

## Organic brown sugar and jaboticaba pulp influence on water kefir fermentation

### Influência do açúcar mascavo orgânico e da polpa de jaboticaba na fermentação do kefir de água

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#### ABSTRACT

Water kefir is considered to be a functional food with probiotic characteristics. It can be improved with addition of organic brown sugar and fruits native to Brazil, such as jaboticaba. The objective of this study was to evaluate the effects of brown sugar type: organic and conventional and the presence or absence of jaboticaba pulp in water kefir fermentation, based on the carbohydrate, organic acid, mineral composition and color profile. The fermentation process was carried out in two stages: with kefir grains, for 24 hours, at 25 °C and after filtration, maintained for 24 h at 25 °C in hermetic bottles, followed by a period of 8h refrigeration, and monitored every 8 h. Scanning electron microscopy of the kefir grains was performed after 24 h. The major changes in physicochemical patterns occurred up to 40 h. The final beverages presented acidic characteristics as a result of the production of lactic, acetic and succinic acids. The total sugar content had a reduction of around 50%. The sucrose content was reduced and the glucose and fructose increased. The use of organic sugar influenced the composition of the minerals. The characteristics related to color showed a tendency to increase over the evaluated times. It was concluded that it is possible to produce beverages fermented by water kefir grains, using organic or conventional brown sugar and jaboticaba pulp, being an alternative for the substitution of soft drinks, since it has no preservatives or food colorings, presents lower content of sugars and can be handcrafted.

**Index terms:** Probiotic; bioprocess; mixed culture; *Myrtales*; tibico.

#### RESUMO

O kefir de água é considerado um alimento funcional com características probióticas. Pode ser melhorado com adição de açúcar mascavo orgânico e frutas nativas do Brasil, como a jaboticaba. Objetivou-se avaliar os efeitos do tipo de açúcar mascavo: orgânico e convencional e da presença ou ausência de polpa de jaboticaba na fermentação de kefir de água, baseada no perfil de carboidratos, ácidos orgânicos, composição de minerais e cor. O processo fermentativo foi conduzido em duas etapas: com grãos de kefir, por 24 horas, a 25 °C e após filtragem, mantido por 24 h a 25 °C em garrafas herméticas, seguida de um período de 8h de refrigeração, e monitorado a cada 8 h. Foi realizada microscopia eletrônica de varredura dos grãos de kefir decorridas 24 h. As principais mudanças nos padrões físico-químicos ocorreram até 40h. As bebidas finais apresentaram característica ácida em decorrência da produção dos ácidos láctico, acético e succínico. Os teores de açúcares totais tiveram uma redução em torno de 50%. O teor de sacarose foi reduzido e os teores de glicose e frutose aumentaram. A utilização de açúcar orgânico influenciou a composição dos minerais. As características relacionadas à cor apresentaram tendência de aumento ao longo dos tempos avaliados. Concluiu-se que é possível produzir bebidas fermentadas por grãos de kefir de água, utilizando-se como substrato açúcar mascavo orgânico ou convencional e polpa de jaboticaba, sendo alternativa para a substituição de refrigerantes, pois não tem conservantes nem corantes alimentares, apresenta menor teor de açúcares e pode ser produzido artesanalmente.

**Termos para indexação:** Probiótico; bioprocesso; cultura mista; *Myrtales*; tibico.

#### INTRODUCTION

The growing interest in food for health improvement has driven the consumption of functional foods. The role of probiotics in modulating immunity against pathogens

is important for prevention and treatment of infectious diseases. The effectiveness of a probiotic depends mainly on its class (presence of different strains of microorganisms), dose and type of feed (Kandasamy et al., 2017). The development of non-dairy probiotic

beverages is particularly attractive due to the absence of milk allergens, cholesterol, and it is accepted by strict vegetarians (vegans) (Morris et al., 2016).

Among the non-lactic fermented beverages, water kefir, produced by a consortium of several bacteria and yeasts (Nikolaou et al., 2019), presents an interesting option, with the advantage of being low cost, safe and free from negative side effects (Alsayadi et al., 2013; Fiorda et al., 2016; Marsh et al., 2014). Brown sugar is the main substrate used for the fermentation of water kefir (Borguini; Torres, 2006). Various vegetables and fruits have been successfully used as kefir substrates, such as: soy, onion, ginger, carrot, apple, pineapple, grape, quince, kiwi, pear, melon, strawberry, pomegranate, tomato and coconut (Corona et al., 2016; Fernandes et al., 2017; Fiorda et al., 2017; Randazzo et al., 2016) as an option for diversification and nutritional enrichment. It is interesting to use fruits of Brazilian origin, such as jaboticaba, chosen in an unprecedented way to be evaluated in the present work.

In the search for routines considered healthier, organic agriculture is a sector in constant expansion, due to its superior sustainability and low environmental impact. Therefore, there has been scientific interest in comparing agricultural systems (Hendgen et al., 2018). Most of these studies focus on soil fertility, yield, crop quality and plant physiology (Banks et al., 2014; Döring et al., 2015; Puglisi et al., 2012). There are still few studies comparing the effect of organic and conventional management on the physico-chemical and sensorial characteristics of the final processed food products. Currently, there is a debate about the positive effects of applying organic practices in different cultures (Reganold; Wachter, 2016; Parpinello et al., 2015; Turinek et al., 2009).

In order to generate more information comparing organic and conventional cultivation, both types of brown sugar were used in this study. Brown sugar results from sugarcane milling, followed by cooking for dehydration. It does not go through the following stages of refinement (as those used for white sugar production), so it conserves vitamins and minerals (Ca, K, Fe, Mg, F), with a sucrose content of about 90%. Organic brown sugar is more nutritious than conventional crystal sugar, presenting respectively 1.35 and 0.01% of ash, 5.6 and 0.05% of reducing sugars, 56.8 and 1.8 mg.kg<sup>-1</sup> of phenolic compounds, 151.6 and 1.0 mg.kg<sup>-1</sup> of amino acids (Bettani et al., 2014) and antioxidant capacity (0.45±0,25 and <0.01 mmol FRAP/100 g) (Phillips; Carlsen; Blomhoff, 2009).

Jaboticaba (*Plinia jaboticaba*) is a Brazilian fruit with high nutritional potential: vitamin C, minerals (K, Mg, P, Ca, Zn, Cu), fibers, polyphenols etc. The main polyphenols identified in jaboticaba are cyanidin- 3- O-

glucoside, delphinidin- 3- O- glucoside, gallic acid, rutin, myricetin, and quercetin. The anti-radical activity is linked to the inhibition of enzymes associated with reactive oxygen (ROS) production, direct quenching of free radicals, chelation of transition metals, or redox inactivation of ROS. Additional attributes of phenolic compounds include modulating the gut microbiota and its metabolites, such as short chain fatty acids (SCFA), which improves glucose tolerance, appetite control, reduction of gut absorption, and lipid synthesis on liver (Silva-Maia et al., 2018).

It shows other beneficial health effects, including inhibition of *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. coli* and mitochondrial protection. It has been useful for the treatment of hemoptysis, cough, bronchitis, asthma, tonsils inflammation, diarrhea and dysentery (Pereira et al., 2018).

Once jaboticaba is fermented during kefir production, it can probably be compared with grape, since both are rich in polyphenols, which are the main contributors for certain organoleptic characteristics of wines, such as color, astringency and bitterness. Taste, bouquet and color can be associated to oxidation and eventual sugar degradation through Maillard type reactions, acidic degradation or even caramelization (Pereira et al., 2013). Therefore, it was used to produce a more nutritious product as well as presenting a more attractive color and flavor.

The sensorial profile is noticed through a combination of factors, and can be analyzed through the interaction of the intensity with respect to time, that is, the persistence of the flavor. Since sucrose is the main substrate supplied for kefir fermentation, the organic acids provided throughout the fermentation may have the ability to provide a more rounded and optimized flavor profile, balancing the sweet taste with the acid, for example, and masking off flavors. Citric acid, often used in industries, has an intense taste but disappears in a short period of time, which works well because it has intensity x time curves almost identical to those of sucrose. Lactic acid, in turn, is considered intermediate, emphasizing the “green” taste, astringency and acidity. They have an advantage over malic acid, for example because they do not come from petroleum-derived raw materials (Pletsch; Raab, 2018).

The objective was to evaluate water kefir fermentation, using jaboticaba pulp and organic or conventional brown sugars as substrates, through physico-chemical parameters, carbohydrate profile, organic acids and mineral composition. The evaluations of these parameters were monitored at different times throughout the fermentation process, and the kefir culture was characterized by scanning electron microscopy (SEM).

## MATERIAL AND METHODS

Experiments and physico-chemical analyzes were carried out at the Department of Food Science and Technology (DCTA-UEL) Microbiology laboratories. The chromatographic analyzes were done in the Laboratory for the Development of Instrumentation, Automation and Analytical Methodology (DIA). The analysis of scanning electron microscopy (SEM) was conducted in the Laboratory of Electron Microscopy and Microanalysis (LMEM/PPPG-UEL). Chemical analyzes of minerals were conducted in the LaborSolo laboratory, in Londrina - PR.

### Formulations for beverages fermented by water kefir

Raw materials used for fermentation were mineral water, organic and conventional brown sugar produced from sugar cane, grown at Lucélia, São Paulo, BR, provided by Planeta Verde; and pasteurized jaboticaba pulp, manufactured by DeMarchi, at São Paulo, São Paulo, Brazil. Water kefir grains were initially obtained by donation from handcrafted cultivation in Londrina - PR.

### Preparation of formulations

Two water solutions, one with organic and the other with conventional brown sugar ( $60 \text{ g L}^{-1}$ ) were autoclaved at  $121 \text{ }^\circ\text{C}$  for 15 min and cooled to  $25 \text{ }^\circ\text{C}$ . Before starting the experiment, water kefir grains were adapted to each of these substrates, transferring  $60 \text{ g L}^{-1}$  to each one of these solutions, completing three cycles (after 24 h the musts passed through a qualitative filter and kefir grains were added to new solutions).

For each one of the four different formulations, 24 reagent bottles were used, totalizing 96 recipients. 30 g of the adapted water kefir grains were aseptically transferred to 470 mL of each reagent bottle. The formulation with organic brown sugar solution (O) contained 470 mL of the solution with the correspondent sugar. The formulation with conventional brown sugar (C) held 470 mL of the matching sugar solution. The third formulation was prepared like the first with the addition of 30 g of jaboticaba pulp (OJ). The fourth formulation was prepared like the second with the addition of 30 g of jaboticaba pulp (CJ).

### Fermentative process

The fermentation of water kefir with mixed culture was performed in two stages without stirring. In the first one (fermentation with grains), the formulations were kept in 500 mL glass reagent bottles with screw caps, which were semi-closed and were maintained at  $25 \text{ }^\circ\text{C}$  for 24 h.

To start the second stage (gasification), the musts passed through a qualitative filter and were bottled in amber glass bottles (12 for each formulation, totalizing 48) with a useful volume of 500 mL, sealed with flip-top lids for retention of the carbonic gas, probably produced by yeasts during fermentation, and kept at  $25 \text{ }^\circ\text{C}$ , for 24 h. After this incubation stage, the 12 remaining bottles were refrigerated for 8 h at  $4 \text{ }^\circ\text{C}$  for stabilization, when the beverages were considered ready for consumption.

### Experimental design

The experiments were conducted using a completely randomized design, in a factorial scheme, with 4 treatments (formulations) and 8 times of fermentation, with 3 repetitions. After the necessary samples were taken for the analyzes at each time, the remaining contents into the reagent bottles or amber glass bottles were discarded. The samples, after being analyzed, were also discarded. The four different formulations were monitored from time 0 (immediately after inoculation with kefir grains and homogenization with other ingredients of the formulations) and analyzed every 8 h, up to 56 h. Thus, in the first step the formulations were analyzed at 4 stages (0, 8, 16 and 24 h), in the second step at 3 stages (32, 40 and 48 h), and finally, the last data were collected at final time, after being refrigerated for 8 h at  $4 \text{ }^\circ\text{C}$ .

Physico-chemical characteristics evaluated were: total soluble solids and pH, with 3 replicates per repetition; titratable total acidity, with 2 replicates per repetition and color parameters, with 5 replicates per repetition. For carbohydrates and organic acids, the data were collected in 2 repetitions, with one evaluation per repetition. For minerals, samples were collected at the initial and final time, with three repetitions; the repetition value being the mean of 2 replications.

### Physico-chemical analysis

Determinations of pH, total soluble solids (TSS) ( $^\circ\text{Brix}$ ) and total titratable acidity (TTA) ( $\text{g L}^{-1}$ ) were performed according to AOAC (2012).

### A - Determination of organic acids

Organic acids (lactic, acetic, citric and succinic) were determined by High Performance Liquid Chromatography (HPLC) in Shimadzu LC 20 A (Kyoto, Japan), consisting of high pressure pump LC-20AT, automatic injector SIL- 20AC HT, RID-10A refractive index detector, SPD-M20A photodiode array detector, CTO-20A column oven and CBM-20A control module. For the analyzes, Shiseido Cap Cell Pak  $5\mu$  C18 MG

250 x 4.6 mm chromatographic column was used. The mobile phase used was a 25 mM sodium phosphate buffer solution, pH adjusted to 2.4 at the flow rate of 1.0 mL min<sup>-1</sup>, the column temperature was maintained at 30 °C and the injection volume was 20 µL. Detection was performed simultaneously on the RID-10A and SPD-M20A detectors at a fixed wavelength of 215 nm and in the 200 to 400 nm scan mode. The data were processed by LC Solutions Software (Shimadzu Corporation, Kyoto, Japan) (Reuter, 2015). Peak identification was performed comparing the retention time of the lactic, acetic, citric and succinic acid standards with those of the peaks observed in the samples analyzed under the same conditions. The results were expressed in mg L<sup>-1</sup>.

### B - Determination of carbohydrates

Carbohydrates were analyzed by HPLC (Shimadzu Corporation, Kyoto, Japan) composed of high pressure pump (LC-20AT); automatic injection (SIL-20AC HT), at the flow rate of 1.0 mL min<sup>-1</sup>, with injection volume adjusted to 20 µL; detector by refractive index (RID-10A); column furnace (CTO-20A) maintained at a constant temperature of 85 °C; (CBM-20A) and Aminex HPX-87P ion exchange column (7.8 x 300 mm in ionic form Pb-2, Biorad, CA, USA). The mobile phase used was ultra pure water (Mili-Q (R)). Data acquisition and integration of chromatographic peaks were performed using LC Solutions software (Shimadzu Corporation, Kyoto, Japan) (Pauli; Cristiano; Nixdorf, 2011). Peak identification was performed comparing the retention time of the sucrose, glucose and fructose standards with those of the peaks observed in the samples analyzed under the same conditions. Results were expressed in mg L<sup>-1</sup>.

### C - Determination of mineral composition

Approximately one gram of each sample was transferred to a 250 mL Erlenmeyer flask. Enough distilled water was added to cover the sample and 10 mL of concentrated nitric acid was added. The samples were placed in a heating plate for approximately 10 min, avoiding the boiling. Then, 4 mL of hydrogen peroxide was added. Thereafter, the digestion step was carried out for approximately 40 min, by the addition of 5 mL of concentrated hydrochloric acid. The material digested at room temperature was passed through quantitative filter paper into a 50 mL volumetric flask and the volume was filled with distilled water. The samples were analyzed in Atomic Emission Spectrometer with Plasma Source for

the following minerals: P, K, Ca, Mg, S, Na, Cu, Fe, Zn, Mn, B, Ni, Cr, Mo, Pb, Co and Cd (ICP-OES) (Perkin Elmer-Optima 8300, USA) (APHA, 2012).

### D - Color analysis

Color analysis was determined on a colorimeter (Konica Minolta, Chroma Meter CR-4000, Japan) with illuminant D65 (daylight), which was calibrated using white plates. Samples were analyzed at an angle of 45° of illumination and 10° of observation at 25 °C. Readings were made directly on the equipment and the values of the luminosity (L\*), red/green coordinate (a\*), yellow/blue coordinate (b\*) expressed at the CIE Lab system. The coordinates a\* and b\* are responsible for saturation or chromaticity (C\*), and the Hue angle identifies the colors in a diagram, which consistently correlate color values with visual perception (Lawless; Heymann, 1998; Minolta, 1994; Vilhalva et al., 2012).

The total color differences ( $\Delta E^*$ ), which measures the color change between the formulations evaluated, and between the different times, were determined by the following equation:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$$

### E - Scanning electron microscopy (SEM)

For SEM analyzes, material was fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 N sodium cacodylate buffer, pH 7.2, for a period of 12 h at room temperature. After the primary fixation, the samples were passed through three washes in sodium cacodylate buffer, post-fixed in 1% osmium tetroxide solution in the same buffer, for 2 h at room temperature and washed again in buffer. Then the material was dehydrated in an increasing alcoholic series and dried to the critical point in the CPD 030 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). The dried samples were mounted on aluminum stubs using carbon tape and covered with gold (Sputter coater - Baltec SCD 050). The images were obtained using the scanning electron microscope FEI - Quanta 200 (Guergoletto et al., 2010). SEM was performed on the grains after 24 h of fermentation.

### Statistical analyzes

The analysis of variance (ANOVA) and Tukey's tests for multiple comparison of the means, at a level of 5% of significance ( $p < 0.05$ ), were made using software R.

## RESULTS AND DISCUSSION

### Acidity

The analysis of variance (ANOVA) for the six evaluated characteristics related to acidity: pH, total titratable acidity (TTA), lactic acid (LA), acetic acid (AA), citric acid (CA) and succinic acid (SA), were significant ( $p < 0.001$ ) for all formulations, times and interaction formulation x time. The coefficients of variation (CV) obtained for these six characteristics related to acidity ranged from 0.77% for pH to 6.06% for TTA. These values indicate optimal experimental precision (Table 1).

Table 2 shows the average of the acidity-related characteristics for four formulations in eight times. Regarding pH, all the formulations evaluated in the final product (56 h) showed a significant difference. Over

time, these values did not differ statistically from 40 h of fermentation, except for kefir prepared with conventional sugar, in which this value was maintained since 48 h. The lowest pH value for the different beverages was reached at 40 h, then the pH remained constant. At the end of 56 h the drink C had the lowest pH (3.71), followed by CJ (3.78), O (3.91) and OJ (3.98). The pH values reached in this study were important because acidity is a limiting factor for the development of pathogenic microorganisms and contributes to product stability (Franco; Landgraf, 2008).

Titratable total acidity and lactic, acetic and succinic acids contents showed increasing values throughout the fermentation time. Citric acid declined up to 16 h. At 56 h, beverages O and C presented higher values of lactic acid and acetic acid and lower contents of citric and succinic acids, when compared to OJ and CJ products.

**Table 1:** Analysis of variance (ANOVA) for the characteristics related to acidity, evaluated in water kefir for the four formulations and in eight times.

Source of Variation	Mean Square <sup>1</sup>					
	pH	Total Titratable acidity (mol 100 mL <sup>-1</sup> )	Lactic acid (mg L <sup>-1</sup> )	Acetic acid (mg L <sup>-1</sup> )	Citric acid (mg L <sup>-1</sup> )	Succinic acid (mg L <sup>-1</sup> )
Formulations (F)	***	***	***	***	***	***
Time (T)	***	***	***	***	***	***
F x T	***	***	***	***	***	***
Residue	0.00	0.00237	0.00147	0.000	0.001	0.0036
Mean	4.05	2.63	1.05	0.0190	0.308	0.300
Coefficient of variation (%)	0.77	6.06	3.65	4.14	4.85	5.51

<sup>1</sup> \*\*\* Significant at  $p \leq 0.001$ .

**Table 2:** Means for characteristics related to acidity in water kefir, for four formulations (F), in eight times.

F <sup>1</sup>	0 h	8 h	16 h	24 h	32 h	40 h	48 h	56 h
pH								
O	5.77 <sup>aA2</sup>	4.45 <sup>aB</sup>	4.10 <sup>aC</sup>	4.04 <sup>bCD</sup>	4.02 <sup>aD</sup>	3.94 <sup>aE</sup>	3.91 <sup>bE</sup>	3.91 <sup>bE</sup>
OJ	4.61 <sup>cA</sup>	4.31 <sup>bB</sup>	4.14 <sup>aC</sup>	4.10 <sup>aCD</sup>	4.07 <sup>aD</sup>	3.97 <sup>aE</sup>	3.99 <sup>aE</sup>	3.98 <sup>aE</sup>
CJ	5.14 <sup>bA</sup>	4.04 <sup>cB</sup>	3.73 <sup>cC</sup>	3.67 <sup>dDE</sup>	3.68 <sup>cCDE</sup>	3.64 <sup>cE</sup>	3.66 <sup>dDE</sup>	3.71 <sup>dCD</sup>
C	4.24 <sup>dA</sup>	4.00 <sup>cB</sup>	3.83 <sup>bC</sup>	3.78 <sup>cCD</sup>	3.79 <sup>bCD</sup>	3.78 <sup>bD</sup>	3.77 <sup>cD</sup>	3.78 <sup>cD</sup>
Total Titratable Acidity (molar acidity 100 mL <sup>-1</sup> )								
O	0.43 <sup>bF</sup>	1.42 <sup>bE</sup>	2.08 <sup>bD</sup>	2.51 <sup>aC</sup>	2.41 <sup>bCD</sup>	3.30 <sup>aB</sup>	4.33 <sup>aA</sup>	4.16 <sup>aA</sup>
OJ	1.49 <sup>aE</sup>	2.05 <sup>aD</sup>	2.38 <sup>abCD</sup>	2.58 <sup>aBC</sup>	2.87 <sup>aB</sup>	2.94 <sup>abB</sup>	4.06 <sup>abA</sup>	3.99 <sup>abA</sup>
C	0.46 <sup>bE</sup>	1.42 <sup>bD</sup>	2.08 <sup>bC</sup>	2.48 <sup>aB</sup>	2.38 <sup>bBC</sup>	2.64 <sup>bB</sup>	4.06 <sup>abA</sup>	3.60 <sup>bA</sup>
CJ	1.39 <sup>aF</sup>	2.18 <sup>aE</sup>	2.41 <sup>aDE</sup>	2.58 <sup>aD</sup>	2.61 <sup>abD</sup>	3.17 <sup>aC</sup>	3.67 <sup>bB</sup>	4.19 <sup>aA</sup>

Continue...

**Table 2:** Continuation.

F <sup>1</sup>	0 h	8 h	16 h	24 h	32 h	40 h	48 h	56 h
Lactic acid (g L <sup>-1</sup> )								
O	0.19 <sup>aE</sup>	0.96 <sup>aD</sup>	1.40 <sup>aC</sup>	1.64 <sup>aB</sup>	1.75 <sup>aAB</sup>	1.72 <sup>aAB</sup>	1.82 <sup>aA</sup>	1.73 <sup>aAB</sup>
OJ	0.18 <sup>aE</sup>	0.55 <sup>bD</sup>	0.86 <sup>bC</sup>	0.94 <sup>bCB</sup>	1.05 <sup>cAB</sup>	1.08 <sup>cA</sup>	1.12 <sup>cA</sup>	1.13 <sup>cA</sup>
C	0.19 <sup>aD</sup>	0.87 <sup>aC</sup>	1.43 <sup>aB</sup>	1.53 <sup>bAB</sup>	1.52 <sup>bAB</sup>	1.57 <sup>bA</sup>	1.58 <sup>bA</sup>	1.43 <sup>bB</sup>
CJ	0.14 <sup>aC</sup>	0.50 <sup>bB</sup>	0.75 <sup>cA</sup>	0.81 <sup>dA</sup>	0.79 <sup>dA</sup>	0.83 <sup>dA</sup>	0.78 <sup>dA</sup>	0.76 <sup>dA</sup>
Acetic acid (g L <sup>-1</sup> )								
O	0.008 <sup>aE</sup>	0.013 <sup>aD</sup>	0.021 <sup>aC</sup>	0.026 <sup>aB</sup>	0.028 <sup>aAB</sup>	0.027 <sup>aB</sup>	0.030 <sup>aA</sup>	0.028 <sup>aAB</sup>
OJ	0.007 <sup>aF</sup>	0.011 <sup>bE</sup>	0.017 <sup>bD</sup>	0.019 <sup>bCD</sup>	0.020 <sup>bCB</sup>	0.021 <sup>bAB</sup>	0.022 <sup>bAB</sup>	0.023 <sup>bA</sup>
C	0.005 <sup>bF</sup>	0.011 <sup>bCE</sup>	0.021 <sup>aD</sup>	0.047 <sup>aC</sup>	0.024 <sup>bBC</sup>	0.027 <sup>aAB</sup>	0.028 <sup>aA</sup>	0.025 <sup>bBC</sup>
CJ	0.005 <sup>bD</sup>	0.009 <sup>cC</sup>	0.016 <sup>bB</sup>	0.018 <sup>bAB</sup>	0.018 <sup>dAB</sup>	0.020 <sup>bA</sup>	0.019 <sup>cA</sup>	0.019 <sup>cA</sup>
Citric acid (g L <sup>-1</sup> )								
O	0.048 <sup>cB</sup>	0.064 <sup>bA</sup>	0.050 <sup>bB</sup>	0.037 <sup>cC</sup>	0.041 <sup>cC</sup>	0.040 <sup>cC</sup>	0.040 <sup>cC</sup>	0.039 <sup>cC</sup>
OJ	0.628 <sup>aA</sup>	0.618 <sup>aAB</sup>	0.597 <sup>aABC</sup>	0.546 <sup>aCD</sup>	0.536 <sup>aCD</sup>	0.556 <sup>aBCD</sup>	0.525 <sup>aD</sup>	0.509 <sup>aD</sup>
C	0.059 <sup>bB</sup>	0.070 <sup>bA</sup>	0.046 <sup>bC</sup>	0.047 <sup>bC</sup>	0.048 <sup>bC</sup>	0.049 <sup>bC</sup>	0.047 <sup>bC</sup>	0.048 <sup>bC</sup>
CJ	0.666 <sup>aA</sup>	0.647 <sup>aAB</sup>	0.586 <sup>aBC</sup>	0.561 <sup>aCD</sup>	0.538 <sup>aCD</sup>	0.556 <sup>aCD</sup>	0.513 <sup>aD</sup>	0.516 <sup>aD</sup>
Succinic acid (g L <sup>-1</sup> )								
O	0.018 <sup>aE</sup>	0.081 <sup>bD</sup>	0.198 <sup>cC</sup>	0.264 <sup>bB</sup>	0.305 <sup>cAB</sup>	0.307 <sup>dAB</sup>	0.345 <sup>cA</sup>	0.319 <sup>bA</sup>
OJ	0.019 <sup>aF</sup>	0.074 <sup>bE</sup>	0.246 <sup>bD</sup>	0.305 <sup>bC</sup>	0.415 <sup>bB</sup>	0.451 <sup>bAB</sup>	0.495 <sup>bAB</sup>	0.528 <sup>aA</sup>
C	0.016 <sup>aF</sup>	0.098 <sup>aE</sup>	0.2781 <sup>bD</sup>	0.303 <sup>bCD</sup>	0.324 <sup>cBCD</sup>	0.372 <sup>cAB</sup>	0.424 <sup>bA</sup>	0.358 <sup>bABC</sup>
CJ	0.017 <sup>aD</sup>	0.107 <sup>aC</sup>	0.331 <sup>aB</sup>	0.397 <sup>aB</sup>	0.490 <sup>aA</sup>	0.565 <sup>aA</sup>	0.592 <sup>aA</sup>	0.565 <sup>aA</sup>

<sup>1</sup>O, kefir with organic brown sugar; OJ, kefir with organic brown sugar and jaboticaba; C, kefir with conventional brown sugar; CJ, kefir with conventional brown sugar and jaboticaba. <sup>2</sup>Different lowercase letters in the same column indicate that there is significant statistical difference ( $p < 0.05$ ) between formulations. Different capital letters on the same line indicate that there is a statistically significant difference ( $p \leq 0.05$ ) between fermentation times.

The analyzes of raw materials: conventional brown sugar, organic brown sugar and jaboticaba pulp, presented values between 0 and 0.15 g L<sup>-1</sup> for the organic acids, except for citric acid in the jaboticaba pulp (0.60 g L<sup>-1</sup>). The four formulations evaluated in this study showed a pH reduction along time. This result was due to metabolites produced during the fermentation process, like organic acids, with the exception of citric acid, which is part of jaboticaba's composition.

Succinic acid is an efficient antioxidant, normalizes energy metabolism and has a role in the tricarboxylic acid cycle and its enzyme components have some crucial roles for basal cell metabolism. A 5 and 10  $\mu$ M concentration of succinic acid resulted in apoptosis in endometrium cancer cells; no such effect was seen in the control cell line (Iplik; Catmakas; Cakmakoglu, 2018). It shows potential to eliminate certain microorganisms such as *Salmonella* (Radkowski; Zdrodowska; Gomółka-

Pawlicka, 2018). The addition of jaboticaba skin extract increased the microbiota of the stomach of rats, especially *Lactobacillus*, *Bifidobacterium* and *Enterobacteriaceae* (Silva-Maia et al., 2018). Our results show an influence of jaboticaba on the 33 times increase of succinic acid amount (Table 2).

Randazzo et al. (2016) used water kefir grains for the static fermentation at 25 °C for 48 h of six different fruit juices from the Mediterranean: apple, grape, kiwifruit, pomegranate, prickly pear and quince. The pH levels reached values of  $4.04 \pm 0.08$ ,  $3.81 \pm 0.04$ ,  $3.48 \pm 0.03$ ,  $3.89 \pm 0.08$ ,  $4.11 \pm 0.07$  and  $3.62 \pm 0.05$ , respectively. These values are close to those we have obtained. In the beverage prepared at room temperature, with 1.2 grams of grains obtained from Pastoral da Criança, Aracaju, Sergipe, Brazil, with 15 g L<sup>-1</sup> of brown sugar, the pH ranged from 6 to 4 after 48 h of fermentation (Silva et al., 2009), therefore, within the expected.

Laureys and Vuyst (2014) reported in their water kefir fermentation study, that initial pH was  $4.85 \pm 0.01$  and reached  $3.45 \pm 0.01$ , after 72 h of fermentation. The decreasing tendency of this measure was slow until reaching the value of  $3.35 \pm 0.01$ , after 192 h of fermentation. Compared with our results, they are in agreement. We did not reach pH so low probably because we evaluated up to 56 h, only. In their work, the production of LA and AA increased, mainly up to 72 h of fermentation, the first one being produced in greater quantity, with  $15 \text{ g L}^{-1}$ , and  $3 \text{ g L}^{-1}$  for the latter. In our work, the maximum was  $1.8 \text{ g L}^{-1}$  LA, for O in 48 h and  $0.0297 \text{ g L}^{-1}$  AA, also for O in 48 h.

Magalhães et al. (2010) obtained pH values between 5.6 and 4.1 for the initial (0 h) and final (24 h) fermentation time, respectively. The TTA ranged from 0.02 to  $0.07 \text{ g } 100 \text{ mL}^{-1}$ . LA varied from 0.2 to  $1.75 \text{ mg mL}^{-1}$ , similar to the results found in our study, for treatments without jaboticaba ( $0.19$  to  $1.8 \text{ g L}^{-1}$  between 0 h and 48 h). The AA started close to zero and reached  $1.4 \text{ mg mL}^{-1}$  (24 h), higher values than those found in this study ( $0.02 \text{ g L}^{-1}$  at 24 h).

Monar et al. (2014) found values of 0.71, 0.86 and  $1.71 \text{ mg mL}^{-1}$  LA at 24, 48 and 72 h of fermentation at  $30 \text{ }^\circ\text{C}$ , respectively. In the experiment with  $42 \text{ g L}^{-1}$  sucrose, Harta (2004) observed that  $8.71 \text{ g L}^{-1}$  LA were produced after 24 h at  $30 \text{ }^\circ\text{C}$ .

Comparing studies found in the literature with the present study, it is observed that all types of beverages have in common the acidic characteristic, whether they are elaborated by different sources of sucrose, fruits, or the combination of fruit and sugar, as expected to occur after the fermentation process.

### Carbohydrates

The ANOVA for four sugar-related characteristics evaluated in water kefir for the four formulations at

eight times showed that there was significant effect ( $p < 0.001$ ) for all traits studied, for all formulations, times and interaction formulation x time. The coefficients of variation varied from 1.61% for total soluble solids to 5.43% for fructose, also indicating optimal experimental precision (Table 3).

Based on analysis obtained from chromatography of raw materials, organic sugar showed higher sucrose ( $53.33 \text{ g L}^{-1}$ ) and lower glucose ( $0.25 \text{ g L}^{-1}$ ) and fructose contents ( $0.40 \text{ g L}^{-1}$ ) when compared to conventional sugar which values were 52.61, 0.93, and  $0.82 \text{ g L}^{-1}$ , respectively. The pulp of jaboticaba presented lower content of sucrose ( $1.5 \text{ g L}^{-1}$ ) and higher of glucose ( $1.7 \text{ g L}^{-1}$ ) and fructose ( $2.6 \text{ g L}^{-1}$ ) when compared to sugars.

Total soluble solids values decreased for the 4 formulations, until 32 h, from then on, the values were stable until 56 h, regardless the type of substrate. The formulation with organic brown sugar (O), statically differed from the others in relation to the TSS at time 56 h, demonstrating a higher rate of consumption by the microorganisms present (reduction of 34%). Formulation C showed the lowest rate (26%), and beverages with jaboticaba (OJ and CJ), presented a reduction of 29% (Table 4).

Over time, sucrose conversion stabilized from 32 h for O formulation and at 40 h for C and CJ. The glucose and fructose contents were increasing, with maximums at 40 and 45 hours, respectively.

The transformation of the components of the medium into products takes place due to the activities of several enzymes that are synthesized by the microorganisms themselves. In the context of mixed fermentation, it is difficult to distinguish precisely which enzymes and specific metabolic pathways are being used. However, it is possible that the hydrolysis of sucrose was probably

**Table 3:** Analysis of variance (ANOVA) for the characteristics related to sugars analyzed in water kefir for four formulations in eight times.

Source of Variation	Mean Square <sup>1</sup>			
	Total Soluble Solids	Sucrose	Glucose	Fructose
Formulations (F)	***	***	***	***
Time (T)	***	***	***	***
F x T	***	***	***	***
Residue	0.0064	1.06	0.00153	21.3
Mean	4.968	23.27	6.229	11.96
Coef. of variation (%)	1.61	4.42	4.33	5.43

<sup>1</sup> \*\*\* Significant at  $p \leq 0.001$ .

carried out by the extracellular invertase enzyme from the yeasts, the intracellular invertase and the glucosucrase enzyme of the bacteria, in theory, resulting in an increase in the reducing sugars glucose and fructose. This fact was verified by reducing the sucrose content of 93, 80, 83 and 79%, for O, OJ, C and CJ, respectively. The increase in glucose (g L<sup>-1</sup>) was 600, 239, 309 and 130%, for O, OJ, C and CJ, respectively; and the increase in fructose (g L<sup>-1</sup>) was 1.115, 206, 139 and 51%, respectively, for O, OJ, C and CJ from the beginning (0 h) to the end of the monitoring (56 h). Therefore, with organic sugar there was more effective fermentation.

Comparing the change in total sugar values, obtained by the sum of the three sugars (sucrose, glucose and fructose), evaluated between the initial and final time and between the four formulations, it can be seen in the ANOVA that mean squares for the sources of variation

formulation and time had a significant effect ( $p < 0.001$ ), and for the interaction formulation x time ( $p < 0.05$ ). The coefficient of variation for this characteristic was 3.24%, indicating optimal experimental accuracy (Table 5).

**Table 5:** Analysis of variance (ANOVA) for total sugars in water kefir, for four formulations, in two times.

Source of Variation	Mean Square <sup>1</sup>
Formulations (F)	***
Time (T)	***
F x T	*
Resíduo	2.40
Mean	47.64
Coefficient of variation (%)	3.24

<sup>1</sup> \* Significant at  $p \leq 0.05$ ; \*\*\* Significant at  $p \leq 0.001$ .

**Table 4:** Means for carbohydrate-related characteristics in water kefir, for four formulations (F), in eight times.

F <sup>1</sup>	Total Soluble Solids (°Brix)							
	0 h	8 h	16 h	24 h	32 h	40 h	48 h	56 h
O	6.17 <sup>ba2</sup>	5.36 <sup>bb</sup>	4.55 <sup>cc</sup>	4.44 <sup>bd</sup>	4.24 <sup>cde</sup>	4.19 <sup>ce</sup>	4.05 <sup>ce</sup>	4.08 <sup>be</sup>
OJ	6.41 <sup>aa</sup>	5.79 <sup>ab</sup>	5.29 <sup>ac</sup>	5.05 <sup>ad</sup>	4.87 <sup>ade</sup>	4.82 <sup>ae</sup>	4.69 <sup>aeF</sup>	4.57 <sup>af</sup>
C	6.10 <sup>ba</sup>	5.45 <sup>bb</sup>	4.84 <sup>bc</sup>	4.66 <sup>bcd</sup>	4.55 <sup>bde</sup>	4.55 <sup>bde</sup>	4.35 <sup>be</sup>	4.51 <sup>ade</sup>
CJ	6.43 <sup>aa</sup>	5.90 <sup>ab</sup>	5.29 <sup>ac</sup>	4.98 <sup>ad</sup>	4.75 <sup>abe</sup>	4.71 <sup>abe</sup>	4.55 <sup>abe</sup>	4.55 <sup>ae</sup>
	Sucrose (g L <sup>-1</sup> )							
O	55.29 <sup>ba</sup>	37.10 <sup>bb</sup>	17.46 <sup>bc</sup>	11.30 <sup>cd</sup>	6.15 <sup>ce</sup>	6.00 <sup>ce</sup>	3.81 <sup>ce</sup>	3.90 <sup>be</sup>
OJ	57.87 <sup>aba</sup>	45.97 <sup>ab</sup>	28.43 <sup>ac</sup>	23.73 <sup>ad</sup>	17.53 <sup>ae</sup>	16.54 <sup>aeF</sup>	13.79 <sup>afG</sup>	11.39 <sup>ag</sup>
C	57.41 <sup>ba</sup>	38.21 <sup>bb</sup>	19.90 <sup>bc</sup>	18.18 <sup>bc</sup>	13.13 <sup>bd</sup>	10.56 <sup>bde</sup>	8.25 <sup>be</sup>	9.84 <sup>ade</sup>
CJ	60.23 <sup>aa</sup>	46.07 <sup>ab</sup>	28.39 <sup>ac</sup>	24.29 <sup>ad</sup>	16.66 <sup>ae</sup>	13.16 <sup>bf</sup>	11.74 <sup>af</sup>	12.46 <sup>af</sup>
	Glucose (g L <sup>-1</sup> )							
O	0.97 <sup>dc</sup>	5.06 <sup>bb</sup>	6.83 <sup>aa</sup>	6.86 <sup>aa</sup>	7.14 <sup>bcA</sup>	7.06 <sup>ba</sup>	6.83 <sup>ba</sup>	6.77 <sup>ba</sup>
OJ	2.3 <sup>bd</sup>	5.15 <sup>abc</sup>	6.96 <sup>ab</sup>	7.36 <sup>abAB</sup>	8.09 <sup>aa</sup>	8.08 <sup>aa</sup>	7.66 <sup>abAB</sup>	7.89 <sup>abAB</sup>
C	1.65 <sup>cd</sup>	4.90 <sup>bc</sup>	5.65 <sup>bb</sup>	6.02 <sup>bab</sup>	6.60 <sup>ca</sup>	6.57 <sup>ba</sup>	6.21 <sup>bab</sup>	6.75 <sup>ba</sup>
CJ	3.19 <sup>ae</sup>	5.69 <sup>ad</sup>	6.63 <sup>ac</sup>	6.71 <sup>ac</sup>	7.78 <sup>abAB</sup>	8.57 <sup>aa</sup>	8.02 <sup>abAB</sup>	7.36 <sup>bBC</sup>
	Fructose (g L <sup>-1</sup> )							
O	1.24 <sup>cd</sup>	12.17 <sup>ac</sup>	14.50 <sup>ab</sup>	15.06 <sup>abAB</sup>	15.85 <sup>aa</sup>	15.60 <sup>abAB</sup>	15.22 <sup>abAB</sup>	15.03 <sup>abAB</sup>
OJ	4.21 <sup>bcd</sup>	8.56 <sup>cc</sup>	10.96 <sup>bcB</sup>	11.66 <sup>bcAB</sup>	12.67 <sup>ca</sup>	12.84 <sup>ca</sup>	12.58 <sup>ca</sup>	12.87 <sup>ba</sup>
C	6.06 <sup>be</sup>	10.22 <sup>bd</sup>	12.23 <sup>bc</sup>	12.78 <sup>bBC</sup>	14.37 <sup>ba</sup>	14.32 <sup>ba</sup>	13.74 <sup>bcAB</sup>	14.48 <sup>aa</sup>
CJ	8.72 <sup>ad</sup>	8.70 <sup>bcd</sup>	10.90 <sup>cc</sup>	11.16 <sup>cc</sup>	12.70 <sup>cb</sup>	14.20 <sup>ba</sup>	13.99 <sup>bab</sup>	13.20 <sup>bab</sup>

<sup>1</sup>O, kefir with organic brown sugar; OJ, kefir with organic brown sugar and jaborcaba; C, kefir with conventional brown sugar; CJ, kefir with conventional brown sugar and jaborcaba. <sup>2</sup>Different lowercase letters in the same column indicate that there is significant statistical difference ( $p < 0.05$ ) between formulations. Different capital letters on the same line indicate that there is a statistically significant difference ( $p \leq 0.05$ ) between fermentation times.

Comparing the four formulations, in relation to total sugars, there was a reduction at the final time to approximately half the values found at the beginning of the process. At the end, the formulation with kefir fermented with organic brown sugar presented the lowest sugar content (25.7 g L<sup>-1</sup>) and the highest reduction (55%), statistically significant (Table 6).

A 350 mL portion of soft drink, relative to the contents of an usual can, provides, for example, an average of 37 g of carbohydrates from sugars, extrapolating the World Health Organization's recommendation (25 g) (WHO, 2015). On the other hand, the formulations analyzed in the present study resulted in values between 9.0 and 11.6 g 350 mL<sup>-1</sup>, acceptable values within these recommendations.

Laureys and Vuyst (2014), in a water kefir fermentation study, started an experiment with a sucrose value of approximately 50 g L<sup>-1</sup>, and it was almost completely consumed after 24 h of fermentation. Glucose and fructose presented an initial concentration of 14 g L<sup>-1</sup>, and fructose showed a maximum value of 25 g L<sup>-1</sup> up to 24 h, after which it started to decrease till its total consumption, in 72 h of fermentation. Glucose, on the other hand, showed reduced levels until near zero, with 48 h of total fermentation. This fact is in accordance with our work, since glucose amounts were lower than fructose. After being available through the hydrolysis of sucrose, the reducing sugars are subjected to the action of glycolytic enzymes, resulting in different metabolites, such as: ethanol, CO<sub>2</sub>, organic acids etc. The higher consumption of glucose in relation to fructose can be justified in part by the greater efficiency that yeasts have in using it as a substrate, which leads to being preferentially catabolized and inhibiting fructose breakage. Also due to its use by the bacteria producing the polymer for the formation of the kefir grains.

The results of this work are similar to those of Magalhães et al. (2010), who accompanied the fermentation of 21 samples of water kefir grains grown in 5% brown sugar solution at 25 °C. Total soluble solids ranged from 5.2 to 4.1 °Brix from the initial time to 24 h of fermentation equivalent to a 21% reduction. For sucrose, values ranged from 40 g L<sup>-1</sup> (0 h) to 28 g L<sup>-1</sup> (24 h), equivalent to a 30% reduction. We obtained 80% for the formulation with organic sugar (O) and 68% for conventional sugar (C), after 24 h and 93% and 83% after 56 h, respectively.

Also, in this work, the reducing sugars showed increasing concentrations, and the fructose content ranged from approximately 1.5 g L<sup>-1</sup> at the initial time to 5.5 g L<sup>-1</sup> (increase of 367%), and glucose, from 1.2 g L<sup>-1</sup> to 3.9 g L<sup>-1</sup> (325% increase) at the final 24 h fermentation time. This is expected, since during fermentation sucrose breakage occurs, with consequent formation of glucose and fructose. In our work, at a 24 h fermentation time, an increase was observed for fructose of 1115% (O), 177% (OJ), 111% (C) and 28% (CJ); and for glucose 609% (O), 216% (OJ), 265% (C) and 110% (CJ).

### Minerals

According to the ANOVA for the minerals analyzed in water kefir for four formulations in two times it was observed that there was a significant effect ( $p < 0.001$ ) for P, K, Mg, S, Cu, Mn and B, significant effect ( $p < 0.01$ ) for Ca and Cr, significant effect ( $p < 0.05$ ) for Zn and Ni and no significant effect for Na and Fe. In relation to the fermentation follow-up time, there were significant effects ( $P < 0.001$ ) for P, Mg, Na, Zn, Mn, B, Ni and Cr, significant effect ( $p < 0.01$ ) for Ca and Fe, significant effect ( $p < 0.05$ ) for Cu and non-significant effect for K and S. The product x time interaction had significant effect ( $P < 0.001$ ) for P, Mg, Mn, B and Ni, significant effect ( $p < 0.01$ ) for Cr, significant effect ( $p < 0.05$ ) for Cu, Fe and Zn and not significant for K, Ca, S and Na.

**Table 6:** Means for total sugars in water kefir, for four formulations, at the beginning and at final fermentation time.

Formulations <sup>1</sup>	Time			
	0h (g L <sup>-1</sup> )	56h (g L <sup>-1</sup> )	% Reduction	56 h (g 350 mL <sup>-1</sup> )
O	57.49 <sup>aA2</sup>	25.70 <sup>bB</sup>	55.30	9.0
OJ	64.40 <sup>bA</sup>	32.15 <sup>aB</sup>	50.09	11.3
C	65.12 <sup>bA</sup>	31.07 <sup>aB</sup>	52.28	10.9
CJ	72.15 <sup>aA</sup>	33.03 <sup>aB</sup>	54.22	11.6

<sup>1</sup>O, kefir with organic brown sugar; OJ, kefir with organic brown sugar and jaboticaba; C = kefir with conventional brown sugar; CJ = kefir with conventional brown sugar and jaboticaba. <sup>2</sup>Different lowercase letters in the same column indicate that there is significant statistical difference ( $p < 0.05$ ) between the formulations. Different capital letters on the same line indicate that there is a statistically significant difference ( $p \leq 0.05$ ) between the fermentation times.

The coefficients of variation of minerals varied from 3.86% for P to 21.68% for Cr. Higher values were observed for Ni (26.9%), Zn (29.7%) and Cu (40.15%), present in low concentrations in the evaluated products, with averages of 0.1277, 0.2403 and 0.2567 mg L<sup>-1</sup>, respectively (Table 7).

The values analyzed for minerals in water kefir for four beverages at 0 and 56 h are shown in Table 8. The levels of P, Ca and Na increased significantly in all four final beverages. P presented higher increases in the formulations with conventional sugar (C: 209% and CJ: 138%), compared to those containing organic sugar (O: 131% and OJ: 67%). For Ca, the results showed percent increases of 32 (CJ), 29 (O), 21 (C) and 17% (OJ). For Cr, there was a significant increase for C: 409%, CJ: 365% and OJ: 103%, but for O it was not significant.

For Na, Fe and B, no significant differences were observed between the 4 formulations in the final products. But there was an increase of 91 (OJ), 101 (C), 114 (CJ) and 201% (O) for Na. The Fe content did not change significantly when the organic sugar was used, but it had a decrease of 22 and 35% with the conventional sugar. These results show it is interesting to use organic sugar. Concerning B, there was a percentage decrease of 67 (C), 64 (CJ) and 34% (OJ) and an increase of 84% for O.

K and S contents did not undergo statistically significant changes until the end of the experiment in the four formulations. The same was observed for Mg and Zn, with

conventional sugar. Mg increased with organic sugar (O: 43% and OJ: 13%), while Zn presented reduction when organic sugar was used (O: 53% and OJ: 50%). For copper there was a significant reduction (62%) for OJ. For Ni, it presented a significant increase of 185% for C and 277% for CJ. For K, it is possible to observe that the formulations with jaboticaba presented statistically superior values, when compared with the same type of sugar. The minerals Mo and Pb were not detected in most of the replicates and Co and Cd were not detected in none of the replicates.

In all the samples evaluated, Na and Fe contents stood out. However, these and the other evaluated elements were classified below the recommended daily intake for a portion of 350 mL, except for Cr (Institute of Medicine, 2006). In general, Cr shows very low oral toxicity, and there are rare, well documented observations of oral toxicity in humans (Lovik et al., 2018). Cr is found, for example, in beer yeast. It plays a vital role in the proper functioning of insulin.

Therefore, it is implicated in mechanisms of action that lead to consequences on the metabolism of proteins, fats and carbohydrates, especially if there is scarcity in the body. Zhang et al. (2017) analyzed the influence of Cr on characteristics related to diabetes and lipid metabolism. They reported that rats fed a Cr-deficient diet generated pups that developed higher body weight, more adipose tissue and high serum levels of total cholesterol (TC) and triglycerides (TAGs).

**Table 7:** Analysis of variance (ANOVA) for thirteen minerals analyzed in water kefir, for four formulations, in two times.

Source of Variation	Mean Square <sup>1</sup>						
	P	K	Ca	Mg	S	Na	Cu
Formulations (F)	***	***	**	***	***	ns	***
Time (T)	***	ns	**	***	ns	***	*
F x T	***	ns	ns	***	ns	ns	*
Residue	0.37	8835	242.2	0.000	0.000	2.38	0.4952
Mean	15.68	449.23	120.76	46.98	38.63	17.76	0.2567
Coef. of variation (%)	3.86	20.92	12.89	15.1	14.04	8.69	40.15

Source of Variation	Mean Square					
	Fe	Zn	Mn	B	Ni	Cr
Formulations (F)	ns	*	***	***	*	**
Time (T)	**	***	***	***	***	***
F x T	*	*	***	***	***	**
Residue	0.344	0.7923	0.0005	0.00806	0.00122	0.00102
Mean	4.448	0.2403	0.3636	0.4973	0.1297	0.1659
Coef. of variation (%)	13.18	29.7	6.02	18.06	26.9	21.68

<sup>1</sup> ns, Not Significant at p>0.05; \* Significant at p ≤ 0.05; \*\* Significant at p ≤ 0.01; \*\*\* Significant at p ≤ 0.001.

**Table 8:** Means for thirteen minerals analyzed in water kefir beverages, for four formulations, in two times.

Formulations <sup>1</sup>	Mineral (mg L <sup>-1</sup> )		Formulations	Mineral (mg L <sup>-1</sup> )	
	0 h	56 h		0 h	56 h
P			Cu		
O	8.59 <sup>bb2</sup>	19.82 <sup>ba</sup>	O	0.669 <sup>aa</sup>	0.367 <sup>aa</sup>
OJ	13.55 <sup>ab</sup>	22.68 <sup>aa</sup>	OJ	0.362 <sup>abA</sup>	0.139 <sup>bb</sup>
C	6.37 <sup>cb</sup>	19.69 <sup>ba</sup>	C	0.117 <sup>ca</sup>	0.150 <sup>ba</sup>
CJ	9.58 <sup>bb</sup>	22.82 <sup>aa</sup>	CJ	0.235 <sup>bcA</sup>	0.153 <sup>ba</sup>
K			O		
O	423.6 <sup>ba</sup>	531.0 <sup>ba</sup>	O	3.74 <sup>aa</sup>	4.27 <sup>aa</sup>
OJ	730.3 <sup>aA</sup>	649.8 <sup>aA</sup>	OJ	5.09 <sup>aA</sup>	4.48 <sup>aA</sup>
C	217.0 <sup>ca</sup>	314.3 <sup>ca</sup>	C	5.14 <sup>aA</sup>	3.32 <sup>ab</sup>
CJ	298.9 <sup>bcA</sup>	420.4 <sup>bcA</sup>	CJ	5.22 <sup>aA</sup>	4.09 <sup>ab</sup>
Ca			Zn		
O	107.9 <sup>abB</sup>	138.7 <sup>abA</sup>	O	0.344 <sup>aa</sup>	0.163 <sup>bb</sup>
OJ	130.6 <sup>ab</sup>	152.9 <sup>aA</sup>	OJ	0.362 <sup>abA</sup>	0.182 <sup>abB</sup>
C	92.9 <sup>bb</sup>	111.9 <sup>ba</sup>	C	0.191 <sup>ba</sup>	0.179 <sup>abA</sup>
CJ	97.7 <sup>bb</sup>	129.3 <sup>ba</sup>	CJ	0.283 <sup>abA</sup>	0.253 <sup>aA</sup>
Mg			Mn		
O	27.50 <sup>cb</sup>	39.42 <sup>ba</sup>	O	0.356 <sup>ca</sup>	0.103 <sup>cb</sup>
OJ	39.56 <sup>bb</sup>	44.59 <sup>abA</sup>	OJ	0.507 <sup>aA</sup>	0.480 <sup>aA</sup>
C	49.88 <sup>aA</sup>	54.32 <sup>aA</sup>	C	0.354 <sup>ca</sup>	0.179 <sup>bb</sup>
CJ	56.31 <sup>aA</sup>	57.76 <sup>aA</sup>	CJ	0.417 <sup>bb</sup>	0.510 <sup>aA</sup>
S			B		
O	28.58 <sup>ca</sup>	31.62 <sup>ca</sup>	O	0.193 <sup>ca</sup>	0.351 <sup>aA</sup>
OJ	36.13 <sup>ba</sup>	33.47 <sup>ba</sup>	OJ	0.639 <sup>ba</sup>	0.424 <sup>ab</sup>
C	44.66 <sup>abA</sup>	40.67 <sup>abA</sup>	C	0.880 <sup>aA</sup>	0.287 <sup>ab</sup>
CJ	48.52 <sup>aA</sup>	42.03 <sup>aA</sup>	CJ	0.811 <sup>abA</sup>	0.292 <sup>ab</sup>
Na			Ni		
O	7.67 <sup>ab</sup>	23.13 <sup>aA</sup>	O	0.176 <sup>aA</sup>	0.128 <sup>bcA</sup>
OJ	12.41 <sup>ab</sup>	23.77 <sup>aA</sup>	OJ	0.105 <sup>abA</sup>	0.081 <sup>ca</sup>
C	11.66 <sup>ab</sup>	23.48 <sup>aA</sup>	C	0.082 <sup>bb</sup>	0.234 <sup>aA</sup>
CJ	11.68 <sup>ab</sup>	24.94 <sup>aA</sup>	CJ	0.052 <sup>bb</sup>	0.196 <sup>abA</sup>
Cr					
O	0.2175 <sup>aA</sup>	0.2267 <sup>aA</sup>			
OJ	0.1127 <sup>bb</sup>	0.2290 <sup>aA</sup>			
C	0.0460 <sup>bb</sup>	0.2343 <sup>aA</sup>			
CJ	0.0493 <sup>bb</sup>	0.2290 <sup>aA</sup>			

<sup>1</sup>O. kefir fermented with organic brown sugar; OJ. kefir fermented with organic brown sugar and jaborcaba; C. kefir fermented with conventional brown sugar; CJ. kefir fermented with jaborcaba. <sup>2</sup>Different lowercase letters in the same column indicate that there is significant statistical difference ( $p < 0.05$ ) between the formulations. Different capital letters on the same line indicate that there is a statistically significant difference ( $p \leq 0.05$ ) between the fermentation times.

Doddigarla, Ahmad and Parwez (2016) reported that supplements such as chromium picolinate reduce glycemia; TC and TAGs, raise high-density lipoprotein cholesterol and improve antioxidant capacity in diabetics by attenuation of insulin resistance. In addition to these characteristics, Huang et al. (2017) also cite the reduction of glycated hemoglobin. Gomes, Rogero and Tirapegui (2005) commented that Cr has been used as a dietary supplement in sports with the proposal to promote muscle mass gain and body fat loss, but proven effects were cases of improvements on lipid profile and type 2 diabetes, because Cr increases insulin sensitivity and inhibits cholesterol synthesis.

Magalhães et al. (2010) did not detect changes in minerals contents (0.2%), between initial and final fermentation time. Luchini et al (2017) compared different brown sugars in organic and conventional systems, and detected levels of Cu, Zn, Mn and Fe below that recommended by the World Health Organization.

Minerals and vitamins are activators for enzymes function, key pieces of biochemical reactions and enable body to perform its metabolic activities. Minerals are vital elements, naturally found in the soil and absorbed by vegetables, which are consumed by animals (Mahan; Escott-Stump, 2010).

Mineral nutrients are recognized as essential for health, accounting for about 5% of adult body weight. Calcium accounts for approximately 50% and phosphorus 25%. The other macro minerals are magnesium, sodium, potassium, chlorine and sulfur and the microminerals iron, zinc, iodine, selenium, manganese, fluorine, molybdenum, copper, chromium, cobalt and boron. The ultra trace elements (eg. Ni) constitute the remaining 25% (Mahan; Escott- Stump, 2010).

Among the chemical elements, hydrogen (H), oxygen (O), carbon (C), nitrogen (N), phosphorus (P), sulfur (S) and selenium (Se) are considered essential for microbial nutrition. P is required by the cell for the synthesis of nucleic acids, phospholipids, and adenosine triphosphate (ATP). It is usually supplied to the cell in the form of phosphate ( $\text{PO}_4^{2-}$ ).

Sulfur is present in the amino acids cysteine and methionine and in a number of vitamins such as thiamine, biotin, lipoic acid, and is usually supplied to the cell in the form of sulfate ( $\text{SO}_4^{2-}$ ). K is required for the activity of several enzymes, while Mg is required for the stabilization of ribosomes, membranes and nucleic acids, and is also necessary for the activity of many enzymes. The microbiological process of mineral formation is called biomineralization (Madigan et al., 2016).

Microorganisms require several metals for growth, usually in small amounts, and these are part of the micronutrients. Lactic bacteria are known for their various needs for these molecules, which even surpass the needs of humans. Among the microminerals, the main one is iron, which plays an important role in cellular respiration. Iron is an essential component of cytochromes and iron and sulfur-containing proteins involved in electron transport reactions (Madigan et al., 2016).

## Color

The ANOVA for five color-related characteristics (L \*, a \*, b \*, C \* and Hue) evaluated in water kefir for four formulations at eight times presented a significant effect ( $p < 0.001$ ) for all five characteristics studied, for all formulations, times and interaction formulation x time. The coefficients of variation obtained for the five studied characteristics related to color ranged from 0.83% to 3.97%, indicating excellent experimental precision (Table 9).

**Table 9:** Analysis of variance (ANOVA) for the color characteristics analyzed in water kefir, for four formulations, in eight times.

Source of Variation	Mean Square <sup>1</sup>				
	L	a *	b *	C *	Hue
Formulations (F)	***	***	***	***	***
Time (T)	***	***	***	***	***
F x T	***	***	***	***	***
Residue	0.0000	0.0619	0.00141	0.00134	0.23
Mean	28.34	6.57	11.21	13.15	57.48
Coef. of Variation (%)	1.12	3.79	3.97	3.73	0.83

<sup>1</sup> \*\*\* Significant at  $p \leq 0.001$

Color variation between the final and the initial products, evaluated by the total color difference ( $\Delta E$ ) between initial time (0 h) and final time (56 h), was 6.4, 8.53, 8.94 and 7.96 for the formulations O, OJ, C and CJ, respectively. There was influence of jaboticaba in color variation, comparing the results of jaboticaba containing beverages with those that did not contain (O x OJ and C x CJ), and the type of sugar was also important (O x C and OJ x CJ) (Table 10).

The averages of the characteristics related to the colors: L\*, a\*, b\*, C and Hue, evaluated in eight times (Table 11), showed a tendency to increase during the eight evaluated times. and that in the period from 0 to 8 h of fermentation the increase was more pronounced for all the characteristics. The formulations without jaboticaba presented values generally superior to those that contained the fruit, except for the characteristic a\*, where the formulation with conventional sugar showed lower values in almost all the times of evaluation.

**Table 10:** Comparison of total color differences ( $\Delta E$ ) between four formulations made by water kefir in eight different times.

Compared formulations <sup>1</sup>	O x OJ	C x CJ	O x C	OJ x CJ
Time (h)				
0	3.45	5.84	3.41	0.92
8	8.42	10.00	3.31	1.20
16	9.05	8.98	3.37	2.16
24	8.47	8.62	2.91	1.26
32	8.57	7.60	3.65	1.11
40	6.93	8.25	3.53	1.85
48	7.16	5.29	3.29	2.21
56	7.03	7.78	2.75	1.50

<sup>1</sup>O, kefir fermented with organic brown sugar; OJ, kefir fermented with organic brown sugar and jaboticaba; C, kefir fermented with conventional brown sugar; CJ, kefir fermented with organic brown sugar and jaboticaba.

**Table 11:** Means of characteristics related to water kefir color, for four formulations (F), in eight times.

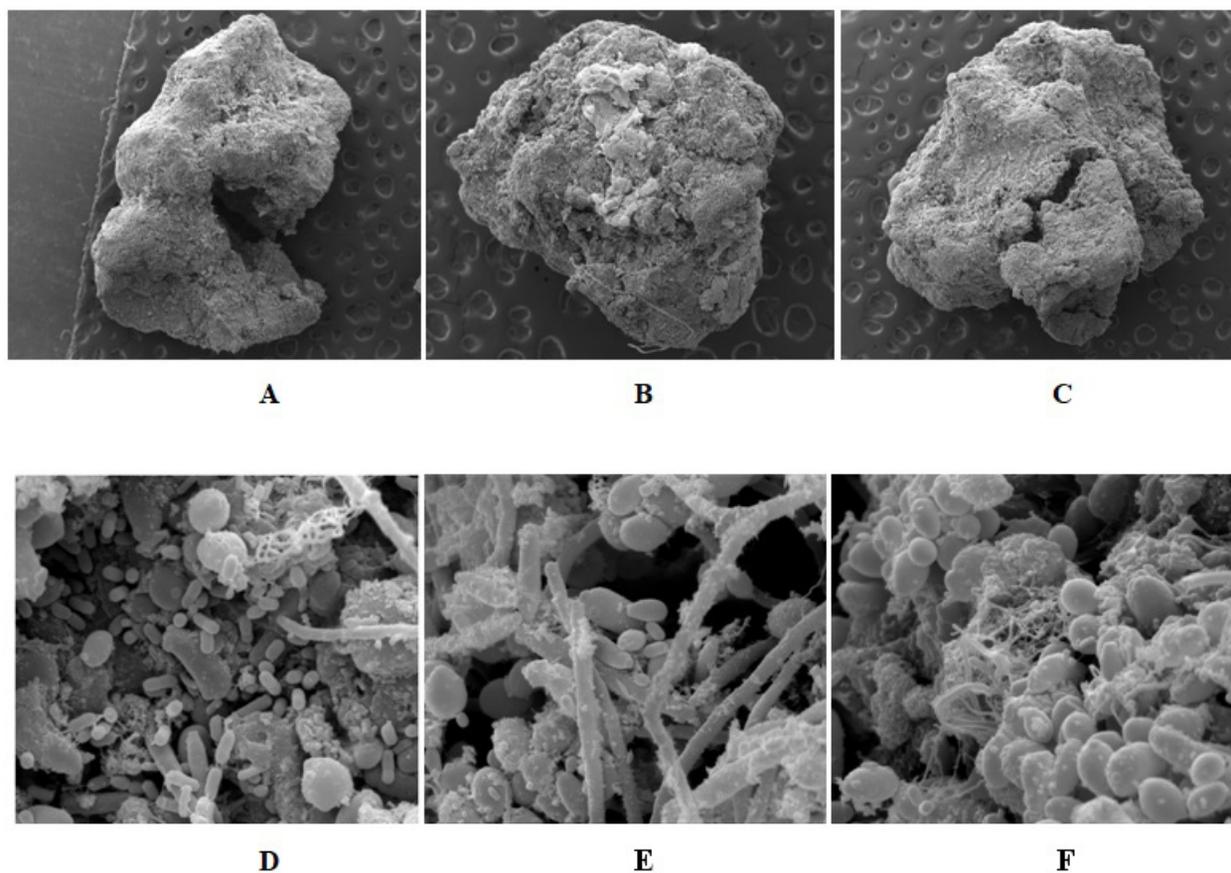
F <sup>1</sup>	** 0 h	8 h	16 h	24 h	32 h	40 h	48 h	56 h
Luminosity								
O	26.04 <sup>ba2</sup>	28.44 <sup>bb</sup>	29.93 <sup>bcd</sup>	30.07 <sup>bcde</sup>	30.33 <sup>bcde</sup>	29.83 <sup>bc</sup>	30.74 <sup>bde</sup>	30.84 <sup>be</sup>
OJ	24.78 <sup>cab</sup>	24.32 <sup>da</sup>	25.18 <sup>dbc</sup>	25.45 <sup>dcd</sup>	25.52 <sup>dcd</sup>	25.79 <sup>dde</sup>	26.38 <sup>def</sup>	26.57 <sup>df</sup>
C	28.36 <sup>aA</sup>	30.97 <sup>aB</sup>	32.23 <sup>aDEF</sup>	31.86 <sup>acde</sup>	31.22 <sup>abc</sup>	32.76 <sup>aef</sup>	31.63 <sup>abcd</sup>	32.91 <sup>af</sup>
CJ	25.43 <sup>bcA</sup>	25.40 <sup>cA</sup>	27.00 <sup>cBC</sup>	26.70 <sup>cB</sup>	26.41 <sup>cB</sup>	27.60 <sup>cCD</sup>	28.35 <sup>cE</sup>	28.00 <sup>cDE</sup>
a*								
O	4.73 <sup>aB</sup>	7.96 <sup>aA</sup>	7.76 <sup>aA</sup>	7.62 <sup>aA</sup>	7.71 <sup>aA</sup>	7.41 <sup>aA</sup>	7.56 <sup>aA</sup>	7.72 <sup>aA</sup>
OJ	3.52 <sup>cD</sup>	6.78 <sup>bC</sup>	7.03 <sup>bBC</sup>	7.15 <sup>abc</sup>	6.94 <sup>bBC</sup>	7.46 <sup>aAB</sup>	7.93 <sup>aA</sup>	7.97 <sup>aA</sup>
C	4.03 <sup>bcD</sup>	6.04 <sup>cA</sup>	5.29 <sup>cBC</sup>	5.38 <sup>bBC</sup>	4.81 <sup>cC</sup>	5.61 <sup>bAB</sup>	4.95 <sup>cC</sup>	5.92 <sup>bAB</sup>
CJ	4.08 <sup>bD</sup>	7.31 <sup>bAB</sup>	7.90 <sup>aA</sup>	7.29 <sup>aAB</sup>	6.62 <sup>bC</sup>	7.19 <sup>aBC</sup>	6.94 <sup>bBC</sup>	7.54 <sup>aAB</sup>
b*								
O	6.04 <sup>bC</sup>	14.27 <sup>aB</sup>	15.83 <sup>aA</sup>	15.72 <sup>aA</sup>	15.41 <sup>aAB</sup>	14.84 <sup>aAB</sup>	16.06 <sup>aA</sup>	15.69 <sup>aAB</sup>
OJ	3.07 <sup>cF</sup>	7.03 <sup>bE</sup>	8.16 <sup>bD</sup>	8.65 <sup>bCD</sup>	8.36 <sup>cD</sup>	9.22 <sup>bBC</sup>	10.40 <sup>cA</sup>	10.11 <sup>bAB</sup>
C	8.43 <sup>aD</sup>	15.21 <sup>aAB</sup>	15.76 <sup>aA</sup>	15.26 <sup>aAB</sup>	13.38 <sup>bC</sup>	15.67 <sup>aA</sup>	14.25 <sup>bBC</sup>	15.90 <sup>aA</sup>
CJ	3.39 <sup>cF</sup>	7.01 <sup>bE</sup>	8.95 <sup>bC</sup>	8.64 <sup>bC</sup>	7.78 <sup>cD</sup>	9.43 <sup>bBC</sup>	10.61 <sup>cA</sup>	10.08 <sup>bAB</sup>

Continue...

**Table 11:** Continuation.

F <sup>1</sup>	** 0 h	8 h	16 h	24 h	32 h	40 h	48 h	56 h
Chromaticity								
O	7.68 <sup>bb</sup>	16.43 <sup>aA</sup>	17.63 <sup>aA</sup>	17.48 <sup>aA</sup>	17.23 <sup>aA</sup>	16.56 <sup>aA</sup>	17.75 <sup>aA</sup>	17.48 <sup>aA</sup>
OJ	4.65 <sup>df</sup>	9.77 <sup>be</sup>	10.77 <sup>cd</sup>	11.22 <sup>bcd</sup>	10.89 <sup>ccd</sup>	11.88 <sup>bBC</sup>	13.06 <sup>cA</sup>	12.88 <sup>bAB</sup>
C	9.35 <sup>ad</sup>	16.35 <sup>aAB</sup>	16.63 <sup>aA</sup>	16.18 <sup>aAB</sup>	14.21 <sup>bc</sup>	16.65 <sup>aA</sup>	15.09 <sup>bBC</sup>	16.96 <sup>aA</sup>
CJ	5.29 <sup>cd</sup>	10.13 <sup>bc</sup>	11.95 <sup>bAB</sup>	11.30 <sup>bb</sup>	10.21 <sup>cc</sup>	11.85 <sup>bAB</sup>	12.71 <sup>cA</sup>	12.61 <sup>bA</sup>
Tone (Hue)								
O	51.97 <sup>bd</sup>	60.76 <sup>bc</sup>	63.85 <sup>bAB</sup>	64.15 <sup>bAB</sup>	63.43 <sup>bb</sup>	63.61 <sup>bAB</sup>	64.79 <sup>bA</sup>	63.86 <sup>bAB</sup>
OJ	41.21 <sup>cf</sup>	46.15 <sup>ce</sup>	49.24 <sup>cd</sup>	50.39 <sup>ccd</sup>	50.25 <sup>ccd</sup>	51.15 <sup>dBC</sup>	52.58 <sup>dA</sup>	51.72 <sup>dAB</sup>
C	64.58 <sup>ad</sup>	68.41 <sup>ac</sup>	71.45 <sup>aA</sup>	70.62 <sup>aAB</sup>	70.26 <sup>aAB</sup>	70.26 <sup>aAB</sup>	70.85 <sup>aA</sup>	69.60 <sup>aBC</sup>
CJ	39.57 <sup>df</sup>	43.98 <sup>de</sup>	48.36 <sup>cd</sup>	49.91 <sup>cc</sup>	49.61 <sup>cc</sup>	52.87 <sup>cb</sup>	56.80 <sup>cA</sup>	53.11 <sup>cb</sup>

<sup>1</sup>O. kefir with organic brown sugar; OJ. kefir with organic brown sugar and jабoticaba; C. kefir with conventional brown sugar; CJ. kefir with conventional brown sugar and jабoticaba. <sup>2</sup>Different lowercase letters in the same column indicate that there is significant statistical difference ( $p < 0.05$ ) between the formulations. Different capital letters on the same line indicate that there is a statistically significant difference ( $p < 0.05$ ) between the fermentation times.



**Figure 1:** SEM of kefir grains grown with brown sugar. A) Organic (60 fold increase). B) Organic and jабoticaba (80 fold increase). C) Conventional (60 fold increase). D) Organic (8.000 fold increase) E) Organic and jабoticaba. (8.000 fold increase) and F) Conventional (8.000 fold increase).

In relation to the Luminosity ( $L^*$ ), it showed a significant increase for all formulations from the 16 h of fermentation. So, they were tending to be clearer, being less pronounced for those that contained jaboticaba, since it presents the dark color from the fruit's peel. There was a significant difference among the beverages formulated with organic sugar and the conventional one, which presented higher values. The parameter  $a^*$  presented increase from the 8th h of fermentation, for O and CJ, demonstrating a tendency to increase the red tone specially in drinks containing jaboticaba, which presented almost double the initial value, in the final product. There was only significant difference in times 0 and 16 h between OJ and CJ, so there is no influence of the type of sugar, in this case.

The greatest increase observed in relation to color was in the coordinate  $b^*$ , which reflects the yellowish tone, and in beverages with jaboticaba these differences were smaller. The formulations containing organic sugar presented higher increase than those with conventional sugar. Color saturation ( $C^*$ ) increased from 8 h to 48 h. It was higher in the formulations with jaboticaba, but the type of sugar did not influence, since the beverages that contained it presented significant difference only in two times evaluated for two unique formulations.

### Scanning electron microscopy (SEM)

SEM was used as a complementary technique of qualitative determination from the observation of images (Figure 1) that evidenced the complex microbial association between bacteria and yeasts present in water kefir grains with irregular shapes and surfaces. We can observe that there is a difference in the grains that were developed with and without jaboticaba and, also, in the microorganisms present.

The images show the existence of yeasts and bacteria, distributed in an organized way, like a network interconnected through corridors of beings and their respective survival mechanisms, such as: development of biopolymers that provide fixation and adaptation to acidic means, and production of metabolites that interact synergistically with the various species present, for example.

Moinas, Horisberger and Bauer (1980), when evaluating water kefir grains images, observed that they consisted of an outer compact layer and an internal spongy structure. The outer layer was more densely populated by the microorganisms than the inner layer, but the dextran (polimer) was more abundant in the inner layer. Pidoux (1989) cultivated domestic sugar kefir grains from the Angers region of France, which were propagated at room temperature by daily transfer into drinking water

solution ( $60 \text{ g L}^{-1}$ ) and came to a similar conclusion. The exterior was covered by a network of blastospore bearing pseudomycelium and trapped among themselves or in bacterial colonies. He attributed these pseudomycelium to *Candida*.

Magalhães et al. (2010) evaluated water kefir grains incubated for 24 h at  $25^\circ\text{C}$  and observed a complex and compact network of biofilm and microorganisms outside the grain, while the interior was composed mainly of unstructured material. The microbiota in the outer portion was dominated by lemon-like yeast cells that were growing in association with long, curved bacteria.

### CONCLUSIONS

It is possible to produce beverages fermented by water kefir grains, using as substrate organic brown sugar or conventional brown sugar and jaboticaba pulp. The final beverages were acidic. The contents of total soluble solids and sucrose decreased, with minimum contents after 45 h of inoculation. The glucose and fructose contents increased, with maximum points at approximately 42 h. The total sugar contents had a reduction of about 50 % throughout the fermentation process. In relation to the variation in the mineral content of all formulations during the fermentation process, the following levels were increased: P, Ca and Mg (in formulations O and OJ), Na and Cr (in OJ, C and CJ formulations) and Ni (in formulations C and CJ); significant differences were not present in: K and S; (in formulations C and CJ), Zn (in formulations O and OJ), Mn (in formulations O and C), and B (in O, C and CJ). This variation may be due to the fact that the minerals have been assimilated by the kefir grain, in the case of reduction in the beverage, or were released from them in case their contents have increased. It is interesting to use organic sugar because the Fe was maintained and there was a decrease with the conventional sugar. The use of jaboticaba in the visual attribute of color is desirable, since it resulted in products with the most vivid color, less luminosity (less color lost), redder and less yellowish. This paper provides a basis for new developments related to water kefir as a functional, low-sugar, fermented beverage with the potential to replace soft drink consumption while promoting food sovereignty and security.

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