fasciculata*, a specie of geographic relevance, (M2, M4, M6, M8, M10, M12, M14), were collected in the municipalities of Bragança (01°03.2728'S; 046°45.7051'W), Capanema (01°11.3711'S; 047°10.6991'W), Nova Timboteua (01°12.3487'S; 047°23.3239'W and 01°02.2052'S; 047°21.1413'W), São João de Pirabas (00°51.5575'S; 047°14.5793'W), Salinópolis (00°37.5162'S; 047°23.3239'W) e Tracuateua (01°04.4734'S; 046°54.2782'W), which compose the Northeast region of the State of Pará. The samples were kept in their original containers, transported at room temperature and analyzed until 12h after collection. The sample size was calculated considering an estimated prevalence of 50% both microbial contamination and physical contaminants, according to Spiegel et al. (2004), taking into account 5% sample error and 95% significance level.

Microbiological analyzes of coliforms, molds and/ yeasts were carried out according to the methodology proposed by the Brazilian legislation (BRAZIL, 2003) of the Agriculture, Livestock and Food Supply Ministry, which officializes the Analytical Methods for Microbiological Analysis for Product Control of Animal Origin and Water and the results obtained were evaluated according to the Annex III, part of this legislation.

The presence of microorganisms of the coliforms group was expressed in Most Probable Number (MPN/g) of coliforms at 35°C, performed by the multiple tube technique.

Fungus and yeast counts were expressed in Colony Forming Unit, with results expressed in CFU/g, using the Spread-plate technique in Potato Dextrose Agar acidified in 10% Tartaric Acid from serial dilutions.

The determination of foreign matter and dirt in the collected honeys were performed by macroscopy and microscopy. For this, the samples were previously treated according to the methodology proposed by Souza & Carneiro (2008), with adjustments in the preparation of the samples, where 200 ml of the honey sample were diluted in 200 ml of distilled water, previously heated (50° C ± 55° C) aiming to decrease the honey viscosity and better visualize the inert particles.

After this previous treatment, samples were analyzed macroscopically, by naked eye visualization using a 1.5 times magnifying glass, as described by Fontes & Fontes (2012), with adaptations in the filtration step, where filter paper was used for dirt retention and better visualization.

To perform microscopy, the previously treated samples followed the methodology proposed by AOAC (1990), using a Diagtech® XTL6445 model stereoscope microscope with a minimum increase of 14 times.

After obtaining the results, data were tabulated using Friedman's Variance Analysis, followed by Pearson's Linear Correlation, applied from the *BioEstat® Software* version 5.3, aiming to determine the direct correlation between dirt prevalence and coliforms at 35° C, dirt and molds and/yeasts and between coliforms and molds and yeasts.

Results and Discussion

The microbiological analysis results of the Most Probable Number of coliforms at 35° C and molds and/ yeast counts can be observed in Table 01.

Table 1: Coliforms at 35° C and molds and/ yeasts counts in samples of commercially available honeys in the Northeast of the State of Pará, Brazil

Sample	Municipality	Honey species	Coliforms at 35° C	Molds and/ Yeast in CFU/g
M1	Bragança	<i>Apis mellifera ligustica</i>	3,6 NMP/g	1,6X
M2	Bragança	<i>Melipona fasciculata</i>	<3,0 NMP/g	8,6X
M3	Capanema	<i>Apis mellifera ligustica</i>	20 NMP/g	1,1 X 10 ²
M4	Capanema	<i>Melipona fasciculata</i>	7,2 NMP/g	2,0 X 10 ¹ est*
M5	Nova Timboteua	<i>Apis mellifera ligustica</i>	<3,0 NMP/g	1,5 X 10 ¹ est*
M6	Nova Timboteua	<i>Melipona fasciculata</i>	7,4 NMP/g	2,0 X 10 ¹ est*
M7	Nova Timboteua	<i>Apis mellifera ligustica</i>	3,6 NMP/g	3,0 X 10 ¹ est*
M8	Nova Timboteua	<i>Melipona fasciculata</i>	3,6 NMP/g	8,0 X 10 ¹ est*
M9	São João de Pirabas	<i>Apis mellifera ligustica</i>	<3,0 NMP/g	1,0 X 10 ¹ est*
M10	São João de Pirabas	<i>Melipona fasciculata</i>	<3,0 NMP/g	1,0 X 10 ¹ est*
M11	Salinópolis	<i>Apis mellifera ligustica</i>	3,0 NMP/g	2,0 X 10 ¹ est*
M12	Salinópolis	<i>Melipona fasciculata</i>	3,6 NMP/g	1,0 X 10 ¹ est*
M13	Tracuateua	<i>Apis mellifera ligustica</i>	<3,0 NMP/g	3,0 X 10 ¹ est*
M14	Tracuateua	<i>Melipona fasciculata</i>	<3,0 NMP/g	2,0 X 10 ¹ est*

*est: Estimated Value, out of the confidence interval.

Microbiological analyzes showed 57.14% of the honey samples analyzed were above the allowed value for the determination of the Most Likely Number of Coliforms at 35 ° C. Only 6 samples were in compliance with the standard required by MERCOSUR (1994), which determines that the limit for this bacterial group in honey is <3,0 MPN/g, indicating the product is in satisfactory hygienic-sanitary quality conditions.

The results obtained by counting molds and/ yeasts showed 78.57% of the honey samples were in agreement with the value established by MERCOSUR, not exceeding the limit of 1.0X10² CFU/g counts.

Different results were presented by several researchers in relation to coliform absence at 35° C in honey samples (IURLINA & FRITZ, 2005; SILVA et al., 2008; ALVES et al., 2009; GALEZ & FERNADEZ, 2009; SOUZA et al., 2009; GOMES et al., 2010; ADJELANE et al., 2014), as well as the studies by Santos & Oliveira (2013), when analyzing *Apis mellifera* honey from seven warehouses in the Jaguaribe Valley region, State of Ceará. They showed no analyzed samples presented contamination by coliforms at 35° C, known as total coliforms as well as the 45° C coliforms, called fecal coliforms.

The high presence of sample with coliforms in the present study indicates the presence of hygienic failures during the process of honey harvest. Such faults may be associated to several factors depending on the bee species that produces honey. For *Apis mellifera* honeys, different from the *Melipona fasciculata* whose

production is made in more rustic nests, the steps of honey removal from the honeycomb, settling and filtering allow foreign matter to serve as a means of contamination for this food. For honeys obtained through beekeeping, stages of honey removal from the honeycomb and hygienic

The results shown in table 1, when evaluated for molds and/yeast determination, presented values within the acceptable limit established by current legislation. The samples (M1 and M2 from the municipality of Bragança and M3 from the municipality of Capanema) presented results in CFU/g of 1.6 X 10² and 8.6 X 10², 1.1 X 10², respectively, falling outside the standard of 1.0 X 10² CFU / g, required by MERCOSUR (1994). The presence of this class of microorganisms serves as an indicator of hygienic deficiencies in the stages that occur during the process of obtaining honey (extraction, decanting, pressing and filtration) as well as in the packages used and, in the places, where honey is fractionated and stored. These factors, associated with improper manipulator handling and the structure of honey houses (warehouses) favor contamination by these microorganisms, which are considered indicators.

Similar results were demonstrated by Souza et al., (2009), who observed counts varying between <1.0 × 10 and 4.4 × 10³ CFU/g in 50.0% of the analyzed honeys,

in the state of Bahia, reinforcing the hypothesis that there is a strong relationship between honey production and microbial contamination. Similarly, Cordeiro et al., (2012) observed formation of mold and yeast colonies in 33.3% of the total of 66 samples. In the studies of Silva et al., (2008) in honey samples from the northern region on the Minas Gerais State Forest Zone showed higher contamination by filamentous molds and yeasts by 2.9x10⁴ CFU/g. In honeys analyzed by Adjelane et al., (2014), yeasts and molds were also present, but at lower levels as well as in the study by Pontara et al., (2012), where growth below 10 CFU/g for molds and yeasts was observed, corroborating with 18.89% of the results observed in the samples analyzed in the present study.

The results obtained from the macroscopic analysis of the collected honey samples showed 50% (7/14) of the samples contained apparent dirt, that is, possible to detect by naked eye. In samples M6, M7, M11 and M13, the presence of whole insects was verified.

By performing the microscopic analysis, several dirt was found, such as: hairs of unknown origin, insect fragments, whole insects, wood fragments and unidentified dirt, as we can see in Table 02.

Table 2: Microscopic analysis on samples of commercially available honeys in the northeast of the state of Pará, Brazil

Samples	Non identified Dirt	Identified Dirt			
		Organic Extraneous Matter			
		Hairs	Insect Fragments	Whole Insects	Wood
M1	Uncountable	1	ND	ND	ND
M2	ND	1	1	ND	ND
M3	ND	1	ND	ND	ND
M4	6	1	ND	ND	ND
M5	ND	ND	2	ND	ND
M6	ND	ND	ND	1	ND
M7	ND	2	ND	112	ND
M8	ND	1	2	1	ND
M9	ND	6	ND	ND	ND
M10	ND	ND	ND	2	ND
M11	4	ND	ND	ND	ND
M12	9	ND	ND	ND	ND
M13	11	ND	1	1	ND
M14	ND	ND	1	ND	1

ND: not detected

Microscopic analysis also revealed the presence of hairs in 50% of the samples (M1, M2, M3, M4, M7, M8, M9), four samples from *A. mellifera* and three from *M. fasciculata* (Figure 1).

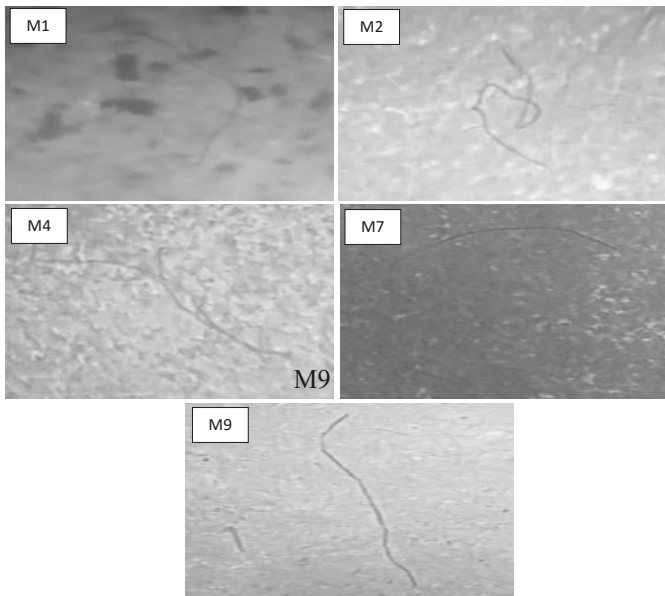


Figure 1: Photomicrography showing contamination by hairs in honey samples (M1, M2, M4, M7, M9), commercially available in the Northeast of the State of Pará, Brazil.

In samples M6, M7, M10 and M13, the presence of whole insects, mainly ants, was detected, and in the M7 sample a total of 112 ants were found (Figure 2).

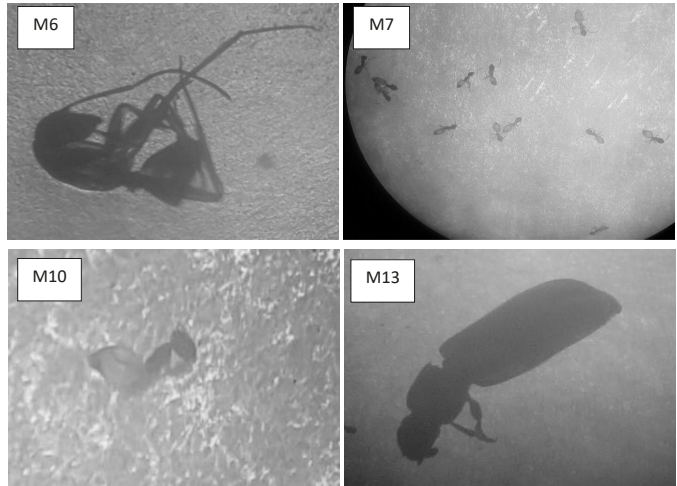


Figure 2: Photomicrography showing physical contamination by whole insects in honey samples (M6, M7, M10, M13), commercially available in the Northeast of the State of Pará, Brazil.

In samples M2, M5, M8, M12, M13 and M14, the presence of several insect fragments, mainly paws, was observed, besides wood fragment linked to sugar crystals (Figure 3).

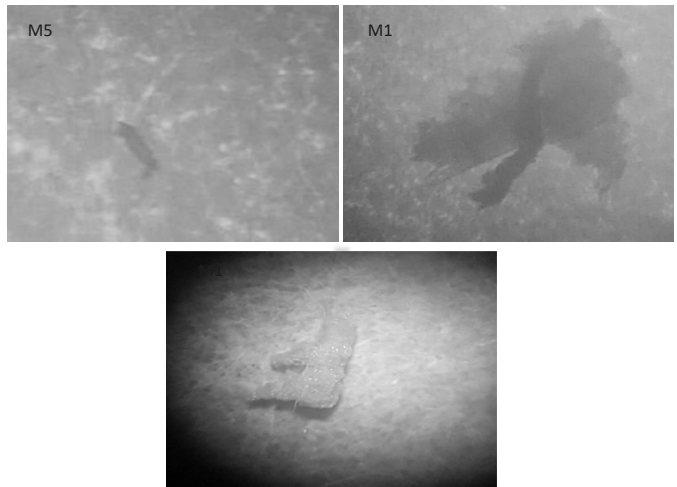


Figure 3: Photomicrography showing the presence of insect fragments (M5 and M6) and sugar granules adhered to a wood splint (M1) in commercially available honeys in the Northeast of the State of Pará, Brazil.

Dirt analysis in honey is a quality parameter required by Brazilian legislation, which deals with foreign matters in food (BRAZIL, 2014). This legislation, which states on macroscopic and microscopic foreign matter in food and beverages, does not establish specific tolerance limits for honey. However, the Technical Regulation on Honey Identity and Quality (BRAZIL, 2000) and the Mercosul Technical Regulation on Honey Identity and Quality (MERCOSUL, 1994) require this food be free of

foreign substances of any kind. Therefore, our results indicate a large part of the honey commercialized in the target region of our study is found to be non-conforming to this criterion.

In this work, the presence of macroscopic dirt was detected in 42.85% of the samples. Nevertheless, it was not possible to observe the presence of macroscopic dirt in the samples corresponding to the Bragança and Capanema municipalities (both in *A. mellifera* and *M. fasciculata* honeys), Nova Timboteua and São João de Pirabas (*A. mellifera* honey) and Salinópolis (*M. fasciculata* honey).

It was also observed that, macroscopic analysis of some of the samples did not present any apparent physical contaminations, but when it was analyzed microscopically it was possible to observe that 100% of the samples were in disagreement with the legislation, because they presented dirt corroborating with the affirmations of Fontes & Fontes (2012), when they indicate the stereomicroscope use contributes to the detection of components capable of passing unnoticed in a direct analysis.

Few studies report macro and microscopy analysis on commercially available honey samples, but they are extremely important parameters for honey quality, since physical contamination is a risk factor for food safety and can lead to damages to consumer health, as well as being a determination required by current legislation. In a dirt survey with 10 samples of bee honeys of the *Apis mellifera* species, Lima et al., (2011) did not find macroscopic materials, but occurrence of dirt in 100% of the samples was verified, when these were analyzed microscopically.

In the studied honeys, it was possible to observe the presence of hairs in 50% of the samples, using the macroscopic technic. The findings of Martins et al., (2014), in commercial honeys in São João do Meriti, state of Rio de Janeiro, using microscopy detected the presence of hairs in one of the five samples of honeys analyzed. Campos et al., (2016), when analyzing the hygienic quality of honey sold in free markets and supermarkets in the city of Belém, state of Pará, observed the presence of hairs and insects such as ants and spiders, results that resemble those described from the analyzed samples in the present study.

The presence of any type of insect or possible contamination vectors may be associated with improper processing practices associated with handling and storage sites and especially the use of inadequate packaging. Brazilian legislation (BRAZIL, 2014) considers insects as cockroaches, ants, flies, among others, extraneous matters indicative of health risks, since they are capable of linking pathogens to food. The authors Fontes & Fontes (2012) point out the structure of fragments of mite and insect exoskeletons is constituted by chitin and the digestion of these components is not possible, since human digestive system does not have the chitinase enzyme, which may cause nutritional implications by occasional ingestion.

Ants were detected at both macro and microscopic levels. The presence of ants is considered common in honey samples, because it is a sweet product and has attractive potential for this type of insect. However, factors related to packaging, storage location and especially an efficient program of good practices in the honey obtaining and processing are responsible for the effectiveness of the control of this type of dirt in honey. Another factor related to the presence of ants in containers that keep honey is the fractionation procedures of the product in

the informal commerce, where used containers are improper because they are reusable, like bottles of glass or plastic drinks, besides not having system for internal protection of this product.

The presence of sugar crystals was not associated in this study with the crystallization phenomenon, which occurs naturally in honey when stored at refrigeration temperatures and it is associated with its purity. The studied samples were purchased in establishments that did not have refrigeration equipment, both for commercialization and storage of the product, as well as transportation to the place of analysis was performed at room temperature. Thus, it can be associated to the presence of sugar crystals, visualized by microscopy, to possible frauds or adulterations by addition of syrups or commercial sugar in the honeys.

In the literature, it was observed the scarcity of information on the detection of sugar crystals by microscopy. In addition, there is no methodology for detection of possible fraud by adding syrup or sugar in honey. However, the finding of the present study corroborates the results observed by Rodrigues et al., (2016), when they studied honey sold in open fairs in the city of Cuiabá, state of Mato Grosso, where they visualized the presence of sugar grains in four of the five analyzed samples, associating this result as adulteration evidence.

Through the Friedman Test ($F_{r2} = 2.8214$), it is possible to observe significant values ($p = 0.2440$), when analyzing the parameters of evaluation of the honey quality studied, determining the direct correlation between dirt prevalence and coliforms at 35° C, dirt and molds and/yeasts. Additionally, from the statistical analysis, it was possible to observe a strong Pearson Linear correlation between dirt, coliforms and molds and/or yeasts present in the honey samples.

Table 3 presents the results where this relation was evaluated through the Pearson correlation.

Table 3: Pearson linear correlation between dirt (D) and coliform (C), dirt (S) and mold and/or yeast (MY) and between coliform (C) and mold and/or yeast (BL) contaminations

Pearson Linear Correlation 211			
	D/C	D/MY	C/MY
n	14	14	14
R	0,1743	0,9763	0,06337
P	0,5513	1,0000	0,8286

The R values, close to 1, show a positive linear correlation

between the presence of dirt and the other microbiological determinations. The results obtained from the Pearson Linear Correlation show a strong positive correlation between dirt and coliform (D/C), dirt and mold and yeast (S/BL) and between coliforms and molds and yeasts (C/MY), thus rejecting the null hypothesis. For this reason, it can be inferred the intensity of dirt presence is directly proportional to the microbiological contamination, which increases the importance of the results presented here, and raises the need for hygienic-sanitary control measures at all stages of the honey production process and commercialization in the areas used here as reference and in other localities of the state of Pará.

Conclusions

The results obtained for the microbiological evaluation and dirt contamination research in commercialized honey in the State of Pará show this food deserves attention in the application of

good beekeeping practices to guarantee the microbiological quality of the product, mainly to improve the quality of the product, production quality, productive management and hygiene practices in the handling of this food.

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