Original Article

MICROBIOLOGICAL QUALITY OF *APIS MELLIFERA* L. HONEY SAMPLES FROM WESTERN PARANÁ, SOUTHERN BRAZIL

Douglas Galhardo[1]*
Regina C. Garcia[1]
Cibele R. Schneider[1]
Sandra M. Ströher[1]
Bruna L. M. Cerny[1]
Emerson D. Chambó[2]

[1]Animal Science Department, State University of Western Paraná, Brazil
[2] Institute of Nature and Culture, Federal University of Amazonas, Benjamim Constant, AM, Brasil

*corresponding author: galhardo.douglas@gmail.com
Received: 03 March 2019; accepted: 20 August 2020

Abstract

Honey from the western region of Paraná receives the seal of origin and in-process denomination of origin because it is mostly produced in permanent preservation areas, adherence to hygiene and sanitary standards, proper management techniques and agro-industrial management. The objective of this study was to verify the microbiological quality of honey produced by beekeepers associated with Cooperative. Sixty-seven honey samples were evaluated for microbiological quality, and the mean values observed to total aerobic mesophiles were 2.52 log CFU/g; *Clostridium* spp. 1.46 log CFU/g; total coliforms 0.78 log CFU/g; fecal coliforms 0.50 log CFU/g; total yeasts 0.46 log CFU/g, and 2.95 log CFU/g total molds. The evaluations showed the incidence of anaerobic mesophilic in 70% of the samples, *Clostridium* spp. 77%; total coliforms 60%, fecal coliforms 62%, molds 83% and 34% yeasts. Five genera of the isolated molds commonly found in honey were *Fusarium* spp. 0.35 log CFU/g; *Aspergillus* spp. 0.52 log CFU/g; *Cladosporium* spp. 1.2 log CFU/g, *Phoma* spp. 0.71 log CFU/g and 0.16 log CFU/g *Penicillium* spp. Multivariate analysis showed the formation of two groups and indicated the heterogeneity in the bacterial load of the samples. Although microbiological analyses were not mandatory for honey, these results demonstrated the hygienic-sanitary quality of the samples of honey produced. For the reduction of secondary-origin microorganisms, the handlers were recommended to follow the sanitary hygiene norms and handling techniques.

Keywords: *Cladosporium*, Denomination of Origen, honeybee, microbiological safety

INTRODUCTION

Because honey is a saturated solution of sugar obtained from nectar which undergoes dehydration of up to 15 to 18% humidity, it is considered a naturally safe food against microorganisms. The high concentration of carbohydrates, as an antimicrobial factor, limits the amount and activity of water to microorganisms, causing a low reduction potential and high viscosity that limits the entry of oxygen into honey. These factors contribute to the stability of honey, which prevents microorganisms from developing and multiplying, resulting in senescence until their mortality (Molen et al., 1997; Wen et al., 2017). Therefore, only a limited number of microorganisms are to expected survive in honey. Yeasts, fungi, and such spore-forming bacteria as *Clostridium botulinum* are found in honey, but their high presence is indicative of immature honey harvest as it presents conditions for them to survive and proliferate (Snowdon & Cliver, 1996). The primary source of these microorganisms are pollen grains, bee-collected nectar and such factors related to the digestive tract of bees and natural environment as dust, air and soil (Kačániová et al., 2009; Anderson et al., 2013; Corby-Harris, Maes, & Anderson,

2014; Wen et al., 2017). The hygiene of the manipulators of honey, honey house, equipment, and physical structure can also be a source of microorganisms (Fernández et al., 2017). The quality control of honey is determined through international regulations for the physical-chemical parameters of moisture, sugars, hydroxymethylfurfural, diastase activity, acidity, electrical conductivity and color (Codex, 2001). However, many countries have their own regulations, which generate some divergences in these parameters (Silva et al., 2016; Pascual-Maté et al., 2018). Although the microbiological analysis of honey is not necessary as routine, it improves the product's food safety and helps to diagnose possible hygienic-sanitary flaws in colony management and product handling. Thus, importance of Brazil's honey production and increased exports, its commercial honey is governed by the laws of the Southern Common Market (MERCOSUR), which accepts a maximum of 100 CFU/g for fungi and yeast and does not allow the presence of *Salmonella* and *Shigella* or of total coliform bacteria in honey.

The western region of Paraná in the year 2016 produced 705 tons, which represents 15.6% of state production (IBGE, 2018). Because beekeeping is important in this region, the beekeepers working together with Beekeeping Cooperative of the West Coast of Paraná, in partnership with the University State of the West of Paraná, ITAIPU-Binational, have been awarded the Indication of Origin (IP) seal, a type of Geographical Indication (GI), with the National Institute of Intellectual Property (INPI, 2017). These institutions and others that have joined the group have been working to obtain, another GI, the origin denomination quality label for honey produced in this region through research that proves its quality and relate the characteristics of the product to its phytogeographic origin (Camargo et al., 2014; Jacobus et al., 2019). In this study, we characterize the microbiological situation of the honey samples to assess whether beekeepers are applying and following food safety rules. Then, Non-metric Multidimensional Scaling analysis was applied to establish multifactorial relationships and observe the behavior of sample clusters from the region.

MATERIAL AND METHODS

Honey sampling

Sixty-seven (n = 67) honey samples from *Apis mellifera* L. collected between October 2016 and March 2017 were supplied from beekeepers of fourteen municipalities in the Western region of Paraná. Fig. 1 shows the geographical origin of the honey samples analyzed: Santa Helena (n = 27), Missal (n = 7), Terra Roxa (n = 7), Marechal Cândido Rondon (n = 7), Diamante do Oeste (n = 4), Entre Rios do Oeste (n = 3), Matelândia (n = 3), Corbélia (n = 2), Toledo (n = 2), Francisco Alves (n = 1), Itaipulândia (n = 1), Palotina (n = 1), Ramilândia (n = 1) and one of São José das Palmeiras.

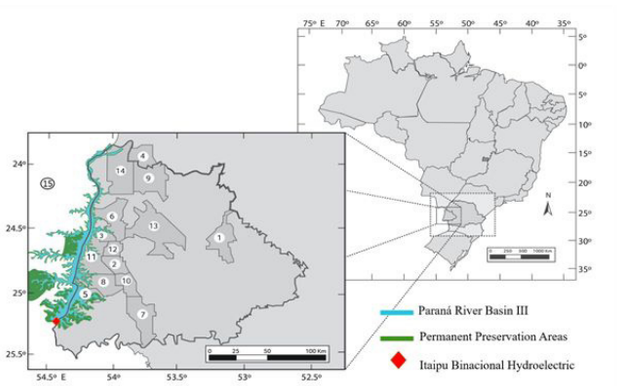


Fig. 1. Location map of the study area: (right) map showing the position of the State of Paraná in Brazil, (left) map showing the position of western region of Paraná, with emphasis on sampling municipalities. 1 - Corbélia, 2 - Diamante do Oeste, 3 - Entre Rios do Oeste, 4 - Francisco Alves, 5 - Itaipulândia, 6 - Marechal Cândido Rondon, 7 - Matelândia, 8 - Missal, 9 - Palotina, 10 - Ramilândia, 11 - Santa Helena, 12 - São José das Palmeiras, 13 – Toledo, 14 - Terra Roxa, as well as the Paraná River Basin III, which borders Paraguay (15).

The sample extraction process was carried out either at the producers' own honey houses or at the cooperative extraction unit, both following international standards due to the export process and the honey origin denomination seal. All samples separate according to producer and period were collected using sterile 500 mL

vials. Each evaluated honey sample represents a sample composed of an apiary. The samples were kept at room temperature without be pasteurized, filtrated or mixed with those of different apiaries and later were delivered to the cooperative. They were collected weekly, sent to the laboratory and stored at 6 to 8°C, because of the tropical climate, until the time of analysis.

Microbiological analysis

Twenty-five grams of each honey sample was homogenized with peptone water 0,1% and decimal dilutions were made 10^{-1}, 10^{-2}, and 10^{-3}. All evaluations were performed according to Silva, Junqueira, & Si (1997) and Sereia et al. (2017). The coliforms were analyzed at 35°C in broth bright green bile lactose 2%, incubated at 35°C for 24 to 48 hours, of fecal coliforms (45°C) and *Escherichia coli* in broth sodium lauryl sulfate, incubated for 48 hours at 44°C. For total aerobic mesophiles bacteria, the standard plate count agar (PCA) was used and incubated at 36°C for 48 hours. The total count of *Clostridium* spp. was determined using the standard reinforced clostridial agar medium, incubated inverted with carbon dioxide system at 35°C for 24 hours. Mold and yeast were counted the use of the culture potato dextrose agar medium acidified with 10% tartaric acid to pH 3.5, 1 g/L of antibiotic (benzylpenicillin) was added and the plates were incubated at 28°C for seven days. The number of microorganisms was determined by the number of colony-forming units per gram of honey (CFU/g) and transformed to $\log_{10}$. Afterwards, in order to identify according to Samson et al. (1996) the genera of the filamentous mold colonies under a microscope. Slides were prepared, and spore grains were removed from the colony border and placed between a blade and coverslip with a drop of lactophenol cotton blue (Nasser, 2004).

Statistical analyses

The values low and high, median, percentiles, quartiles, the mean and standard deviation for the microbiological communities were calculated. Variable data were analyzed by multivariate factor analysis. The Non-metric Multidimensional Scaling (NMDS) was used to test the differences in the parameters among the different honey samples. Subsequently, the Euclidean distance was used with the normalized data and the command "metaMDS" to generate random and interactive processes and to find the best possible solution. The value of the measured NMDS adjustment was evaluated by the stress value. The cophenetic correlation coefficient was calculated to estimate the fit between the dissimilarity matrix and the generated dendrogram (Estevinho et al., 2016). Statistical analyses were performed with statistical software R, version 3.0.2.

RESULTS

Microbiological analyses showed that all samples presented values within the range determined by the RDC-12 technical regulation of 2001. The microbial counts in the samples are shown in Tab. 1. In general, the mean incidence of aerobic mesophilic bacteria, yeasts, and fungi was lower than that reported by other studies (Tab. 1). The total count of aerobic mesophilic had a mean value of 2.52 log CFU/g (Tab. 1), ranging from 0 to 4.4 log CFU/g among samples from different locations (Tab. 2).

The total yeast and mold counts, present mean values of 0.46 log CFU/g and 2.95 log CFU/g suggest adequate management by the great majority of beekeepers (Tab. 1). However, the Entre Rios do Oeste and Itaipulândia samples had a mean between 7.1 and 8.9 log CFU/g for molds. Regarding the sanitary quality of the samples, was a reduced incidence of *Clostridium* spp. 1.46 log CFU/g, total coliforms 0.78 log CFU/g, and 0.50 log CFU/g fecal coliforms (Tab. 1). Despite the reduced microbial load in the evaluated samples, the incidence of aerobic mesophilic was observed in 70% of the samples, *Clostridium* spp. in 77%, total coliforms in 60%, fecal coliforms in 62%, total yeasts in 34% and total molds in 83% (Tab. 1).

Table 3. Shows the genera of isolated fungi. The genus that presented the highest incidence was *Cladosporium* spp., 77% of the total of fungi,

Table 1.
Microbial analyses of the 67 samples of honey from the west State of Paraná, Southern Brazil

Parameters	%	Low	High	Median	Q1[a]	Q3[b]	Mean ±SD[c]
Aerobic mesophiles [d]	70	0	4.48	2.70	0	4.40	2.52 (±1.85)
Clostridium spp. [d]	77	0	3.40	1.30	1.00	2.60	1.46 (±1.05)
Total coliforms [d]	60	0	2.32	0.96	0	1.30	0.78 (±0.66)
Fecal coliforms [d]	62	0	1.81	0.48	0	0.85	0.50 (±0.47)
Total yeasts [d]	34	0	2.15	0	0	1.00	0.46 (±0.68)
Total molds [d]	83	0	9.00	2.85	1.00	4.62	2.95 (±2.44)

% = percentage of incidence of microorganisms in 67 honey samples evaluated. [a]Q1: 25% of the samples are between medians and quartiles1. [b]Q3: 75% of the samples are between the median and quartiles3. [c]Mean and standard deviation (SD). [d]Colony forming unit per gram of honey (log CFU/g).

Table 2.
The mean values of microbial analyses of the 67 samples of honey from the beekeepers in 14 municipalities in the west State of Paraná, Southern Brazil

Location	Aerobic mesophiles	*Costridium* spp.	Total coliforms	Fecal coliforms	Total yeasts	Total molds
Santa Helena (n=27)	2.6	1.5	0.5	3.1	1.0	0.6
Missal (n=7)	1.6	1.5	0.3	4.5	1.0	0.5
Terra Roxa (7)	3.4	1.3	0.5	1.7	1.0	0.1
Marechal Cândido Rondon (n=7)	3.2	1.7	0.3	0.7	0.3	0.4
Diamante do Oeste (n=4)	0.5	2.2	1.2	3.5	0.4	0.7
Entre Rios do Oeste (n=3)	4.4	1.1	0.7	5.7	0.9	0.7
Matelândia (n=3)	2.3	1.5	0.3	2.5	0.4	1.1
Corbélia (n=2)	1.2	1.2	1.1	1.0	0.0	0.4
Toledo (n=2)	1.4	1.2	0.0	5.6	0.6	0.4
Francisco Alves (n=1)	4.4	1.0	0.0	1.0	0.0	0.0
Itaipulândia (n=1)	4.4	0.0	0.0	2.0	1.4	0.0
Palotina (n=1)	0.0	1.0	0.0	8.9	1.2	0.0
Ramilândia (n=1)	4.4	1.3	0.0	4.8	1.2	0.0
São José das palmeiras (n=1)	0.0	1.7	1.7	1.0	0.0	0.8

Colony forming unit per gram of honey (log CFU/g).

representing. This may be due to the cosmopolitan characteristics. The other fungi *Phoma* spp. had an incidence of 49%, *Aspergillus* spp. 31%, *Fusarium* spp. 22%, and *Penicillium* spp. 13% (Tab. 3).

Multivariate analysis

NMDS analysis visualized the formation of a general structure of the bacterial groups in the honey samples and indicated a similarity between them (Fig. 2). A e stress value of 0.154 indicated that the distance between the points in the ordering chart is representative of

Table 3.

Genera of molds identified in honey samples from the west of the State of Paraná, Southern Brazil

Parameters	%	Low	High	Median	Q1[a]	Q3[b]	Mean ±SD[c]
Fusarium spp.[d]	22	0	5.00	0	0	0	0.35 (±0.83)
Aspergillus spp.[d]	31	0	3.30	0	0	1.00	0.52 (±0.88)
Cladosporium spp.[d]	77	0	5.30	1.00	1.00	1.57	1.20 (±1.03)
Phoma spp.[d]	49	0	3.30	0	0	1	0.71(±0.87)
Penicillium spp.[d]	13	0	3.24	0	0	0	0.16 (±0.50)

% = percentage of occurrence of molds in 67 samples evaluated. [a]Q1: 25% of the samples are between medians and quartiles1. [b]Q3: 75% of the samples are between the median and quartiles3. [c]Mean and standard deviation (SD). [d]Colony forming unit per gram of honey (log CFU/g).

the degree of similarity between the samples and the bacterial communities. Samples from the upper left quadrant had higher values for molds, yeasts, *Fusarium* ssp., *Penicillium* spp., *Cladosporium* spp., *Aspergillus* spp., *Phoma* spp. and fecal coliforms. The right quadrant showed higher values for *Clostridium* spp. and aerobic mesophilic (PCA), and, the lower part between the two quadrants presented the highest values for total coliforms.

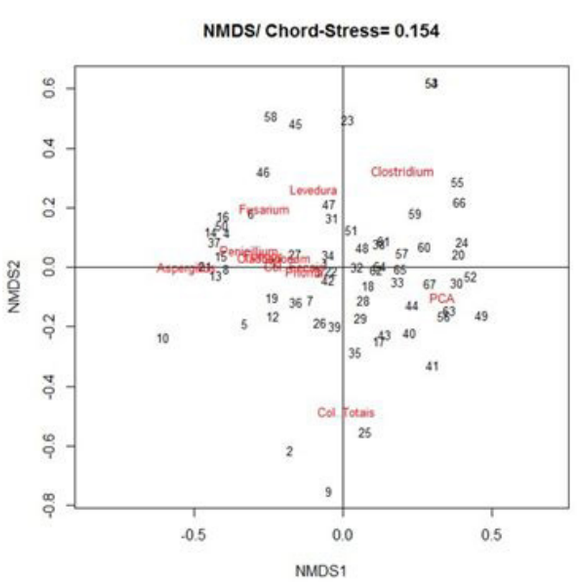


Fig. 2. Shows Non-metric Multidimensional Scaling (NMDS) for the similarity of the microorganisms of 67 honey samples. Each number refers to a sample and the acronyms are the different microbial communities which generates a chord distance matrix with a stress value of 0.154.

However, NMDS was not sufficient to distinguish whether the separation of the cluster was due to some microbial communities showing a degree of overlap. Thus, the application of the method of the width of the silhouette determines the optimal point for the formation of two groups (Fig. 3). Occurring a cophenetic correlation coefficient (CCC) of 0.76 between the original similarity matrix and the resulting matrix of similarity. Estevinho et al. (2016) believes this CPCC value is reasonable as a representative factor of the hierarchy.

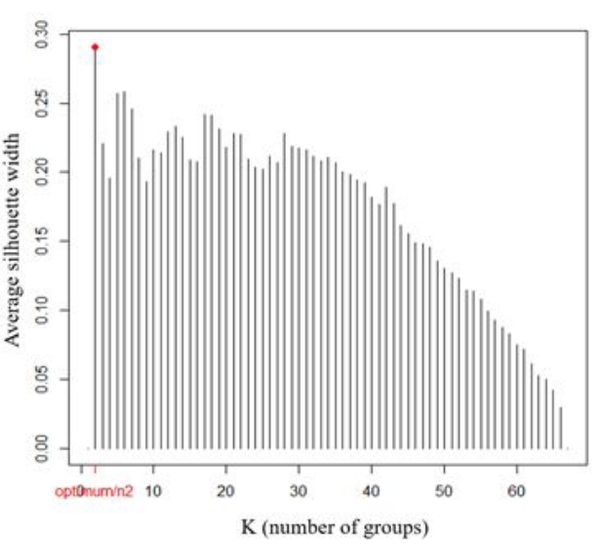


Fig. 3. The bar chart shows the average width of the silhouette for k = 2 groups for 67 samples.

After the ordination of the NMDS graph and the application of the silhouette-width method, the formation of two groups was determined. Together with the clustering analyses (UPGMA), using the Euclidean distance, the difference of the groups between the samples was determined more accurately.

The new NMDS projection (Fig. 4) demonstrated that despite the large projection area, there was a similarity between the microbiological parameters. Although the profiles were visually separable, the samples were separated into two groups. The largest group presented a group of sixty-four samples covering all the microbiological parameters except for total coliforms, which were grouped in the group within the circle, since these three samples presented the highest values of total coliforms with the other parameters.

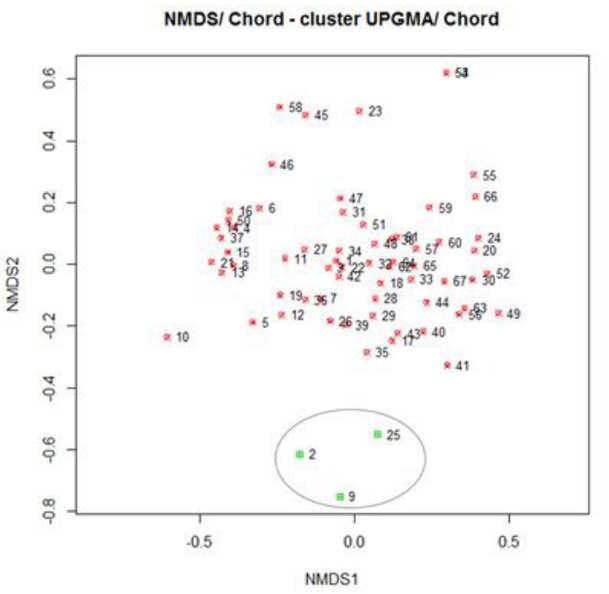


Fig. 4. UPGMA clustering of a string distance matrix between two groups on an NMDS ordering chart for microbiological data of 67 honey samples (CC = 0.76).

DISCUSSION

Honey is a saturated solution of sugar, which limits the quantity and activity of water for the microorganism, whose development and multiplication is prevented its, leading to its senescence until its mortality (WEN et al., 2017). Other antimicrobial factors of honey are low protein content, acidity, and reduced oxidation-reduction potential together with the high viscosity that limits the entry of oxygen into the honey (SILVA et al., 2016). This research showed that the honey produced by beekeepers was very similar with some exceptions, which are being corrected with technical monitoring. The observed values of the microbial population of aerobic mesophiles from this study are below those found in the literature. The samples of honey from Benin ranged from 2.25 to 2.46 log CFU/g (Azonwade et al., 2018) and those of organic honey from Portugal around $1.3x10^{2} \pm 75.5x10^{1}$ CFU/g (Estevinho et al., 2012). These values demonstrate that the incidence of these microorganisms occurs even in regions with high sanitary control.

The microorganisms found in this study for aerobic mesophiles, molds, and yeasts are probably related to the primary contamination by the intestinal contents of the bees, the environment of the colony and the plants foraged by them (Anderson et al., 2013; Wen et al., 2017). These same authors affirmed that a microbial flow occurs inside the colony, because they observed the same microorganisms in the flowers, the bee bread, the environment of the hive and the intestine of the bees.

This study indicated the incidence of *Clostridium* spp., the most important micro-organism for human health. The contamination may have occurred due to foraging, air, dust or energy supply provided by beekeepers during winter (Snowdon & Cliver, 1996). Jay, Loessner, & Golden (2005) reported *Bacillus* and *Clostridium* may be important bacterial contaminants of sugar cane (*Saccharum officinalis*) and beet (*Beta vulgaris*), which are used in the winter feeding of colonies in this region.

The incidence of *Clostridium* spp. was also observed by Pucciarelli et al. (2014), in 70% of the samples when evaluating the growth of this microorganism in the honey of *Tetragonisca angustula*, although they were not able to isolate *Clostridium botulinum* in the same samples through biochemical tests. This suggests that in the present study the

incidence of *Clostridium* spp. does not indicate the presence of *C. botulinum*. Although the antibacterial conditions honey with low pH (3.4 to 5.5) and low water activity (0.5 to 0.6) acts as an antimicrobial factor inhibiting the survival of bacteria and is frequently associated with infant botulism (Aureli, Franciosa, & Fenicia et al., 2002). Due to such characteristics, the World Health Organization (WHO, 2002) and National Health Surveillance Agency (Anvisa, 2011) do not recommend honey for infants and children under 12 months of age, because they have limited intestinal microflora and low immunity.

The low yeast value demonstrated limited growth of these microorganisms, indicating the good hygienic-sanitary quality of the samples because yeasts are transferred to honey through primary and secondary contamination (Snowdon & Cliver, 1996). Furthermore, physicochemical analyses indicated low moisture content (<20%) of the collected honey, which makes bacterial growth difficult because the above 21% moisture is known to favor the proliferation of these microorganisms which survive in acidic conditions, cause fermentation and the formation of carbon dioxide and alcohol and reduce the quality of honey (Snowdon & Cliver 1996; White, 2010). Sereia et al. (2010) evaluated the hygienic-sanitary quality of organic and non-organic honey samples from the Paraná River islands and observed that organic honey presented lower quality than conventional honey to hygienic-sanitary quality and that the humidity of the samples was the main factor for the reduced product quality.

The incidence of these microorganisms indicated secondary contamination for the sanitary quality (total coliforms and fecal coliforms) and safety (*Clostridium* spp.) of the samples (Snowdon & Cliver, 1996). The values of coliforms indicated direct or indirect contamination of fecal origin since the natural habitat of coliforms in the enteric tract of humans and animals and the absence of these microorganisms indicate adequate hygiene during collection, extraction, and handling of honey (Sanz et al., 1995).

Total coliforms and fecal coliforms in the literature (Pucciarelli et al., 2014) were present in a greatest variety in honey samples in Argentina, Italy (Sinacori et al., 2014) and Mexico (Vázquez-Quiñones et al., 2018) and absent in the samples evaluated from Spain (Sanz et al., 1995), Argentina (Iurlina & Fritz, 2005), Portugal Estevinho et al. (2012) and Mexico Fernández et al. (2017). Therefore, this is first study on honey in the process of designation of origin in Brazil and provides information for future work to reduce these microorganisms in honey. Increased microbiological values in the locations furthest from the cooperative (Santa Helena municipalities) such as Terra Roxa, Marechal Cândido Rondon, Entre Rios do Oeste, and Francisco Alves may be due to less technical assistance and training of beekeepers. These skills are important as good production practices improve the quality of the honey produced. Evaluation microorganisms at different points in honey processing (honeycombs, honeycomb uncapping, honey extractor, honey sump, and honey drum), demonstrated that in eight honey extraction units, when good manufacturing standards were not followed observed contamination increased per coliforms at all points of evaluation (Fernández et al., 2017). Sereia et al. (2011) concluded the same in the evaluation of organic honey production systems and reported a reduction of molds and yeasts on samples of honey processed adequately to those whose beekeepers did not follow good hygiene practices, by Administrative Rule No. 367 of September 4, 1997 (Brasil, 1997).

The genera of molds identified in this study corroborate the data of the literature, since the genus *Penicillium*, *Cladosporium*, *Aspergillus*, and *Alternaria*, are considered common microorganisms in honey samples (Nasser, 2004; Kacaniová et al., 2009; Wen et al., 2017). Sinacori et al. (2014) and Knazovická et al. (2015) verified that the microflora of multifloral honey had higher rates of biodiversity and species richness compared to monofloral ones. The highest percentage of *Cladosporium* spp. observed may be due to this genus having a cosmopolitan distribution with 772 names (Ducan et al., 2004), and its spores can be found in the air, soil, water, and plants (Ogórek et al., 2012).

Kačániová et al. (2009) reported a high incidence (25,000 CFU/g) of the genus *Cladosporium* and the genus *Penicillium* (12.66%), in honey, *Cladosporium* (13.27%) and *Penicillium* (72.24%) in the environment of the colony. With an increase in molds and yeasts in the enteric tract of the bees between the season of the summer and the winter, suggesting a microbial flow within the colony (Kačániová et al., 2009).

Wen et al. (2017) evaluated the microbial load in the first fifteen days of honey maturation and observed that while the ripening process and reduction of the moisture content of honey occurred there was a reduction in the quantity and richness of microorganism species. Besides, these authors pointed out that, after five days of maturity, there was a reduction of the genera *Cladosporium*, *Alternaria*, *Aspergillus*, *Penicillium*, *Phoma* while yeast after that period remained in a small quantity. This suggested that samples collected ahead of time, with high moisture content, become more favorable to the multiplication of these microorganisms which leading honey deterioration.

Thus, it was concluded that the majority of the samples were within the parameters accepted by the legislation. The largest microbial communities were of mesophilic aerobes and molds. The analyses showed that honey samples are similar to one another for most microbial communities. These results indicate that honey produced in the western region of Paraná are very similar. The results reported in this study show a sampling of the situation of the bee chain in the region, and with this information it is possible to set up an activity plan to improve technology transfer to beekeepers.

ACKNOWLEDGMENTS

We thank the beekeepers associated with the Beekeeping Cooperative of the Western Coast of Paraná (COOFAMEL) for the samples provided. This work was financially supported by the Brazilian National Council for Scientific and Technological Development (CNPq) for financing our project (Process No. 443302/2016-2) and the Coordination for the Improvement of Higher Education Personnel (CAPES). The Research Support Foundation of the State of Amazonas (Fundação de Amparo à Pesquisa do Estado do Amazonas - FAPEAM) for the financial support to the project coordinated by Emerson Dechechi Chambó (Notice 004/2017 - PPP).

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