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Characterization of yateí (*Tetragonisca fiebrigi*) honey and preservation treatments: dehumidification, pasteurization and refrigeration

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Abstract

A complete study on the microbiological and physico-chemical properties of yateí honey (*Tetragonisca fiebrigi*) was carried out, focusing on the quality standards that are necessary for its commercialization. The results showed that physico-chemical and microbiological parameters of *T. fiebrigi* honey differed from standard values of *Apis mellifera*, but not from other stingless bees honey from South America. Yateí honey showed the presence of fecal contamination (*Escherichia coli*), and a seasonal influence in microbiological parameters, acidity, pH, sucrose and diastase activity. On the other hand, three preservation treatments were carried out and evaluated for 90 days in *T. fiebrigi* honey: refrigeration, pasteurization and dehumidification. Pasteurization and dehumidification of yateí honey eliminated fecal contamination while in refrigerated honey *E. coli* survived in time (8-90 days), unlike the samples kept at room temperature (<3 days). Physico-chemical parameters of yateí honey changed in time after the treatments, specifically, HMF was present after 90 days in honey treated with heat or dehumidified, making it a key parameter of yateí honey quality.

Key-words: yateí honey, microbiological parameters, physico-chemical parameters, preservation treatments.[1]

1. Introduction

The potential of using stingless bee honey by the food, pharmaceutical and cosmetic industries has been growing over the past two decades (Ávila, Beux, Ribani, & Zambiazi, 2018). The most commercially exploited stingless bee honey in Argentina is *Tetragonisca fiebrigi* (Schwarz, 1983), from the Meliponini subfamily, commonly called by locals' yateí. Their habitat covers the tropical and subtropical regions of the American continent from Argentina, up to Mexico (Pucciarelli et al., 2014). Stingless bee farming has increased its popularity among beekeepers because bees do not sting, it is easier to extract the honey, pollen, and propolis compared with the extraction of traditional *A. mellifera* (Abd Jalil, Kasmuri, & Hadi, 2017).

Microbiological and physico-chemical quality standards have been laid out for *A. mellifera* honey by the International Honey Commission (2009). Many have reported that stingless bee honey did not meet these quality standards, stressing the need for an exclusive standard of its own (Biluca et al., 2014; Biluca, Braghini, Gonzaga, Costa, & Fett, 2016; Carvalho et al., 2014; Chuttong, Chanbang, Sringarm, & Burgett, 2016b; Moniruzzaman, Chowdhury, Rahman, Sulaiman, & Gan, 2014; Pucciarelli et al., 2014; Vit, Bogdanov, & Kilchenmann, 1994). However, due to the insufficient knowledge of its composition (Nordin, Sainik, Chowdhury, Saim, & Idrus, 2018) and the variety of stingless bee species, establishing quality standards for Melipona honey is difficult. Therefore, a characterization of yateí honey from Argentina is necessary to contribute in the elaboration of quality standards for commercialization.

One of the challenges in meliponiculture is the high water content in most stingless bee honeys (Souza, 2008; Vit, 2005). If honey is kept at room temperature, it will ferment despite the best hygienic harvesting practices (Nogueira-Neto, 1997). Thus, to increase post-harvest stability and extend the shelf life of this type of honey different preservation methods have been proposed, including dehumidification, pasteurization and refrigeration. Direct refrigeration (4-8 °C) of hermetically sealed containers after harvesting is the simplest and most recommended method. However, this method can cause crystallization of sugars and alter the organoleptic

1 **Abbreviations:** HMF Hydroxymethylfurfural, DN Diastase Number, CB Coliform Bacteria, MY mould and yeast

properties of honey, turning it whitish and more viscous (Venturini, Sarcinelli, & da Silva, 2007).

Pasteurization is another viable option to eliminate pathogenic microorganisms and maintain honey at room temperature, without fermentation, whereas the taste and texture are preserved (Nogueira-Neto, 1997; Venturini et al., 2007). The disadvantage of this process is that some natural enzymes are lost, such as glucose oxidase (Nogueira-Neto, 1997). Finally, the process of dehydration consists of extracting water from honey (Alves, da Silva Sodre, Souza, Lopes de Carvalho, & Fonseca, 2007; Nogueira-Neto, 1997). The advantages are that honey can be stored at room temperature until consumption, without fermentation, and the natural substances and aroma of honey are not lost. An important disadvantage is that honey becomes more viscous than usual, resembling honey of *A. mellifera* (Patricia Vit, 2005; Patricia Vit, Pedro, & Roubik, 2013).

The objectives of this work were to characterize yateí honey from Misiones, Argentina, and observe changes in time in treated honey for preservation (refrigeration, pasteurization and dehumidification). This work was focused in microbiological and physico-chemical analysis, specifically those analyses that are standard for *A. mellifera*. To our knowledge, this is the first work that focuses on three different treatments for a stingless bee (*T. fiebrigi*) honey preservation and focuses on changes in time (until 90 days). By this way, the results will contribute in future decision making of standardization in quality control of yateí honey in Argentina.

2. Materials and Methods

2.1. Microbiological and physico-chemical characterization of yateí honey

A total of 35 yateí honey samples were aseptically extracted (10-55 mL) with sterile syringes during March-April 2016 (25.71% of samples) and in a second instance during November 2016 (74.29% of samples). These samples were provided by different yateí honey bee producers from Misiones Province, Argentina. Honey was transported in cold and protected from light to the laboratory, and kept at 5±1 °C.

Microbiological (coliform bacteria -CB-, *E. coli*, *Salmonella* spp. and mould and yeast-MY) and physico-chemical (pH, acidity, moisture, hydroxymethilfurfural -HMF-, diastase number -DN-, insoluble solids and ash) analyses were done according to methodologies established in the Argentinian Food Code (CAA, 1998). and to standards AOAC Methods (AOAC, 1990) . The

extraction with syringes was significantly burdensome since the honey pots of the hives were very small, and the honey volume extracted was limited. Therefore, the standard work methodologies were modified to use reduced volumes. These modifications were validated using samples of *A. mellifera* honey acquired in the market and no significant differences were observed. Sugars (sucrose, fructose and glucose) were quantified by high-pressure liquid chromatography (HPLC).

2.2. Honey preservation treatments

A second honey sampling (n=3) was conducted during the month of February 2017. In this case, honey was collected by runoff (Nogueira-Neto, 1997), obtaining between 700-800 mL of honey per hive. Honey was fractionated in sterile bottles containing 100 mL each, with hermetic closure, to perform the following preservation treatments:

- Refrigeration: honey was stored in a refrigerator at (6±1) °C.

-Pasteurization: The jars were opened and heated in a thermostatic bath at a water temperature of (74±2) °C. The honey was homogenized continuously throughout the treatment until the coldest point (center of the bottle) reached 72 °C. Afterwards, they were kept 15 seconds at this temperature and then they were transferred to another water bath at room temperature (24±2) °C. Homogenization continued until the honey reached the bath temperature of 24 °C (Nogueira-Neto, 1997). After the process, the bottles were hermetically sealed and kept at room temperature sheltered from light.

- Dehumidification: the bottles with honey were covered with a sterile gauze and placed in a semi-closed container containing silica gel. An approximate ratio of 1:6 (v/v) of silica gel/container was used to maintain the relative humidity between 17-19%, measured with a digital hygrometer. The dehumidification temperature was maintained at (33±1) °C. After 3 days, percentage of relative humidity of the honey reached between 18-19%, similar to that of *A. mellifera* (Vit et al., 1994). Once the process was finished, the bottles were hermetically sealed and kept at room temperature, protected from light.

-Control: Hermetically sealed bottles, without preservation treatment, were stored at room temperature protected from light.

All treatments were carried out in triplicate and honey samples were withdrawn at the beginning (t_0) and after 30 (t_1), 60 (t_2) and 90 (t_3) days of storage to determine MY, pH, acidity

(AOAC 962.19), humidity (AOAC 969.38B), HMF (AOAC 980.23), DN (AOAC 958.19) and sugars (by HPLC).

2.3. Escherichia coli assay

To confirm the ability of the preservation treatments to prevent fecal contamination, samples of yateí honey were challenged with a wild autochthonous *E. coli* strain isolated in this work. Two samples of yateí honey from two different hives were inoculated, henceforth MA and MB. Briefly, 10 mL overnight culture in triptein soy broth (TBS, Britania) was centrifuged (16,000 *x*g, 5 min) and cells washed with sterile physiological solution (FS=0.85% NaCl) and centrifuged again. Harvested cells were re-suspended in the same FS to obtain a final concentration of *ca.* 5×10^7 CFU/mL. This suspension was inoculated at 0.1% (v/v) in 25 mL of honey contained in airtight glass jars. Sub-samples of MA and MB were then subjected to refrigeration, pasteurization or dehumidification treatments as previously described. Untreated honey inoculated with *E. coli* was reserved as control. All samples were kept at room temperature (25-35 °C), except the refrigerated samples at (6±1) °C, and analyzed in triplicate at 0, 2, 8, 15, 27, 33, 40 and 57 days. The counts of *E. coli* were developed on VRBG agar incubated at (44±1) °C, 24-48 h. Results were expressed as colony-forming units per gram of honey (CFU/g).

2.4. Statistical analysis

For the first stage, basic statistical tests were used: Normality tests and determination of arithmetic means and medians. The nonparametric Mann-Whitney test was used to assess the variability between honey harvested in spring (November) and autumn (March-April). These analyses were carried out with the Minitab15 program (Minitab Statistic Program).

For the honey preservation treatments, an analysis of variance of two factors with repeated measures was carried out (2-way ANOVA), being one factor the treatment in honey (Control, Refrigeration, Pasteurization and Dehumidification), and the second factor time (t_0, t_1, t_2 and t_3), considering the latter factor as repeated measures. The condition of normality and homogeneity of variances were checked with Shapiro-Wilk and Levene tests, respectively (Sokal & Rohlf, 1995)· . Some variables were transformed using the logarithm or the square root to meet the assumptions of the ANOVA. If the ANOVA model used was significant, Tukey HSD tests were performed post-hoc to detect significant differences. The preservation treatments data was

148 statistically analyzed with software Rstudio 1.2.1335 (R-3.6.1. (R Core Team, 2013)). Since the
149 content of HMF could not be detected in most of the samples, no statistical analysis was
150 performed. All data is presented as mean±standard deviation.
151
152 **3. Results and discussion**
153 *3.1. Microbiological analysis*
154 In honey, microorganisms may originate from pollen, the digestive tract of bees, dust, air,
155 earth and nectar. These are primary sources of contamination and are very difficult to control. A
156 secondary contamination source may be during manipulation of honey by the producer, which can
157 be controlled with good manufacturing practices (Snowdon & Cliver, 1996). Results obtained in
158 this work revealed that CB were present in a large number of samples (71%) in yateí honey, with a
159 median value of 1.90 Log CFU/g (Table 1). *Escherichia coli* was positive in 3 samples (<10%) that
160 showed CB, but no other pathogens such as *Salmonella* spp. and *Shigella* spp. were detected. MY
161 were also observed in a large number of samples (77%) reaching count values between 3-4 Log
162 CFU/g. The fecal and fungal contamination of yateí honey was higher than the upper limit
163 established by the Codex Alimentarius and CAA for *A. mellifera* honey (absence of CB, MY≤2
164 Log CFU/g). This contamination is related with a primary source because the samples were
165 collected in aseptic conditions. It was very surprising to found *E. coli* in yateí honey since no
166 consulted literature reported its presence (Almeida-Anacleto, 2007; Oliveira, 2011; Pucciarelli et
167 al., 2014; Souza et al., 2009). Only a low percentage (≤10%) of samples evaluated by Pucciarelli *et*
168 *al.* (2014) and Almeida-Anacleto (2007) reported CB in *Tetragonisca* honey, but not *E. coli.*
169 According to Nogueira-Neto (1997), stingless bees can be in contact with feces of warm-blood
170 animals, since bees have variable activities with very dirty habits that could cause fecal
171 contamination of honey and hives in general. Nogueira-Neto (1997) also points out the presence
172 of fecal contamination and *E. coli* in batumen samples of *Melipona quadrifaciata.*
173 In the case of fungal contamination, other studies conducted in Latin America also reported
174 high counts (3-4 Log CFU/g) in honey from Melipona bees, including *Tetragonisca* species, in a
175 high percentage (≥60%) of samples (Almeida-Anacleto, 2007; Alves, Lopes de Carvalho, Souza,
176 da Silva Sodre, & Marchini, 2005; Pucciarelli et al., 2014; Souza et al., 2009). According to
177 Teixeira *et al.* (2003), yateí honey is a conducive environment to the survival of the yeast
178 *Starmerella* (*S.*) *meliponinorum*, described for the first time in melipona nests (Rosa et al., 2003).

The authors reported high counts in honey samples (*ca*. 4 Log CFU/mL) and pollen supplies (*ca.* 6 Log CFU/mL), and suggested that the *S. meliponinarum* would be metabolically active and able to grow at the expense of honey sugars (Teixeira et al., 2003). It should be noted that other osmotolerant yeasts, such as *Candida apícola, S. bombícola* and *Zygosaccharomyces* spp. could be present in stingless bee honey (Rosa et al., 2007).

The fecal and fungal contamination of numerous yateí honey samples here analyzed was higher than the upper limit established by the Brazilian Food Code (ADAB, 2014) and recently by the CAA (2019). The CAA recommends the absence of *E. coli* and less than 4 Log CFU/g of MY in *T. fiebrigi* honey. The unacceptable samples with MY were harvested in spring, presenting a significantly higher median than in autumn, as CB (Table 2).

3.2. Physico-chemical analysis

Physico-chemical parameters (Table 1) showed that yateí honey has an acidic pH (~4) and a median acidity of 30 meq/kg. However, certain honey samples showed values further from the median (70-130 meq/kg) and correspond to samples obtained in autumn. The acidity value corresponds to the balance of organic acids present in honey, which varies according to the floral composition and the bee species (Ávila et al., 2018). A high range of acidity values of yateí honey from Misiones was already observed by Pucciarelli et al. (2014), who also observed high values (130-160 meq/kg). Souza et al. (2008) found in honey of stingless bees from Brazil, Venezuela and Mexico a similar range of values (77-109 meq/kg). This may be due to the harvest time, the maturity of the honey, and/or climatic factors that may favor chemical, enzymatic and microbiological reactions capable of forming acidic compounds in honey (Souza, 2008; Vit et al., 2013). Furthermore, the acidity in yateí honey from Misiones is higher than that found in *A. mellifera* (Lira, Sousa, Lorenzon, Vianna, & Castro, 2014; Pucciarelli et al., 2014; Vit et al., 1994), which is found in other melipona honeys and reflected in its pH and taste (Fuenmayor, Diaz-Moreno, Zuluaga-Dominguez, & Quicaza, 2013), and in its stability against microorganisms (White, 1975). According to Alves et al. (2005) , the pH is affected by the nectar, the cephalic secretion of the bees while they carry the nectar to the hive, the origin of the honey and the concentration of different ions. The mean value found in yateí honey in Misiones (Table 1) was similar to that found in Meliponini species (Souza, 2008), but lower than that observed in *A. mellifera* (Almedia-Muradian, 2013; Lira et al., 2014).

Moisture values found in this work (Table 1) are within the range of values found in honey from Melipona bees from Brazil (Almedia-Muradian, 2013)· and from Venezuela (Vit et al., 1994)· . Although it is higher than that stipulated by CAA for *A. mellifera* honey (max. 20%), which was previously observed in honey from Melipona (see citations in Vit et al., 1994)· . In Brazil, a threshold of <35% humidity has been proposed for stingless bee honey (Villas Boas & Malaspina, 2005)· . Honey's moisture content has been reported to be dependent on the environmental factors during harvesting and storage. Furthermore, Crane (1992)· has emphasized that honey from stingless bees are generally more acidic and contain more water than *A. mellifera*, and that, for reasons not yet clarified, these are more resistant to decomposition by fermentation. It has been suggested that the resin present in the wax used in the hives could be present in the honey and may be acting as a biocidal agent, preventing fermentation (Lira et al., 2014)· .

The content of sugar in yateí honey (Table 1) presented a maximum higher than the value proposed for stingless bee honey in Venezuela -50 g/100g- (Vit et al., 1994)· , but within the range proposed in Brazil – 58.0-75.7 g/100g- (Souza, 2008)· . The mean content of reducing sugars is similar to data from Almeida-Anacleto (2007)· , (48.66-57.94 g/100g) and Rodrigues et al. (1998)· (58.19 g/100g for *T. angustula*), and lower than *A. mellifera* honey (Almedia-Muradian, 2013; P Vit et al., 1994)· . On the other hand, the content of sucrose in yateí honey from Misiones in general was low, and non-detectable in some samples with the technique used (Table 1). In previous studies this sugar was not detected in yateí honey form Misiones using the same detection methodology (Pucciarelli et al., 2014)· . The sugar present in honey comes from the nectar, which can contain sucrose, glucose and fructose in different proportions, depending on the floral species. During honey maturation, the invertase unfolds the sucrose and its presence is a sign of immaturity or adulteration of honey (Moreira & De Maria, 2001)· . The results obtained in this work showed that the honey extracted in spring had a lower sucrose content (Table 2), suggesting it is more mature than those harvested in autumn.

The diastase enzyme is a heat sensitive enzyme, that is why, in general, it is recommended as a honey quality test. Misiones yateí honey was highly variable in the DN, with a range between 3.55 and 45.95 DN, and a high standard deviation (Table 1). A high variability of the DN has been observed in honey of stingless bees from Guatemala (Dardon, Maldonado-Aguilera, & Enriquez, 2013)· , although values so high found in Misiones' yateí honey (23-46 DN) were not observed. These high values correspond to the samples taken during autumn, showing a strong seasonal

influence (Table 2). On the other hand, the high variability found in DN in yateí honey here evaluated, and in other honey samples of Meliponas and *A. mellifera* (Souza, 2008)· has called into question the use of this index as an indicator of honey quality due to the great variation observed, even when freshly extracted (Chuttong, Chanbang, Sringarm, & Burgett, 2016a)·. The exclusion of this analysis has been suggested as being a redundant, misleading and variable test (Souza, 2008)· and it has been suggested to replace the determination of the DN by invertase activity, also present in honey (Bonvehí, Torrentó, & Raich, 2000)·. Another parameter of quality of honey is the amount of HMF (Nordin et al., 2018; Vilhena & Almedia-Muradian, 1999)·. It is a six-carbon heterocyclic organic compound containing aldehyde and alcohol functional groups, formed by the Maillard reaction from the decomposition of fructose, which indicates aging and heating of the honey (Gonnet, 1963; Gonzalez, 2002; White, 1975)·. Factors that affect HMF content are storage condition, pH, and adulteration of honey with simple sugars from an external source (Pasias, Kiriakou, & Proestos, 2017)·. In honey samples analyzed in this work, HMF concentration was null in the majority (57%), and high values (21, 49, 54 and 96 mg/kg) were found in 4 samples, which could not be related with any factor evaluated in this work. So far, the highest value of HMF detected in stingless bee honey was 78.5 mg/kg from Mexico (Dardon et al., 2013)·. In general, low values of HMF (less than 2 mg/kg) are found in Melipona and Tetragona honey (Almedia-Muradian, 2013; Lira et al., 2014; Vit et al., 1994)·, and even values below the quantitation limit (Biluca et al., 2016)·. The low amounts of HMF can be caused by several factors, such as the origin, the honey-producing species, climate, harvest time, pH, floral origin, and good management practices (Ávila et al., 2018; Carvalho et al., 2014; Chuttong et al., 2016a; Lira et al., 2014)·.

Ash is constituted mainly by salts of calcium, sodium, potassium, magnesium, iron, chlorine, phosphorus, sulfur and iodine (Almedia-Muradian, 2013)·. The content of ash and minerals depend both on the botanical and geographical origin, as well as on the bee species (Carvalho et al., 2014; Lira et al., 2014; Souza, 2008; Vit, 2005; Vit et al., 2013)·. The majority of ash content values in yateí honeys analyzed (Table 1) were within the standard Codex Alimentarius values proposed by Vit et al. (2004)·, with a maximum of 0.5 g/100g for honeys of *A. mellifera*, *Melipona*, *Scaptorigona* and *Tetragonisca*. 17% of the samples presented values higher than the standard (Fuenmayor et al., 2013)·. On the other hand, quantification of insoluble solids is a quality parameter used to verify the purity of honey and the efficiency of the extraction process

(Leite & Santos, 2001). The concentration of insoluble solids determined in yateí honey from Misiones showed a high variability (Table 1), but in general it was lower than the standardized value for *A. mellifera* honey (less than 0.1%).

3.3. Seasonal analysis

Microbiological and physico-chemical parameters that presented seasonal differences (Table 2) were briefly approached, a more global analysis is developed here.

The physico-chemical parameters analyzed are influenced by several factors, principally by nectar and flora (Fonseca et al., 2006; Lage et al., 2012; Vit et al., 1994). In stingless bees, pollen-foraging activity reflects the influence of climatic parameters, and ambient temperature is one of the most important abiotic factors regulating the timing of food collection (Aleixo et al., 2017). Roubik (1982) showed that the amount of pollen and honey stored in colonies of Melipona stingless bees varied greatly across time and concluded that flowering seasonality influenced the foraging activity of bees, characteristic of tropical environments where flowering, and consequently resource availability, is strongly associated with seasonal variations in rainfall (van Schaick, Terborgh, & Wright, 1993). In Misiones, the rainy season begins in spring and ends in April-May (Fontana, 2014), which are the months that honey samples were harvested in this work and may have influenced in the bees' activities, honey production and composition. Vossler et al. (2014) found that foraging activity of *T. fiebrigi* in the Chaco region, Argentina, was governed by random factors such as local differences of flower availability, which depended on the season and not on type of forest. Furthermore, the microbiological parameters presenting seasonality could be due to the appropriate conditions given in the honey that presented seasonality as well (pH, acidity and sucrose). This is the first work, to our knowledge, that presents a seasonal difference in physico-chemical and microbiological parameters of honey of Melipona species, however, more studies would be necessary to standardize harvesting times of yatei honey in Misiones and other regions.

3.4. *Honey preservation treatments*

Results of MY in preserved yateí honey showed significant differences between treatments (F=7.944, p=0.009). The highest value was observed in the refrigerated sample while similar values were observed in the pasteurized and dehumidified samples (Table 3). None of the

303 treatments presented differences respect to control. The impossibility of pasteurization and
304 dehumidification processes to eliminate fungi and yeasts was related to the presence of sporulated
305 forms, resistant to adverse conditions such as heat and dehydration. On the other hand, refrigeration
306 treatments had a protective effect on fungi and yeasts as observed by Martinez (2013)·.
307 Moisture values were lower in dehumidified samples ($F_{treatments}$=63.12, p=6.54×10^{-6}. Table
308 3) and were maintained over time (F=1.31, p=0.29), which indicates the effectiveness of the
309 dehumidification method in terms of maintaining the humidity percentage. As there is less water in
310 dehumidified honeys, we observed variations in the concentration of reducing sugars and in pH,
311 mainly, as described below.
312 pH showed a high interaction between the analyzed factors ($F_{treatment:time}$=21.11, p=2.5×10^{-9}). In all treatments there was a decrease at the end of the experiment (t_3), higher in dehumidified
314 honey (Fig.1-A). On the other hand, refrigerated and pasteurized honeys showed the same
315 behavior: an increase in t_1 and t_2, and then a decrease in t_3. The acidity was similar in almost all
316 samples (57-70 meq/kg) except in control where a significant increase was observed over time
317 ($F_{treatment:time}$=2.32, p=0.048. Fig.1-B). Hence, pH in control, refrigeration and pasteurization honeys
318 had a similar tendency over time, but changes in free acidity were only observed in untreated
319 honeys. This is an indication that the treatments had an effect on the processes that cause free
320 acidity change in time. The acid production of honey is given by the enzymatic action of glucose
321 oxidase and by the fermentative processes developed by the microorganisms present in the matrix
322 (Ávila et al., 2018; Martinez, 2013)·. Sanz and Gradillas (1995)· and White (1975)· described
323 that fermentation of honey depends on the initial contamination, storage time, temperature and
324 moisture content, this being the main factor. Perez-Perez et al. (2007)· observed fermentation in
325 honey of *T. angustula* kept at 30 ºC, but did not manifest in those that were kept in refrigeration or
326 subjected to pasteurization processes. There is a possibility that the fermentation process is
327 affecting the acidity in untreated yateí honey from Misiones over time. Although this needs further
328 studies to be corroborated, since Pucciarelli *et al.* (2014)· observed that acidity in yateí honey
329 from Misiones was correlated with chemical and enzymatic reactions, and not with fermentation.
330 The 2-way ANOVA analysis of DN showed a significant interaction between the factors
331 ($F_{treatment:time}$=3.78, p=0.0044). Untreated honeys ($F_{control}$=11.38, p<<0.05) showed a decrease in the
332 enzyme activity at t_2, although it did not differ from t_0 (Fig.1-C). On the other hand, in pasteurized
333 honeys ($F_{pasteurization}$=19.42, p<<0.05), the decrease was observed at t_1 and then increased to initial

values. At the end of the experiment (F_{t3}=9.51, p=0.00514) the untreated honey samples had the highest value of DN (Fig.1-C). The high variability found in DN treated honeys corroborates the disagreements in using DN as an index of quality in honey. Moreover, Tosi *et al.* (2008)· analyzed the diastase enzyme against several heat treatments, and observed that it was not an appropriate index for honeys treated at different temperatures.

The concentration of HMF was detected over time only in the pasteurized honey at t_3 (4.9±2.3 mg/100g) and dehumidified honey at t_2 (4.4±1.9 mg/100g) and t_3 (3.7±0.7 mg/100g). In untreated and refrigerated honeys HMF was not detected. This gives an indication that it can be a reliable parameter for honey stored with the previous treatments. In addition, it indicates that refrigeration is a treatment that prevents the production of this compound until 180 days, time in which the experiment was carried out. Karabournioti and Zervalaki (2001)· observed some degree of resistance to the thermal effect according to the type of monofloral honey involved, and concluded that the amount of HMF is the best evaluation on the harmful effect of heat treatment on honey because it is not present in the fresh product. Chuttong *et al.* (2016a)· also suggested HMF as an indicator of storage quality since they found it to be the key parameter most affected by storage time and temperature.

Reducing sugars showed a significant variation in the concentration of preserved honeys ($F_{treatment}$=4.708, p=0.035), specifically in dehumidified honey, which showed a higher concentration of sugar (Fig.2-A). In time there was a significant variation as well (F_{time}=6.130, p=0.003), observing a decrease in concentration at t_1, which was then recovered (Fig.2-B). Sucrose concentration was constant in time and did not show significant differences between each treatment ($F_{treatment}$=0.021, F_{time}=2.42, $F_{treatment:time}$=1.804; p>0.05), with an average value of (1.2±0.2) g/100g. The changes of reducing sugars observed in time could be due to several factors. First, the enzymes found in honey, mainly invertase, are responsible of hydrolyzing sucrose giving glucose and fructose as products (White, 1975)· , although sucrose did not show significant changes. Second, HMF is formed mainly from fructose, and the appearance of this product in time in treated honeys may be related to the changes in the concentration of reducing sugars. Finally, fermentation processes have been related to a decrease in total sugar concentration in *T. angustula* honey kept at 30ºC for 30 days (Pérez-Pérez et al., 2007)· . A combination of these processes may be affecting sugar concentration in preserved yateí honey from Misiones, although these results should be thoroughly studied in the future.

365

366 *3.5. E. coli assay*

367 Results of treated yateí honey inoculated with *E. coli* showed that pasteurization and
368 dehumidification were effective to abolish honey fecal contamination since immediately after the
369 treatments *E. coli* was not detected (Fig.3). After two days of storage at room temperature (25-35
370 °C), no development of *E. coli* was observed in control samples while refrigerated samples showed
371 positive counts. Cooler temperatures allowed *E. coli* survival until day 8 in MA and almost two
372 months after the inoculation in MB, with a gradual decrease in the counts (Fig.3). These results
373 showed that yateí honeys do not have the proper environment for survival and/or multiplication of
374 vegetative cells such as *E. coli*. This is due to the adverse conditions: high osmolarity and acidity,
375 low pH and the presence of antimicrobial compounds such as hydrogen peroxide and phenolic
376 compounds (Vit et al., 2009)· . However, it seems that the antimicrobial effect of yateí honey
377 decreases with lower temperatures. This result could be associated with a lower activity of glucose
378 oxidase responsible of the generation of hydrogen peroxide, as the main antimicrobial in honey
379 (Poli et al., 2018)· which needs to be studied in the future.

380

381 **Conclusion**

382 The characterization of yateí honey from Misiones carried out in this work will be a useful
383 tool for future quality standardization. In general, the micorbiological and physico-chemical limits
384 of yateí honey are out of the standards for *Apis* honey production, and must be revalued for yateí
385 honey. In addition, values of the parameters analyzed do not differ from those observed in other
386 stingless bees studied in America, but the differences found in honeys harvested in different
387 seasons must be studied in the future in detail. On the other hand, according to the microbiological
388 values obtained, the preservation treatments did not reduce significantly MY, but dehumidification
389 and pasteurization treatments did prevent *E. coli* growth. However, the presence of HMF in time in
390 preserved honeys must be taken into account in heat treated samples as a parameter of quality since
391 it is the parameter that is most affected by temperature and storage time.

392

393

394 **Acknowledgements**

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Conflict of interest. The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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Figure captions

Figure 1. pH (A), free acidity (B) and Diastase Number (C) in yateí honey samples submitted to different treatments. Same symbols indicate no significant difference (p>0.05).

590 **Figure 2.** Reducing sugars concentration of yateí honey samples with different treatments
591 (A) and in time (B). Different letters indicate significant differences (p<0.05).
592 **Figure 3.** Survival of *Escherichia coli* in refrigerated yateí honey samples (MA y MB) over
593 time.

Table 1. Microbiological and Physico-chemical parameters obtained in honey from *Tetragonisca fiebrigi* of Misiones (n=35).

Parameters	Median	Q1[1]	Q3[2]	Minimum	Maximum	Mean	Standard deviation	Variance	A-square	*p*-value[3]
Moulds and yeasts (CFU/g)	8.19E+03	1.22E+03	2.52E+04	≤ 1.00E+02[4]	7.26E+04	1.60E+04	1.91E+04	3.66E+08	2.520	0.005
Coliform bacteria (CFU/g)	8.00E+01	1.00E+01	2.60E+02	≤ 1.00E+01[5]	1.26E+03	1.86E+02	2.72E+02	7.38E+04	4.070	0.005
pH	3.970	3.710	4.470	3.390	4.730	4.058	0.421	0.178	0.760	0.043
Acidity (meq/kg)	30.000	20.000	40.000	20.000	130.000	41.143	29.583	875.126	4.510	0.005
Moisture (%)	24.600	23.800	25.000	21.400	25.000	24.309	0.827	0.684	1.960	0.005
Sucrose (g/100g)	0.506	0.142	0.989	0.000[6]	2.134	0.683	0.665	0.442	1.330	0.005
Reducing sugars (g/100g)	54.302	49.682	58.526	42.892	73.407	54.996	5.995	35.942	0.380	0.377
Ash (g/100g)	0.375	0.268	0.475	0.150	1.240	0.410	0.208	0.043	1.530	0.005
Insoluble solids (g/100g)	0.020	0.014	0.033	0.002	0.081	0.027	0.021	0.000	1.440	0.005
Hydroxymethylfurfural (mg/kg)	0.000	0.000	3.357	0.000	96.805	7.477	19.911	396.430	8.350	0.005
Diastase number (DN)	9.389	6.014	15.080	3.547	45.948	12.865	10.105	102.105	2.710	0.005

[1] First quartile (Q1); [2] Third quartile (Q3); [3] *p*-value: normal distribution ≥ 0.05; [4] Detection limit of the techinque (≤ 100 UFC/g); [5] Detection limit of the techinque (≤ 10 UFC/g); [6] The detection limit of the technique is represented with zero.

Table 2. Mann-Whitney analysis of microbiological and physical-chemical parameters in yateí honey harvested in spring and autumn.

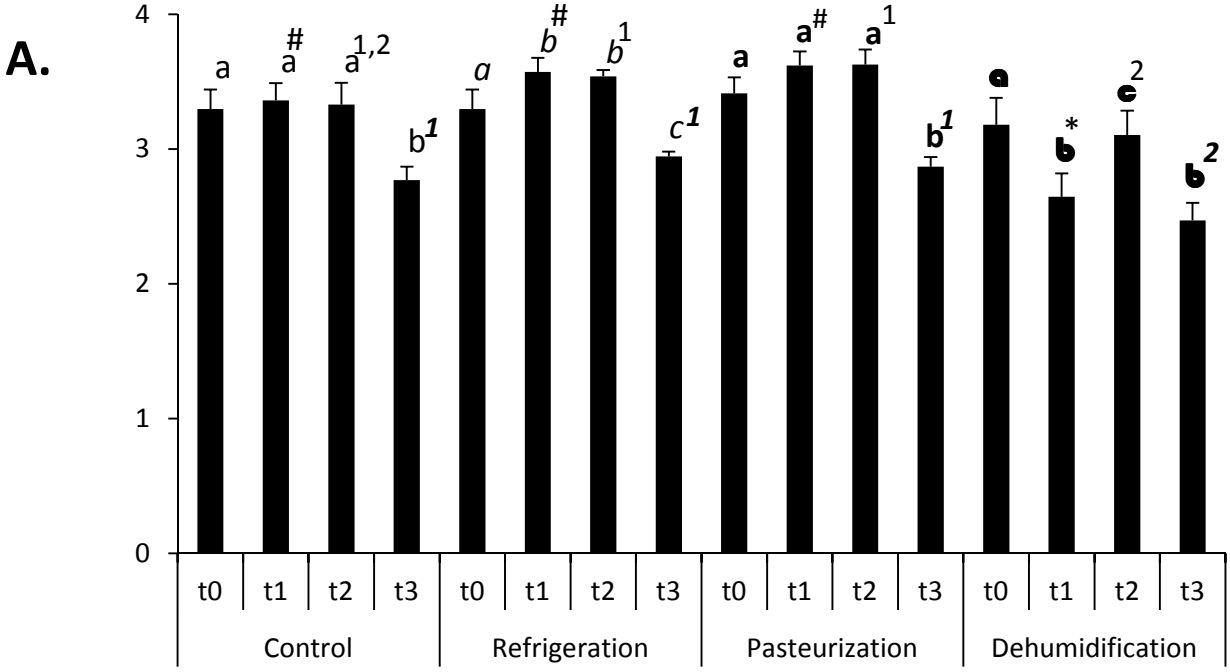
Parameter	Median		*p*-value*
	Spring (n=25)	Autumn (n=9)	
Moulds and yeasts (CFU/g)	4.16	2.78	**0.0280**
Coliform bacteria (CFU/g)	2.04	0.00	**0.0004**
pH	4.16	3.66	**0.0127**
Acidity (mEq/Kg)	30.00	70.00	**0.0001**
Moisture (%)	24.60	25.00	0.4979
Sucrose (g/100g)	0.24	0.99	**0.0160**
Reducing sugars (g/100g)	54.35	53.84	0.6963
Ash (g/100g)	0.36	0.38	0.5259
Insoluble solids (g/100g)	-	-	-
Hydroxymethylfurfural (mg/Kg)	0.00	2.42	0.3910
Diastase number (DN)	8.30	24.62	**0.0003**

*In bold are the significant parameter values (p<0.05).

Table 3. Mould and yeasts and moisture content in yateí honey submitted to different treatments.

Parameters	Treatments*			
	Control	**Refrigeration**	**Pasteurization**	**Dehumidification**
Mould and yeast (CFU/g)	$208 \pm 124^{a,b}$	245 ± 94^{a}	108 ± 76^{b}	125 ± 114^{b}
Moisture (g/100g)	24.3 ± 0.5^{a}	24.1 ± 0.7^{a}	24.2 ± 0.5^{a}	19.5 ± 0.3^{b}

*Different letters indicate significant differences (p<0.05).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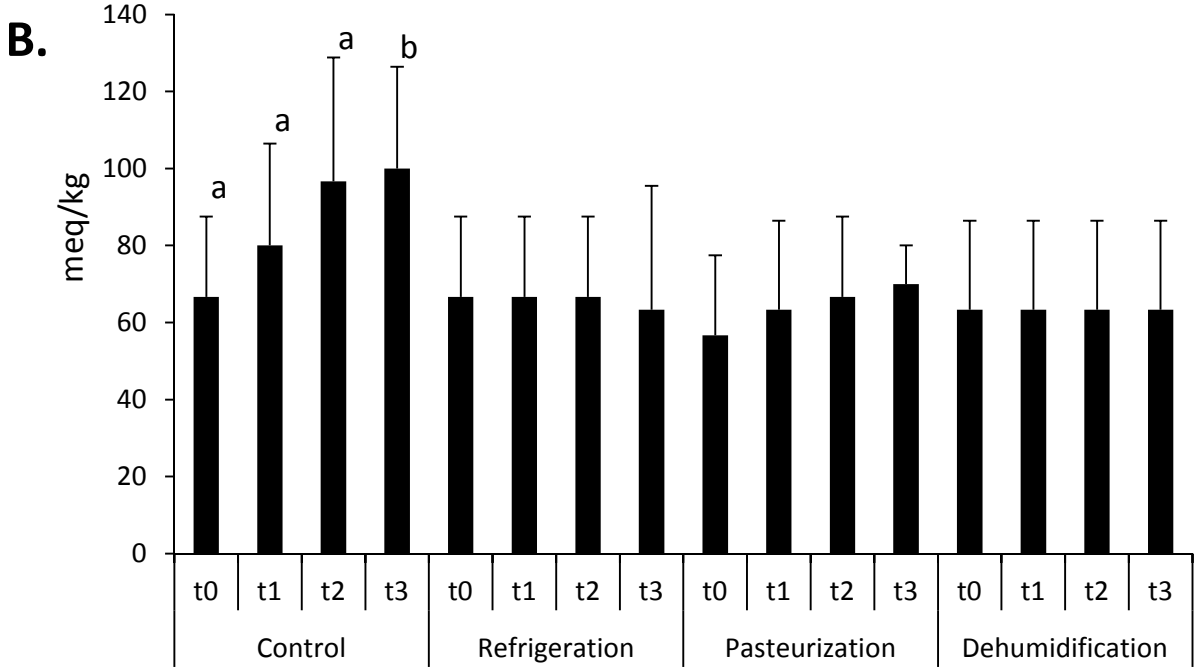
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Control
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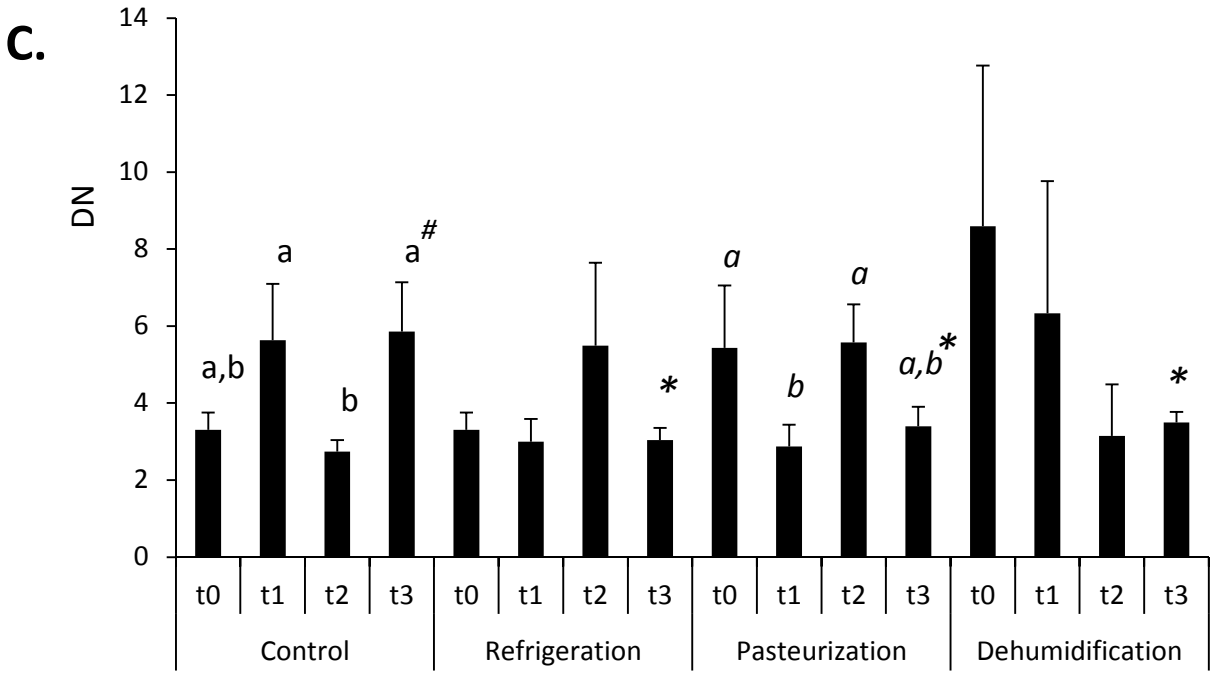
Pasteurization
Dehumidification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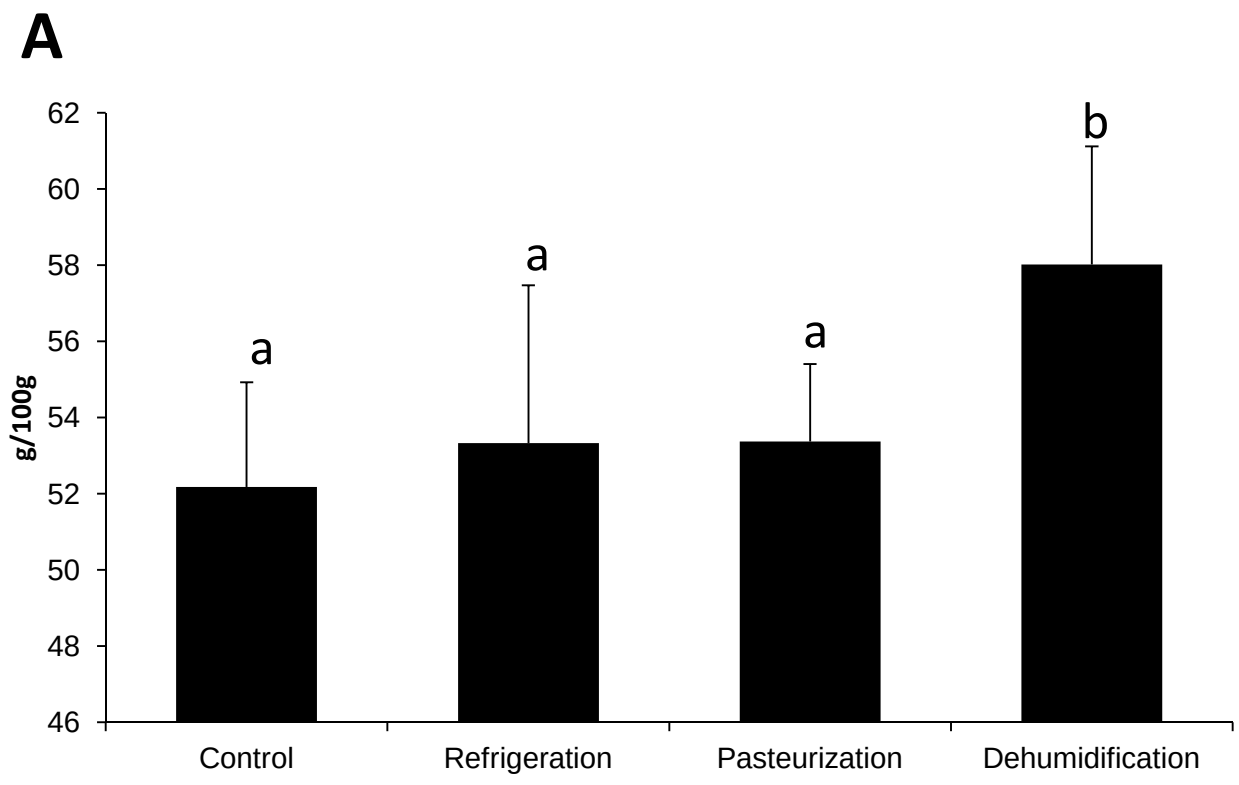
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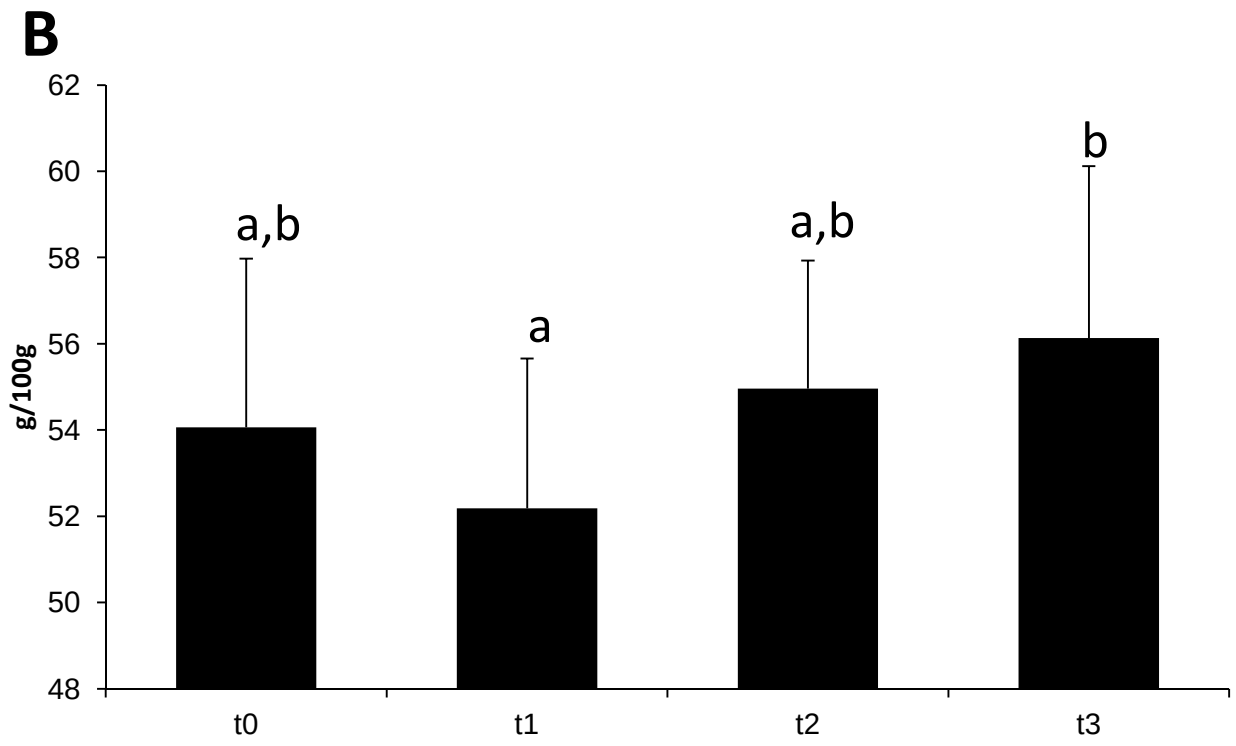
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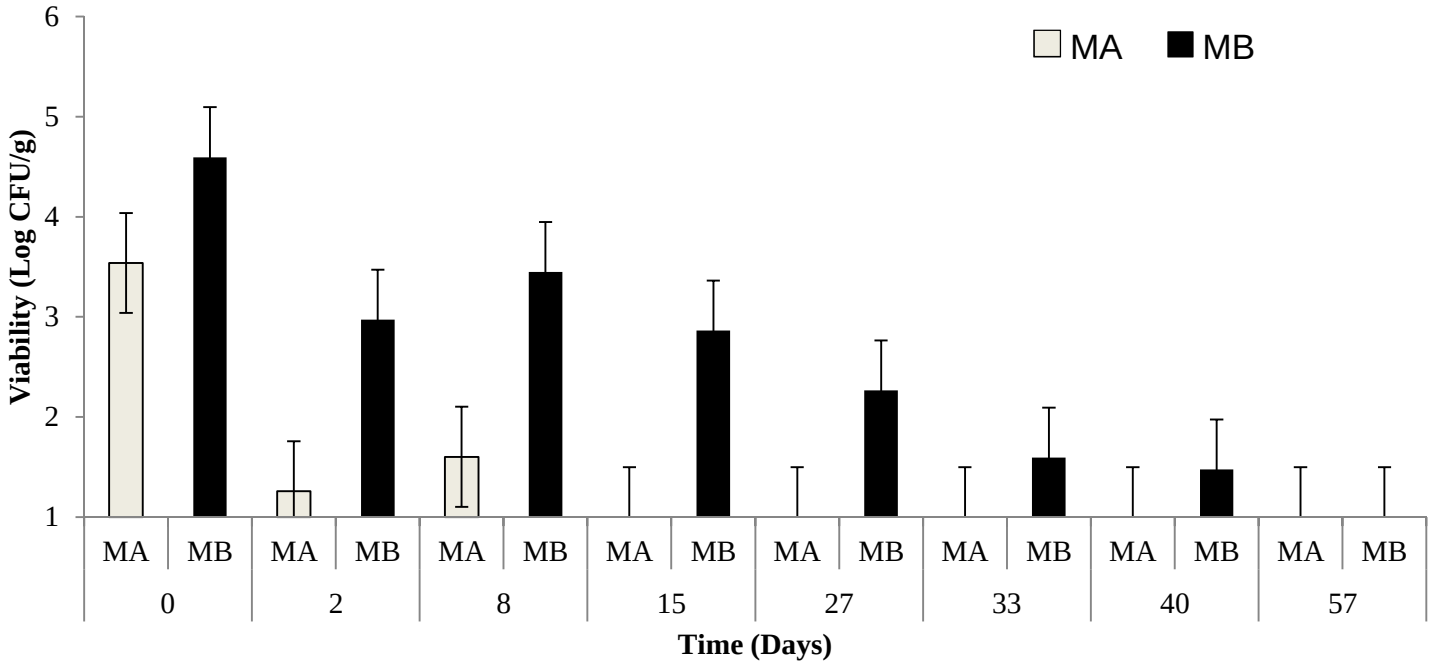
t2
t3


MA
MB
Viability (Log CFU/g)
6
5
4
3
2
1
MA MB MA MB MA MB MA MB MA MB MA MB MA MB MA MB
0 2 8 15 27 33 40 57
Time (Days)

Highlights

- Honey standard criteria for *Tetragonisca fiebrigi* differed from the standard values of *Apis mellifera* honey
- Microbiological parameters showed differences in the season of harvesting.
- Diastase activity, pH, acidtity and sucrose depended on the season of harvesting
- Dehumidification and pasteurization treatments prevented *E. coli* growth
- pH, acidity, reducing sugars and HMF changed with the preservation treatments

Authors contribution. ABP and AMD conceived this research and designed experiments. NS participated in the design of the experiment. ABP, BV and AMD performed experiments. NS, ABP, BV and AMD performed analysis. NS and AMD wrote the paper. ABP participated in the revision of the paper. All authors read and approved the final manuscript.