CUPUASSU AS A POTENTIAL SUBSTRATE FOR FERMENTATION USING KEFIR GRAINS

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ABSTRACT
Kefir grains is complex probiotic culture and in this study it was evaluated the production of fermented (by kefir grains) beverages using milk and cupuassu (Theobramagrandiflorum) pulpin different formulations (g/g). The obtained beverages were evaluated towards pH, soluble solids concentration (SS, °Brix), fermentation yield (Y, g/100g) and cellular growth (Δm, g/100g). It was observed that the higher cupuassu concentration the lower the pH reduction and yield and the higher the cellular growth. There was no significant difference among the replicates but there was a significant difference among the formulations. The beverages were stable during the refrigerated storage (4°C) for 28 days. The pH was established between 3.5 and 4.5 and the SS presented small variations. The cupuassu pulp proved to be a good potential for the production of fermented beverages using kefir grains, even without milk in the formulation.

Keywords: Cellular growth, Fruit pulp, Fermentation yield, Tukey test.

1. INTRODUCTION

Kefir is a fermented beverage with a light acid taste, effervescent and with low alcohol level all resulted from the metabolic activity of microorganism presented in the kefir grains, which is a complex and specific mixture of lactic bacteria and yeast in a polysaccharide matrix known as kefiran. This matrix has irregular grains shape with size between 0.5 and 3.5 cm and the color can vary according the microbiology composition, origin region and substrate using for the cultivation. The beverage obtained from the kefir fermentation is different from the others fermented milk because it contains carbonic gas and ethanol (Leite et al., 2013; Lopitz-Otsoa et al., 2006; StepaniaandFetlińsk, 2002; Weschenfelder et al., 2011).

The double fermentation that occurs in products using kefir produce beverages rich in acetic, formic, gluconic, lactic, propionic and succinic acids; carbonic gas, B vitamins, polysaccharides, different aldehyde, isomalic alcohol traits and acetone(Moreira et al., 2008; Weschenfelder et al., 2011). Due to its complex composition, the kefir fermented beverages are known in homemade medicine due to their probiotic effects, meaning live microorganisms which benefit health when they are ingested in appropriated amounts (Santos et al., 2012).

Kefir grains are very versatile because they can be cultivated in different medium as sugar solution, fruit juice and milk (Santos & Basso, 2013). There are many relates about different fruits used to give taste and flavor in fermented beverages with kefir, like: pineapple (Brandão et al., 2006), acai (Zoellner et al., 2009; Nogueira et al.,2016), acerola (Cruz et al., 2009), mangaba, siriguela (dos Santos, 2012), cocoa (Anastacio et al., 2013), soursop (Matos, 2009), mango (Santos et al.,2008), peach (Kempka et al., 2008), umbu (Santos et al., 2006), etc.

Brazil is a large country with different weather and it has great diversity of fruits that can be explored for the development of new products with the addition of new flavors. One of them is cupuassu (*Theobramagrandiflorum*), a very popular fruit in the Amazon and also well accepted in other regions of the country. Cupuassu is around 15 cm long by 10 cm of diameter and about 30 g/100g of it is pulp and it can be used in different food preparations like ice cream, juice, jam, sweet, yogurt, etc. The seeds can also be used to chocolate-type manufacture due to its similarity with the cocoa beans (da Silva Lannesand Medeiros, 2003).

The Brazilian legislation recommends minimum standards for cupuassu pulp: ascorbic acid (18mg/100g), pH (2.60), acidity (1.50 g citric acid/100g), total sugar (6.0g/100g) e SS (9°Brix). These specifications indicated that cupuassu is a fruit rich in ascorbic acid, sugar and organic acids and volatile esters (Brasil, 2000; Franco and Shibamoto, 2000). The high acidity and the great sugar concentration classified the cupuassu as a good substrate for kefir fermentation but so far it was not found any work in relation to this. However, kefir and cupuassu seeds have been applied in another type of product, a “chocolate” cake formulation with cupuassu powder as the substitute of cocoa powder and kefir as the substitute of industrial ferment (Esteller et al., 2006).

Therefore, the aim of this study was to evaluate the production of a mixed beverage with milk and cupuassu, in different formulations, fermented by kefir grains. The obtained beverages were evaluated in relation to their physical chemical proprieties in order to determine the feasibility of cupuassu as a substrate for a probiotic beverage such as kefir.

2. MATERIAL AND METHODS

2.1. Kefir culture and raw materials

The kefir culture used was kindly provided by the Bioprocess Laboratory of Zootecnic and Food Engineering Faculty of University of São Paulo, *campus* Pirassununga (São Paulo, Brazil). The substrates, UHT whole milk and frozen pulp of cupuassu, were purchased in supermarkets of Mogi Guaçu (São Paulo, Brazil).

2.2. Substrate preparation

The substrates for kefir cultivation were initially prepared using different proportions of UHT whole milk and defrosted cupuassu pulp as the following formulations (milk/cupuassu = g/g): F1 = 100/0; F2 = 90/10; F3 = 70/30; F4 = 50/50; F5 = 30/70; F6 = 10/90 and F7 = 0/100, which were defined based on a previous study using the same kefir culture, but with a different fruit, acai (Nogueira et al., 2016). The formulations were homogenized in a blender for 2 min to obtain homogeneous pastes. 100 g of each formulation, in triplicate, was weighed into glass flasks using a semi-analytical scale and the kefir grains were added in a concentration of 5 g/100g relative to the substrate and the fermentations were conducted in sequence.

2.3. Fermentation

After inoculation, the flasks were capped with towel paper to protect the substrate and to permit the release of gases. The flasks were kept at room temperature (~25°C) - in order to reproduce a home-made procedure - for 24 hours without agitation (Nogueira et al., 2016). The results obtained were compared using the Tukey test to verify if there were significant differences among the formulations evaluated and to select those that presented the best conditions for the culture growth and the best physicochemical characteristics. Additionally, the honestly significant difference (*HDS*) was calculated in order to determine which formulations were statistically different at 95% confidence. The calculation of *HDS* requires the constant value of the Tukey test (q)performed, the mean squares of the residue ($MS_{Residue}$) and the number of different trials (n) as it can be observed on Equation 1.

$$HDS = q\left(\frac{MS_{Residue}}{n}\right)^{0.5} \quad \text{(Eq. 1)}$$

After the first experiment, four formulations (milk/cupuassu = g/g) were selected: F1 (100/0), F3 (70/30), F5 (30/70) and F7 (100/0) to be evaluated with a higher number of replicates (n = 5). At this stage the data was again compared by Tukey test to check if there were significant differences among formulations.

All samples collected had the kefir grains separated from the fermented beverage with the aid of a sieve and both parts, the fermented beverage and the kefir grains, were physicochemically characterized as described below.

2.4. Storage

After the first experiments, the obtained fermented beverages (F1 to F7) were stored under refrigeration at 4°C in glass flasks for 28 days in order to assess the beverage stability as a function of the refrigerated storage time (Irigoyen et al., 2005); *pH* and the concentration of soluble solids

(*SS*) were evaluated right after fermentation (0 h) and after 28 days of storage. Similarly, at the second set of experiments, with the formulations F1, F3, F5 and F7, the fermented beverages were again stored under the same conditions described above and it was evaluated: *pH*, *SS*, titratable acidity and ash content.

2.5. Physicochemical characterization

The analytical methods used were based on the methodology described in the Manual of Food Analysis of the Adolfo Lutz Institute (IAL, 2008).The *pH* and the soluble solids concentration (*SS*, °Brix) were both performed by a direct measurement in a pH meter (Digmed, Model D20, São Paulo, Brazil) and in a portable refracto meter (Instrutemp, ITREF model 25, São Paulo Brazil), respectively. The mass (g) of kefir grains was measured at the beginning (m_{ko}) and the end (m_{kf}) of fermentation in a semi-analytical scale and were used to determine the cell growth (Δm, g/100g) according to Equation (2). The mass (g) of the substrate (m_s) and mass of fermented beverage (m_f) were also measured on a semi-analytical scale and were used to determine the fermentation yield (Y,g/100g) according to Equation (3).

$$\Delta m\left(\frac{g}{100\,g}\right) = 100\left[\frac{m_{kf}-m_{ko}}{m_{ko}}\right] \qquad \text{(Eq. 2)}$$

$$Y\left(\frac{g}{100\,g}\right) = 100\left[\frac{m_f}{m_s}\right] \qquad \text{(Eq. 3)}$$

The titratable acidity was measured with 5.0 mL of each sample volumetrically pipette and transferred to flasks of 250 mL containing distilled water and phenolphthalein (indicator),the samples were titrated with NaOH 0.1 mol/L until the appearance of a pink color. The consumption volume of the titrant was used to calculate the acid concentration in the samples expressed as g lactic acid/100 mL of fermented beverage. To determine the ash content, 5 mL of each sample were transferred to pre-weighed porcelain crucibles and were evaporated in an oven at 70°C and then calcined in a muffle at 550°C for 3 hours. The cooled samples were weighed on an analytical scale and the values used to determine the ash content in g/100mL.

3. RESULTS AND DISCUSSION

3.1. Evaluation of the fermentation parameters

The first set of experiments, performed with 7 different formulations (F1 to F7), resulted in values of *pH* (Fig. 1a), concentration of soluble solids (*SS*, Fig. 1.b), the fermentation yield (*Y*, Tab. 1) and the cell growth of kefir culture (Δm, Tab. 1).

Figure 1a shows the data obtained for *pH* before and after fermentation, and according to this figure, for all formulations there was a *pH* decrease during the fermentation time, which was expected since the formation of lactic acid by the lactic bacteria presented in the kefir culture. However, it is possible to see that, for the formulations with higher concentrations of cupuassu, the *pH* reduction was lower. This result can be explained because these formulations had already a low *pH* at the beginning of the process and they were not affected so strongly by the lactic acid produced during the fermentation. Similar behavior towards the *pH* was also observed by Nogueira et al. (2016) during the kefir fermentation of mixtures containing whole milk and acai, however, the *pH* values in the production of kefir fermented beverage of whole milk and cupuassu (3.4 to 4.8) were slightly lower than withacai (3.8 to 6.6).Weschenfelder et al. (2011) evaluated the kefir

fermentation of just milk and it was obtained *pH* varying from 3.59 to 3.79 after 24 h, also, at room temperature and 144 h of maturation at 7°C.

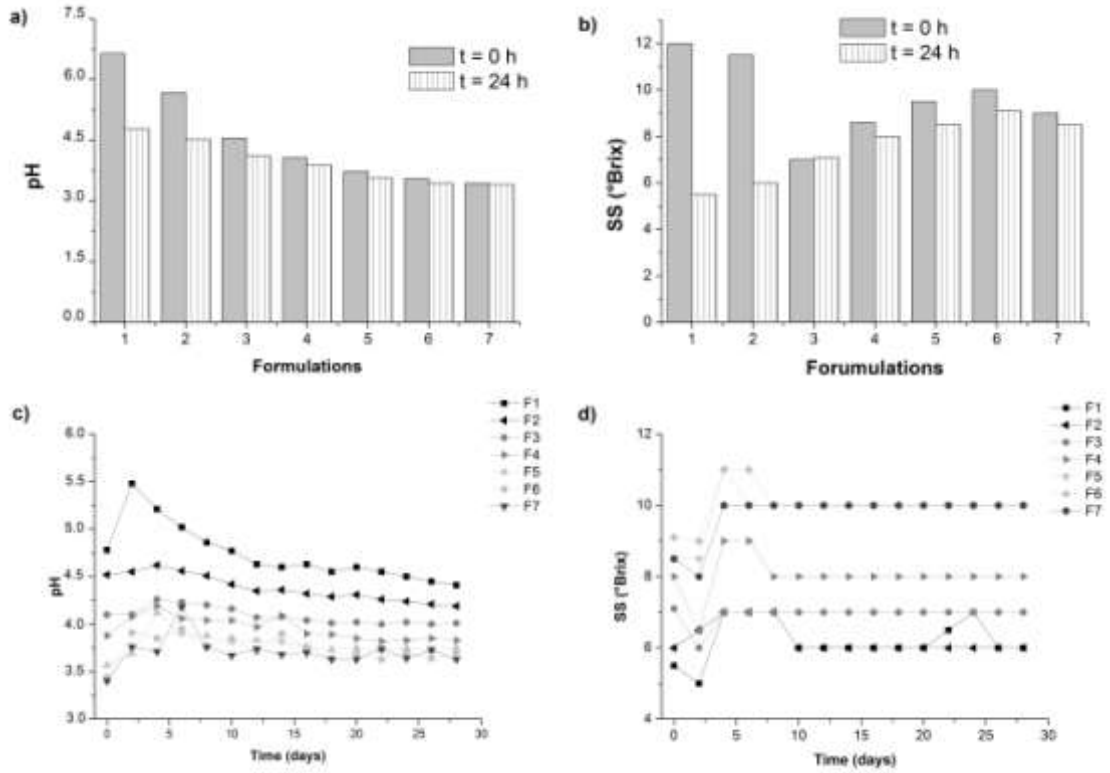

Figure 1.*pH* [**a)** and **c)**] and soluble solids concentration (*SS*, °Brix) [**b)** and **d)**] obtained for the fermented beverages with whole milk and cupuassu pulp in different concentrations [milk/cupuassu = g/g: F1 = 100/0; F2 = 90/10; F3 = 70/30; F4 = 50/50; F5 = 30/70; F6 = 10/90 and F7 = 0/100] and a kefir inoculum of 5 g/100g. The average values (3 replicates) for each formulation before (0 h) and after fermentation (24 h) are presented as bars [**a)** and **b)**] and the obtained values during storage at 4°C are represented as symbols and straight lines (to simply guide the eyes) [**c)** and **d)**].

Table 1. Average values (*n*=3 replicates) for the fermentation yield (*Y*, g/100g) and the cellular growth (*Δm*, g/100g) for beverages using whole milk and cupuassuin seven different formulations fermented by 5 g/100g of kefir at room temperature (~25°C), without agitation and for 24 h.

Formulations (milk/cupuassu= g/g)	***Y* (g/100g)***	***Δm* (g/100g)***
F1 : 100/0	$(91.34 \pm 2.31)^{a}$	$(43.11 \pm 6.05)^{d}$
F2 : 90/10	$(90.41 \pm 2.85)^{a}$	$(41.20 \pm 2.76)^{d}$
F3 :70/30	$(83.68 \pm 8.50)^{a.b}$	$(70.41 \pm 19.85)^{d.e}$
F4 : 50/50	$(84.86 \pm 3.25)^{a.b}$	$(36.41 \pm 8.74)^{d}$
F5 :30/70	$(77.10 \pm 8.75)^{b.c}$	$(69.25 \pm 7.55)^{d.e}$
F6 : 10/90	$(80.54 \pm 4.71)^{a.b.c}$	$(87.21 \pm 19.36)^{e}$
F7 :0/100	$(70.17 \pm 12.66)^{c}$	$(81.05 \pm 38.94)^{e}$
HSD (p = 0.05)**	11.76	31.77

*Conditions marked with different letters show significant differences at 95% of confidence (*p* = 0.05).

** HSD = Honestly significant difference

On Figure 1b the initial and final *SS* values revealed that the formulations with lower cupuassu concentration presented a greater decrease in *SS* than the ones with lower milk. The initial *SS* (basically sugars) presented in the substrate can be used for both cell growth and acid formation and comparing the data of the *pH* (Fig.1a), *SS* (°Brix, Fig. 1b) and the cellular growth (*Δm*, Tab. 1) it is possible to observe that in formulations with higher concentrations of milk the *SS* were more used for acid production than for the cell growth. Whereas for formulations with higher concentration of cupuassu, it was observed an inverse relationship: lower acid production and a greater *Δm*. This fact probably occurred due to the presence of monosaccharides (glucose and fructose) in the cupuassu pulp, which are easier assimilated by yeasts than the lactose from the whole milk.

The average values (triplicate) obtained for the fermentation yield (*Y*, g/100g) and *Δm*(g/100g) of each formulation can be seen in Table 1. From these data it was calculated the analysis of variance (ANOVA) for both responses (Table 2). This analysis allows determining the level of confidence in which it is possible to identify a significant difference among the replicates and the formulations (Rodrigues and Iemma, 2015).

Table 2. Analyze of variance (ANOVA) for the fermentation yield (*Y*, g/100g) and the cellular growth (*Δm*, g/100g) for beverages of whole milk and cupuassu pulp fermented (24 h/~25°C) by kefir in seven different formulations.

		Y **(g/100g)**				***Δm*** **(g/100g)**				
Source	**DF**	**SS**	**SM**	***F***	***p***	**DF**	**SS**	**SM**	***F***	***p***
Replicates	2	1465.78	732.89	2.54	0.12	2	236.67	118.34	2.99	0.09
Formulations	6	7687.53	1281.26	4.44	0.01	6	998.23	166.37	4.21	0.02
Residue	12	3460.37	288.36			12	474.3	39.52		
Total	20	12613.68				20	1709.2			

DF = degree of freedom, SS = sum of squares, SM = square means, F = F-test, p = significance level.

The ANOVA showed that, considering the significance level of 5% (p<0.05), there was no significant difference among the replicates conducted in the same conditions neither to *Y* nor to *Δm*. Regarding to the different formulations evaluated, it was noticed the opposite, *i.e.*, the *p*-values were lower than 0.05, indicating a significant difference among formulations considering 95% of confidence. The value of the honestly significant difference (*HSD*) to *Y* was calculated 11.76 g/100g and 31.77 g/100g for *Δm*.

The seven formulations were compared to each other, this information is also displayed in Table 1, wherein the samples labeled with different letters are statistically different at the confidence level used. The behaviors towards *Δm* and *Y* were similar to those observed in the fermentation of mixtures of milk and *açaí* with the same kefir culture by Nogueira et al. (2016).

In general, it is possible to see (Tab. 1) that there was a decrease in the yield of fermented beverage (*Y*) and an increase in cell growth (*Δm*) with the cupuassu concentration increase. This result is explained by the fact that a higher cell concentration leads to higher substrate consumption. In general, higher cupuassu concentrations were also more favorable to *Δm*(Tab. 1) probably because the lower pH, which is favorable to some species present in kefir grains but also due to the

fact that cupuassu is a richer source of monosaccharide which are fermentable by certain yeasts easier than lactose present in milk.

From the results obtained in this first set of experiments, four formulations (F1, F3, F5 and F7) were repeated, but with a larger number of replicates (n= 5). The average values for *pH* (Fig. 2a) and *SS* (Fig. 2b), before and after the fermentation, and the results for Y (Tab. 3) and Δm (Tab 3) besides the ANOVA for the responses Y and Δm (Table4) were equally obtained. According to these results, this second set of experiment was reproducible in relation to the first one. The *pH* and *SS* profiles (Figs.2aand 2b) showed similar behaviors among the analyzed conditions. According to Tables 3 and 4, as well as in the first experiment, there was no significant difference among the replicates and there was significant difference among formulations for both responses (p = 0.05). The trends of Y and Δm responses were also the same; the increase in the concentration of cupuassu led to an increase of Δm and, consequently, to a reduction in Y. This result suggests that cupuassu is a more favorable substrate for the development of the kefir culture used than whole milk under the evaluated conditions.

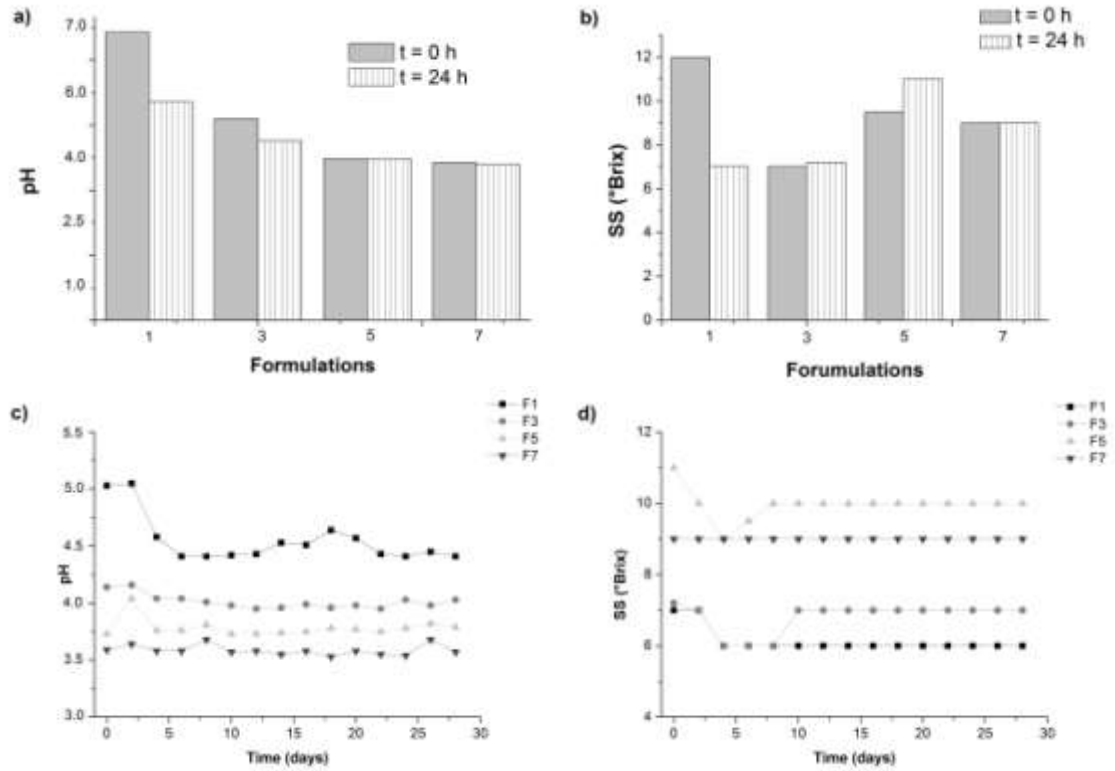

Figure 2.*pH* [**a)** and **c)**] and soluble solids concentration (*SS*, °Brix) [**b)** and **d)**] obtained for the fermented beverages with whole milk and cupuassu pulp in selected concentrations [milk/cupuassu = g/g: F1 = 100/0; F3 = 70/30; F5 = 30/70 and F7 = 0/100] with a kefir inoculum of 5 g/100g. The average values (5 replicates) for each formulation before (0 h) and after fermentation (24 h) are presented as bars [**a)** and **b)**] and the obtained values during storage at 4°C are represented as symbols and straight lines (to simply guide the eyes) [**c)** and **d)**].

Table 3. Average values (n = 5 replicates) for the fermentation yield (Y, g/100g) and the cellular growth (Δm, g/100g) for beverages using whole milk and cupuassu in four different formulations fermented by 5 g/100g of kefir at room temperature (~25°C), without agitation and for 24 h.

Formulations (milk/cupuassu = g/g)	***Y* (g/100g)***	***Δm* (g/100g)***
F1 : 100/0	$(89\pm2)^{a}$	$(37\pm6)^{c}$
F3 : 70/30	$(79\pm8)^{b}$	$(43\pm12)^{c}$
F5 : 30/70	$(83\pm3)^{a,b}$	$(44\pm8)^{c}$
F7 : 0/100	$(80\pm4)^{b}$	$(136\pm46)^{d}$
HSD (*p* = 0.05)**	6.9	75.8

*Conditions marked with different letters show significant differences at 95% of confidence (p = 0.05)

** HSD = Honestly significant difference

Table 4. Analyze of variance (ANOVA) for the fermentation yield (Y, g/100g) and the cellular growth (Δm, g/100g) for fermented beverages using milk,cupuassuand kefir.

		***Y* (g/100g)**				***Δm* (g/100g)**				
Source	**DF**	**SS**	**SM**	***F***	***p***	4	2330.3	582.6	0.98	0.45
Replicates	4	119.4	29.8	1.19	0.36	3	33451.9	11150.6	18.7	$<10^{-4}$
Formulations	3	294.7	98.2	3.93	0.04	12	7141.8	595.1		
Residue	12	299.4	24.9			19	42923.9			
Total	19	713.5				4	2330.3	582.6	0.98	0.45

DF = degree of freedom, SS = sum of squares, SM = square means, F = F-test, p = significance level.

3.2. Analysis of stability during refrigerated storage

The obtained fermented beverages (after 24 h of fermentation and without the kefir grains) were stored at 4°C and samples were collected each 2 days for *pH* and *SS* evaluation during the storage. Figures 1c and 1d show the behaviors of both responses for the first set of experiments. *pH* for all formulations showed different levels of variation along time; formulations with the highest initial *pH* had an increase in acidity while the formulations initially more acid showed a reduction. After 14 days of storage *pH* almost did show any variation indicating a microbiological stability of the beverage. All formulations achieved balance between *pH* values of 3.5 and 4.5, depending on the formulation. The stability of *pH* in this range is very interesting due to the fact that *pH*< 4.5 does not allow the growth of some pathogenic microorganisms, so, the obtained products presented a good conservation during storage. Towards *SS* there was some variation in the beginning of storage time, but after 10 days practically there was no change. These results were very similar to Irigoyen et al. (2005) who determined the stability not just based on pH and SS but also in relation to lactose and fats during 28 days of refrigerated storage.

In the second set of experiment, with 5 replicates, the *pH*(Fig. 2c) and *SS* (Fig 2d) presented similar results in relation to the first set of experiment. In relation to the titrable acidity (Table 5), the acidity of F1 (only milk) after fermentation was much higher than before but considering F7 (only cupuassu) the acidity (2.08 g/100mL) is moreclose to the cupuassu pulp before the

fermentation (1.5 g/100mL in citric acid which is equivalent to 2.1 g/100mL in lactic acid). The titrable acidity are also in accordance to the *pH* profiles (Figures 1a, 1c, 2a and 2c). Weschenfelder et al. (2001) obtained a kefir fermented beverage of milk with acidities ranging from 1.42 to 2.67 g lactic acid/100 mL.

Table 5. Titrable acidity (g lactic acid/100g) and ash (g/100g) content, for beverages of whole milk and cupuassu in selected formulations fermented by 5 g/100g of kefir at room temperature (~25°C),after fermentation (t = 0 day) and after refrigerated storage at 4°C (t = 28 days).

	Titrable acidity (g lactic acid/100g)		**Ash (g/100 g)**	
Formulations (milk/cupuassu= g/g)	**t = 0 day**	**t = 28 days**	**t = 0 day**	**t = 28 days**
F1 :100/0	(0.63±0.02)	(0.86±0.02)	(1.00±0.03)	(0.78±0.09)
F3 :70/30	(1.08±0.02)	(1.56±0.03)	(0.90±0.00)	(0.70±0.01)
F5 :30/70	(1.76±0.05)	(1.98±0.06)	(0.91±0.06)	(0.49±0.08)
F7 :0/100	(2.08±0.19)	(2.07±0.02)	(1.05±0.19)	(1.56±0.20)

The ash content (Tab. 5) was relatively higher and it was possible to observe that formulations with higher cupuassu concentration showed higher ash content. de Souza Pereira et al. (2013) obtained 0.13g/100g of minerals in cupuassu pulp, meanwhile just 4 minerals were evaluated (Cu, Fe, Zn and Mn). Costa (2006) observed that cupuassu is very rich in potassium, which can increases the ash content of the product. Baú et al. (2014) produced a kefir fermented beverage with soybean aqueous extract with and without soybean fibers, the ash content obtained by these researchers (2.30 and 1.69 g/100g) were similar to the ones obtained in this study.

4. CONCLUSION

The production of a kefir fermented beverage with whole milk and cupuassu pulp in different formulations showed that the fermented beverages resulted in physicochemical characteristics with similar behaviors and small variations among the replicates. In general, when more cupuassu pulp was applied in formulation, higher values of cellular growth, soluble solids concentration, titrable acidity and ash content were obtained which means a differentiated product in relation to the traditional kefir fermented milk beverages. Therefore, cupuassu pulp, an "exotic" fruit, is a potential substrate for the development of a new probiotic beverage.

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