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Production and characterization of mead from the honey of *Melipona scutellaris* stingless bees

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Mead is a traditional alcoholic beverage obtained by fermenting must and can offer a solution to honey over-production and a way of valorizing honey of lower quality. The purpose of this study was to produce and characterize mead with different levels of sugars and alcohol obtained from honey from *Melipona scutellaris*. The honey used for mead preparation was analysed in order to ensure that it met the required quality standards. It was found that the alcoholic content and volatile acidity were outwith the limits established by Brazilian law. Mead legislation is based on the product obtained from *Apis mellifera* ('honey bee') honey and these results indicate the need to re-evaluate the standards established for this product in order to incorporate mead produced from honey from stingless bees of the genus *Melipona*.

Keywords: alcoholic beverages; fermentation; yeast; melipona; mead

Introduction

Throughout history, societies have learned how to make fermented beverages using sugar sources from in their local habitats. In pre-industrial times, honey was the main source of sugar in the population's diet *(1)*. The honey produced by stingless bees, particularly *Melipona scutellaris*, is highly appreciated because of its characteristic flavour and aroma, more fluid texture and slow crystallization *(2)*, such that it is one of three most important bee species in Brazil. *M. scutellaris*, popularly known as uruçu bee, is widespread in both urban and rural environments of the northeast of Brazil (from Bahia to Pernambuco) where it plays a pivotal role as a pollinator as well as in production of honey *(3)*.

Mead, is an alcoholic beverage containing 4–14% (*v*/v) ethanol *(4)*, is one of the oldest alcoholic beverages consumed by man. However, in recent years its production has decreased, partly owing to a lack of scientific information since its production is mainly empirical *(5)*. Currently, this drink is consumed in England, Poland, Germany, Slovenia and especially in Ethiopia and South Africa *(6)*.

The production of traditional mead is based on yeast-borne fermentation of honey diluted in water ('must') *(6)*. Production is influenced by several factors, such as the composition and type of honey *(7)*, yeast lineage *(8)* and availability of essential nutrients *(9)*. A primary problem in mead production is sluggish fermentations. These difficulties reflect the low levels of nitrogenous substances and minerals in the honey, which are required for the growth of yeast, and the acidic pH of the fermentative broth, which affects the rate of the process *(8)*. It should be noted that the amount of nitrogen in the honey must also affects the sensory quality of the final product, with the formation of unpleasant aromatic compounds *(10)*. The lack of uniformity of the final product and refermentation by yeasts and bacteria are still frequent problems in the production of mead *(11)*. Therefore, strict control of fermentation is important.

As the shelf-life of honey from *M. scutellaris* is reduced owing to the high moisture content, the development of new products, including mead, presents an opportunity to take advantage of this honey, making meliponiculture a more profitable activity. Accordingly, the main objective of this work was the production and characterization of two types of mead obtained from the honey of *M. scutellaris*.

Material and methods

Physicochemical characterization of honey sample

In this study for the production of mead, we used a sample composed of 23 colonies of *M. scutellaris*, collected in a meliponary at Sauípe, Entre Rios, Bahia, Brazil. To characterize the honey, reducing sugars and apparent sucrose were determined according to the modified Lane and Eynon method *(12)*, together with diastase activity, water activity, hydroxymethylfurfural, pH and acidity *(12)*. Electrical conductivity and ash content were determined as previously described *(13)* and colour according to Vidal and Fregosi *(14)*.

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Mead production

Preparation of honey must The honey must was prepared by diluting the honey in commercial bottled water. The must mixture was supplemented with commercial nutrients (Enovit; 100 g hL^{-1}) and subsequent addition of sulphurous anhydride 6% (*v*/v) (8 g hL^{-1}) and tartaric acid (Sigma-Aldrich) until a pH of 3.3 was obtained. The honey must was pasteurized at 65°C for 10 min and immediately cooled. To characterize the honey must before fermentation, the physicochemical parameters were determined: total soluble solids (°Brix), pH, assimilable nitrogen *(15)*, total acidity *(16)* and reducing sugars *(17)*.

Yeast The commercial yeast *Saccharomyces cerevisiae* 'Fermol® Reims Champagne (Pascal Biotech®)', recommended for the production of white wines, was used.

Fermentation The fermentations were performed at room temperature (25°C) for 13 days in glass containers with a capacity of 10 L containing 6 L of honey must. The bottles were shaken three times a day to avoid yeast sedimentation.

Sweet mead So as to avoid the complete consumption of sugars and obtain a sweet mead, the fermentation was prematurely interrupted with the addition of brandy. The amount of spirit added was determined according to the procedure described in the literature *(18)*. All assays were performed in triplicate.

Dry mead To produce dry mead, the fermentation proceeded until the consumption of all the fermentable sugars. Fermentation was monitored daily by quantifying the following parameters: cell biomass, cell viability and reducing sugars.

Cell biomass

Cell biomass was evaluated by measurement of optical density at 640 nm. When necessary, the samples were diluted with honey must, which was used as a blank.

Cell viability

Cell viability was determined by quantifying colony forming units in solid media. Plates were incubated at 25°C for 3–5 days.

Reducing sugars

Reducing sugars were quantified by the 3,5-dinitrosalicylic acid method according to the published procedure *(17)*, using glucose as standard. The results are expressed as g L^{-1}.

General oenological parameters

The oenological parameters pH, titratable acidity, tartaric acid, volatile acidity, total sulphur dioxide and alcohol content were determined as previously described *(16)*. The yeast assimilable nitrogen was determined according to Aerny *(15)*. The ethanol yield of the fermentation was further determined according to the equation:

$$\underline{Y}\,(\text{ethanol/sugars})\,(\%) = \left[\text{ethanol produced}\left(\text{g L}^{-1}\right)/\text{sugars}\left(\text{g L}^{-1}\right)\right]\times 100$$

Quantification of glucose, fructose, ethanol, glycerol and acetic acid by HPLC

Glucose, fructose, ethanol, glycerol and acetic acid were analysed by high-performance liquid chromatography *(8)*. The Varian HPLC system was used, equipped with a Rheodyne 20 μL injector and a Supelco Gel C-610H column (300 × 7.8 mm) at 35°C, coupled to a refractive index detector, RI-4, from Varian. The mobile phase used consisted of 0.1 % (*v*/v) phosphoric acid at a flow rate of 0.5 mL/min. The data was integrated by Varian's Star Chromatography Workstation. Glucose, fructose, ethanol, glycerol and acetic acid were quantified based on peak area and comparison of the calibration curves with the corresponding standards.

Total phenolic compounds

The total phenol content in the mead was estimated according to the method of Folin–Ciocalteau *(19)*. Mead (0.5 mL) was mixed with 0.5 mL Folin–Ciocalteau reagent (10% *w*/*v*) and 0.5 mL Na_2CO_3 (75 g L^{-1}), and kept in the dark at room temperature for 1 h. The absorbance at 760 nm was then read on a spectrophotometer (Helios). To obtain the calibration curve ($y = 0.434x + 0.0025$; $r^2 = 0.997$), standard solutions of gallic acid (0.01–0.08 g L^{-1}) were used. The total phenol content was expressed in mg gallic acid/mL mead equivalents (GAE mL^{-1}).

Total flavonoids

The total flavonoid content was determined according to the previously published *(20)*. A 2.5 mL aliquot of mead was added to 2.5 mL of 2 % $AlCl_3$ and allowed to stand for 1 h. The solution was mixed and the pink colour was measured at 420 nm. A standard curve was prepared with quercetin. ($y = 28.24x + 0.111$, $R^2 = 0.999$). The results were expressed as mg of quercetin/mL mead equivalents (QE mL^{-1}).

Antioxidant activity

Two methods were used to evaluate the antioxidant activity: evaluation of the free radical blocking effect of DPPH (2,2-diphenyl-1-picrylhydrazyl) based on the published methodology *(21)* and evaluation of the reducing power *(22)*.

Statistical analysis

All tests were performed in triplicate and the results were expressed as means ± standard deviation. Variance analysis (ANOVA) was applied to test significant differences between mead samples. Tukey's test was used to identify differences between mean values obtained in dry and sweet mead ($p \leq 0.05$).

Results and discussion

The honey used in the production of mead was characterized by physicochemical analysis. The results for most parameters, except for diastase activity, were in accordance with the limits established by Ordinance no. 207 *(23)*. The mean values obtained by the physicochemical parameters analysed for *M. scutellaris* honey were 28.0 ± 0 g 100 g^{-1} (humidity), 0.8 ± 0 aw (water activity), 4.4 ± 0 (pH), 31.5 ± 0 meq kg^{-1} (total acidity), 0.5 ± 0 mg kg^{-1} (hydroxymethylfurfural), 4.5 ± 0.1 gothe scale (diastase activity), 65.2 ± 0.1 g 100 g^{-1} (reducing sugars), 1.9 ± 0 g 100 g^{-1} (apparent sucrose), 0.5 ± 0% (ash), 289.0 ± 0 μS cm^{-1} (electrical conductivity) and light amber (colour). These results corroborate those obtained previously *(24–26)*. Quantitative differences in diastase enzyme content in honey of different flower origins may be related to the effects of substances from the flora (nectar)

and/or natural honey. The determination of this enzyme is an important parameter to evaluate the quality of the honey, owing to its sensitivity to heating. As an index of freshness, it decreases with the storage time and with the heating of the honey *(27)*.

Cell viability

Figure 1 presents the colony forming units as a function of time. Yeast entered the stationary phase at 72 h after inoculation. The final biomass was reached at 192 h. This suggests that in the production of mead from honey of *Apis mellifera* the stationary phase occurred at 72 h and the fermentation ended at around 200 h *(8)*.

Consumption of reducing sugars

The consumption of reducing sugars as a function of time are summarized in Fig. 1. A progressive increase in the consumption of reducing sugars was observed up to 192 h, and then it was slow. The fermentation ended at 312 h, which is longer than that observed by Pereira et al. *(28)* in obtaining mead from honey originating from *A. mellifera* with free and immobilized cells, in both cases ending at 120 h after inoculation. Also *(10)*, the fermentation of pollen-enriched mead lasted 192 h. However, the duration of the fermentations conducted by Fernandes et al. *(29)* using residual honey was higher (75 days) than the values obtained in this work.

The rate of fermentation was enhanced with commercial nutrients (Enovit) containing ammonium sulphate, diphasic ammonium phosphate and vitamin B1. Fermentations that occur in the absence of yeast growth factors may be prolonged or 'stuck'. With the addition of nutrients, honey ferments faster, without undesirable flavours resulting from slow fermentation processes *(30)*.

It should be noted that the residual sugars obtained in this study (6.0 g L^{-1}) were lower than those determined by Pereira et al. *(28)* (30 g L^{-1}) and higher (<0.6 g L^{-1}) than those determined by Roldán et al. *(10)*. Also *(7)*, these residual substances are non-fermentable sugars such as trehalose, isomaltose and melezitose.

Physicochemical analyses of honey must and mead

Table 1 presents the results obtained in the physicochemical analyses performed on the honey must and the two types of mead, compared with the Brazilian standards regarding mead *(4)* and liqueur wine *(31)*. It is observed that the physicochemical characteristics of the honey must produced from *Melipona* honey were identical to those determined by Pereira et al. *(8)* in the must prepared from *Apis* honey, with the exception of nitrogen and the total soluble solids (°Brix). The first parameter was higher in *Melipona* honey, while the second was lower.

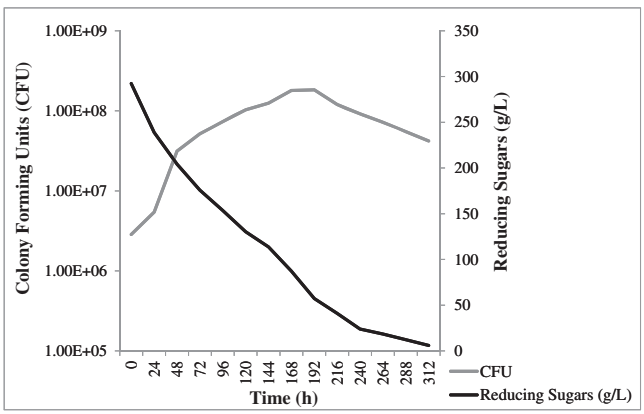

Figure 1. Variation of colony forming units of *Saccharomyces cerevisiae* and reducing sugars as a function of fermentation time for dry mead.

The pH is one of the most important characteristics of mead because it influences colour and has a pronounced impact on taste. Wines with high pH are more susceptible to oxidative and biological change, since the content of active sulphur dioxide is proportionally lower *(32)*. The pH values decreased during the conversion of the honey must into mead. The reduction of pH in the dry mead (3.1 ± 0) was less marked than in sweet mead (3.3 ± 0), since in the production of sweet mead the fermentation was stopped by means of the addition of brandy. There were differences between the final pH values obtained in the two types of mead. According to Sroka and Tuszynski *(11)*, during the first days of fermentation of honey must the main acids that are produced are acetic and succinic. These acids are responsible for the reduction of the pH value, which remains practically unchanged until the end of the fermentation. Our results are corroborated by the observations of Mendes-Ferreira et al. *(9)*, who found an average value of 3.3 ± 0.2 in mead supplemented with 5 g L^{-1} potassium tartrate, 3 g L^{-1} malic acid, 1.0 g L^{-1} of diammonium phosphate (DAP) and Pereira et al. *(33)*, who found a value of 3.6 ± 0 following fermentation by *S. cerevisiae* ICV D47 cells immobilized in 2% alginate. These researchers also observed a reduction in pH values during mead production.

Proteins, peptides and amino acids are the main nitrogenous compounds in musts and wines. Of these, amino acids are the most studied and best known. They serve as nutrients for yeasts during alcoholic fermentation, and their concentration and composition in wines and musts plays an important role in the aromatic characteristics *(34)*.

The amount of nitrogen in the honey must reduced from 203.0 ± 0 to 24.1 ± 3.6 and 32.7 ± 4.0 mg L^{-1} in dry mead and sweet, respectively, with no significant difference ($p \leq 0.05$) between the meads. These results are in agreement with the observations in the literature *(28,33)*, which also verified the presence of residual nitrogen when supplementing the honey must with DAP. This may be related to the method used to determine the nitrogen that quantifies other compounds not assimilable by yeast, in particular the amino acid proline. Indeed, the formaldehyde method used has a recovery rate of only 23% for proline *(35)*, and that amino acid represents 50–85% of the total nitrogen content of the honey of *A. mellifera (36)* and is not used by *S. cerevisiae*.

The total acidity increased throughout the fermentation, reaching a maximum value of 7.8 ± 0 g L^{-1} tartaric acid for the dry mead and 6.0 ± 0.1 g L^{-1} tartaric acid for the sweet mead. Although there are differences in the total acidity between the two types of mead, the values obtained are in accordance with the Brazilian legislation *(4)* for mead, which establishes a minimum content of 3.4 g L^{-1} and a maximum content of 9.8 g L^{-1}. The pH and acidity are relevant properties in wine, as they influence the fermentative performance, sensorial characteristics, stability and colour *(37)*.

The values obtained for the volatile acidity, at the end of the fermentation, were 1.6 and 1.0 g L^{-1} acetic acid for dry and sweet mead, respectively. According to the Brazilian Legislation *(4)*, the value found for dry mead was higher than the legislated maximum value is 1.2 g L^{-1}. Volatile acidity is most often produced during must fermentation by yeasts and other microorganisms, with acetic acid being the predominant constituent. During wine storage, high values of volatile acidity can be found, indicating

Table 1. Physicochemical characteristics of honey must and mead (dry and sweet). On the right rows are displayed the values established by the Brazilian legislation for mead *(3)* and liqueur wine *(30)*

Physicochemical parameters	Honey must (mean ± SD)		Legislation mead	Legislation liqueur wine
Brix°	28.20 ± 0.10		—	—
pH	3.40 ± 0.10		—	—
Total acidity (g L^{-1} tartaric acid)	4.65 ± 0.04		—	—
Initial nitrogen (mg L^{-1} YAN)	203.00 ± 0.02		—	—
Reducing sugars (g L^{-1})	292.38 ± 0.10		—	—
	Dry mead	*Sweet mead*		
pH	3.11 ± 0.01[b]	3.26 ± 0.01[a]	—	—
Final nitrogen (mg L^{-1} YAN)	24.11 ± 3.56[a]	32.67 ± 4.04[a]	—	—
Ethanol (% v/v)	16.04 ± 0.05[b]	20.00 ± 0.00[a]	4.00–14.00	14.00–18.00
Total Acidity (g L^{-1} tartaric acid)	7.83 ± 0.04[b]	6.01 ± 0.13[a]	3.45–9.76	3.45–9.76
Volatile acidity (g L^{-1} acetic acid)	1.56 ± 0.00[b]	0.97 ± 0.02[a]	Max. 1.20	Max. 1.20
Sulphurous total (mg L^{-1})	52.91 ± 3.91[b]	64.85 ± 3.91[a]	—	Max. 350.00
Reducing sugars (g L^{-1})	5.97 ± 0.21[b]	157.75 ± 8.05[a]	—	Max. 20.010

Mean values with different letters in the same row are significantly different ($p < 0.05$).
YAN, Yeast assimilable nitrogen.

microbiological contamination by contaminating organisms, which transform ethanol into acetic acid *(38)*.

The levels of total SO_2 ranged from 52.9 ± 3.9 to 64.9 ± 3.9 mg L^{-1} for dry and sweet mead, respectively. These values are in accordance with the Brazilian Legislation for wines, which allows a maximum of 350 mg L^{-1} of total SO_2 *(31)*, but this parameter is not legislated for in mead *(4)*. In winemaking, sulphur dioxide is a popular additive because of its antioxidant and antimicrobial properties, allowing the growth of desirable yeasts and inhibiting the growth of undesirable bacteria and yeasts *(39,40)*. In recent years there has been a growing tendency to reduce SO_2 in musts and wines because of the potential risks of sulphites to human health *(39)*.

The richness of wine is expressed in alcoholic strength by volume. The maximum ethanol production occurred during the first 72 h of fermentation, and in the dry mead, about 50% of ethanol had already been produced at this time. The volumetric alcoholic strength (% vol.) in the dried mead was 16.0 ± 0.1% and in the sweet mead was 9.3%, increasing to 20.0 ± 0%, after brandy, in the latter case. According to the Brazilian legislation *(4)* for mead, these values are above those permitted, since this drink must have alcohol content between 4 and 14 °GL *(4)*; however according to Iglesias et al. *(6)*, mead is an alcoholic beverage containing between 8 and 18% alcohol by volume.

The results obtained for the metabolic products are presented in Table 2 and Fig. 2. They show that glucose and fructose were metabolized by the yeasts during the exponential and stationary phase. In the latter case the consumption was partial owing to the interruption of the fermentation by the addition of brandy. The low concentration of residual sugars suggests that the mead produced is less susceptible to. In addition, the preferential consumption of glucose compared with fructose was confirmed. Similar results were observed by Fleet et al. *(41)*, Berthels et al. *(42)* and Gomes et al. *(43)*, which indicates that, although glucose and fructose are consumed throughout the fermentation process, *S. cerevisiae* has a preference for glucose.

Glycerol increased progressively throughout the fermentation, reaching final values of 9.4 ± 0.1 g L^{-1} for dry mead and 8.7 ± 1.7 g L^{-1} for sweet mead (Fig. 2), without significant differences between the two products (Table 3). The concentrations of glycerol indicated in the literature for wines, the contents obtained in both meads are in accordance with the legislations. For example, Rankine and Bridson *(44)* indicate that in Australian wines the concentration range of glycerol should be between 1.4 and 9.9 g L^{-1}.

Table 2. Final concentration (g L^{-1}) of glucose, fructose, ethanol, acetic acid and glycerol of the dry mead and sweet mead produced using honey *Melipona scutellaris*

Parameters	Mead		
	Honey must	Dry	Sweet
Fermentation time (h)	0.00	312.00	144.00
Glucose (g L^{-1})	144.14 ± 5.45	4.90 ± 0.41[b]	50.5 ± 2.07[a]
Fructose (g L^{-1})	149.31 ± 5.61	5.45 ± 0.56[b]	99.99 ± 3.29[a]
Glycerol (g L^{-1})	0.00	9.44 ± 0.10[a]	8.67 ± 1.72[a]
Acetic acid (g L^{-1})	0.00	1.10 ± 0.01[a]	1.01 ± 0.03[a]
Ethanol (g L^{-1})	0.00	16.04 ± 0.02[b]	19.55 ± 0.67[a]
Y (ethanol/sugars) (%)	0.00	54.7 ± 0.36	0.00

Mean values with different letters in the same row are significantly different ($p < 0.05$).

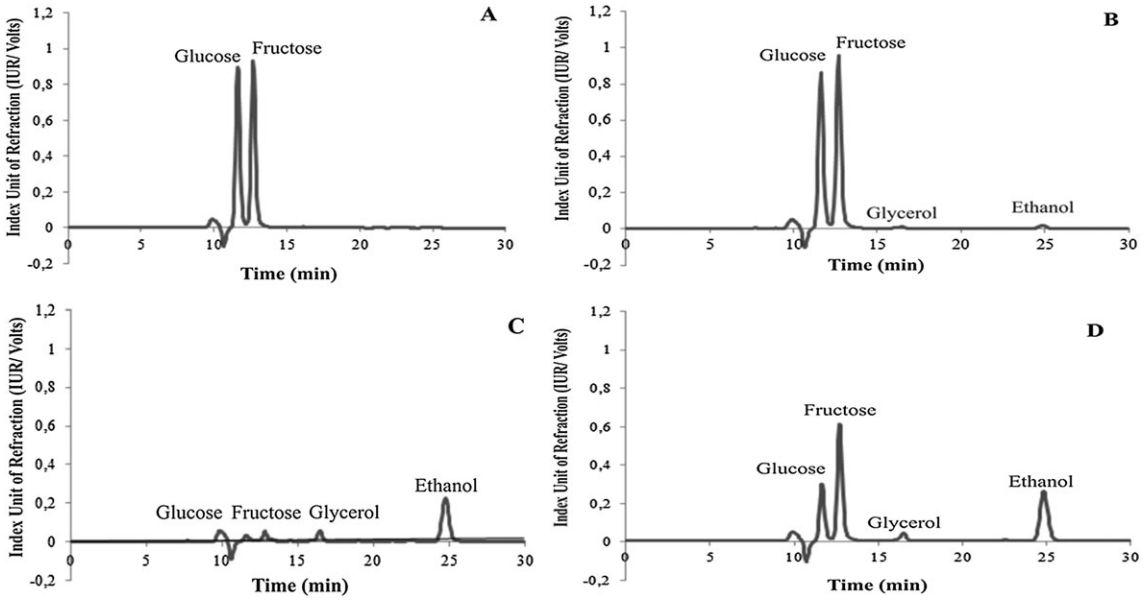

Figure 2. Concentration of glucose, fructose, glycerol, acetic acid and ethanol determined by high performance liquid chromatography (HPLC) on the honey must of *Melipona scutellaris* (Hymenoptera: Apidae: Meliponina) at the initial time (A), after 48 h of fermentation (B), in the dried mead at 312 h of fermentation (C) and in sweet mead at 144 h of fermentation (D).

Table 3. Mean values of total phenolic compounds, total flavonoids, IC_{50} of the samples dry and sweet mead samples

Parameters	Mead	
	Dry	Sweet
Total phenolic compounds (mg GAEs mL^{-1})	2.67 ± 0.130[a]	2.47 ± 0.070[a]
Total flavonoids (mg QE mL^{-1})	0.01 ± 0.002[a]	0.01 ± 0.002[a]
Antioxidant activity by reducing power (mg mL^{-1})	0.57 ± 0.030[b]	0.43 ± 0.020[a]
Antioxidant activity DPPH blocker effect (mg mL^{-1})	1.54 ± 0.005[a]	1.41 ± 0.005[a]

Mean values with different letters in the same row are significantly different ($p < 0.05$).
GAE, Gallic acid mead equivalent; QE, quercetin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

The values obtained for this parameter by Gomes et al. *(43)* in mead varied from 5.4 to 7.0 g L^{-1}. Glycerol has important sensory implications in wines *(45–47)*, contributing mainly to the beverage body, texture and persistence *(46)*. In high concentrations it also influences the sweetness of the drink *(45–48)*, contributing to the softness of the wine.

In relation to acetic acid, the concentrations obtained were 1.1 ± 0 and 1.0 ± 0 g L^{-1} in the dry and sweet mead, respectively. In both products, the final values were within the legal limit, which is 1.1 g L^{-1} *(49)*. The ethanol content was 16.0 ± 0% for dry mead and 19.6 ± 0.7% for sweet mead. Although ethanol is a primary metabolite produced in the exponential phase, *(5)*, its production was still observed throughout the stationary phase. No differences were observed in the ethanol content determined by HPLC and the alcoholic titre evaluated by the method recommended by Organisation International De La Vigne e Du Vin *(16)*.

The yield of fermentation (ethanol/sugars) was 54.7%. This value was higher than that reported in the literature *(50)* for other alcoholic beverages, for mead produced with different strains of yeasts *(29)*, using residual honeys *(10)* and in mead supplemented with bee pollen. This suggests that the efficiency of the fermentation process was high, corroborating the observations of Steckelberg et al. *(51)*. According to these authors, for industrial application of yeast the fermentative yield must be >46%.

Total phenolic compounds

The values obtained for the phenolic compounds were 2.7 ± 0.1 mg (GAEs) mL^{-1} in the dry mead sample and 2.5 ± 0.1 mg (GAEs) mL^{-1} in the sweet mead sample (Table 3). For total flavonoids values of 0.01 ± 0 mg (QE) mL^{-1} and 0.01 ± 0 mg (QE) mL^{-1} were obtained for the dry and sweet mead samples, respectively. There were no significant differences between the content of total phenols and total flavonoids in the two types of mead.

The total phenolic compounds is an important component of wines, as it contributes to the sensorial and qualitative characteristics like flavour, aroma and astringency, and acts as an antioxidant agent *(52,53)*.

Antioxidant activity

From the analysis of Table 3, the values obtained for EC_{50} by the method of reducing power were 0.6 ± 0 mg mL^{-1} for dry mead and 0.4 ± 0 mg mL^{-1} for sweet mead. By the DPPH• (2,2-

diphenyl-1-picrylhydrazyl) blocking effect method, EC_{50} values of 1.5 ± 0 and 1.4 ± 0 mg mL^{-1} were observed for the dry and sweet mead, respectively. Sweet mead showed higher antioxidant capacity, probably owing to the higher concentration of ethanol and residual sugars.

Owing to the relationship between oxidative stress and the appearance of characteristic pathologies, in recent years great emphasis has been placed on the consumption of foods that have antioxidant activity *(54)*. The results obtained in our work for antioxidant activity are comparable with those observed *(55–62)* in red and rosé wines.

Conclusion

The dry and sweet mead produced from the honey of *M. scutellaris* presented slightly different characteristics when compared with each other, with some values exceeding the parameters established by the Brazilian legislation for mead. In dry mead, despite having a volatile acidity higher than that established by legislation, the acetic acid content was within the legislated limits for this drink. Sweet mead had an alcoholic content above that established by legislation, since it was intended to produce a beverage with characteristics similar to those of liqueur wines. There was no significant difference in total phenolic and total flavonoid content between the two types of mead. The beverages produced showed antioxidant activity comparable with those of other beverages. Considering that the legislation for mead is based on honey from *A. mellifera*, these results indicate the need to re-evaluate the standards established for this product in order to incorporate mead produced from the honey of stingless bees.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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