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Using *Moringa oleifera* Lamarck seed extract for controlling microbial contamination when producing organic cachaça

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A R T I C L E I N F O

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A B S T R A C T

This study investigated the use of seed extract from *Moringa oleifera* Lamark (MO) for both clarifying the sugarcane juice and removing fermentation contaminants that originated during the 24 h storage of the freshly cut stalks. The addition of the MO seed extract during the juice clarification step decreased the total microbial population by 41.66% compared to the other treatments. The non-stored raw material had less lactic acid bacteria and yeasts counts in the fermentation process, resulting in 8.8% lower glycerol content compared to the stored raw material. The concentrations of congeners in both raw materials decreased by 5.27% after liming and by 10% after using the natural extract with flocculation activity. It can be concluded that the clarification process combined with the use of the seed extract from *Moringa oleifera* Lamarck can potentially control microbial contaminating during fermentation and increase the alcohol content in the cachaça.

1. Introduction

Cachaça is a distilled spirit made from fermented sugarcane juice, produced exclusively in Brazil (BRASIL, 2014). Cachaça corresponds to approximately 50% of the distillates consumed in Brazil, making it the most consumed spirit in the country and ranking third as the most consumed distilled beverage in the world (IBRAC, 2015; SEBRAE, 2016). Consumption and search for quality products that meet the food safety requirements according to national and international standards have been increasing. Therefore, there is a need for optimizing ethanol production processes to meet consumers' increasing demands.

Several bacteria of the genus *Bacillus* and *Lactobacillus* as well as contaminating yeasts such as *Schizosaccharomyces*, *Debarymomyces*, *Candida*, *Pichia*, and *Kloeckera* can be found in the raw material, sugarcane juice. At levels above 10^8 CFY/mL, these microorganisms are undesirable since they consume the fermentable sugars (e.g., sucrose, fructose, glucose) present in the sugarcane juice to produce compounds (e.g., lactic acid, higher alcohols) that are detrimental to the quality of cachaça (BRASIL, 2014). In addition, wild yeasts have low multiplication rates, low ethanol tolerance, and low fermentation efficiency (Alcarde, 2014; Amorim, 2005).

Contamination of sugarcane juice starts at harvesting, when sugarcane stalks can be colonized by yeasts and bacteria present in the environment (e.g., soil, harvesting equipment), beginning the deterioration process by forming undesirable compounds and by increasing the numbers of microbial contaminants (Cardoso, 2013). Within hours (3–6 h), total fermentable sugars and pH decrease while juice acidity increases (Krishnakumar et al., 2013).

Juice clarification is a process where the sugarcane juice is heated to 105 °C for a short residence time in the presence of a clarifying agent or flocculant (e.g., lime, synthetic polymers) for the removal of impurities, such as organic acids, gums, lipids, inorganic compounds, among others (Albuquerque, 2016). However, the increasing demand for organic cachaça is driving researchers to introduce greener alternatives for juice clarification. *M. oleifera* Lamark, with flocculation activity similar to synthetic polymers, is a green and economic promising alternative (Costa et al., 2015; Macri et al., 2014). In addition to flocculant activity, the MO seeds have proteins with antifungal and antibacterial activity. These proteins belong to the 2S family of albumins, are stable to pH change, and have a 98 °C melting point. (Ullah et al., 2015; Freire et al., 2015).

This research evaluated the use of Moringa seeds as a potential "green" clarifying agent during sugarcane juice clarification and its

Abbreviations: MO, *Moringa oleifera* Lamark seed extract; RS, reducing sugars; TRS, total reducing sugars; POL, polarization; RRS, total residual reducing sugars; CFU, colony-forming units

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antimicrobial properties for controlling microbial contaminants during the fermentation of clarified sugarcane juice to cachaça freshly harvested stalks. The chemical composition of cachaça was also evaluated following regulations set by Normative Instruction N° 28 (BRASIL, 2014). It is noteworthy to mention that this is the first work of its kind using *Moringa oleifera* Lamarck for clarifying and controlling microbial contaminants in sugarcane juice for the production of cachaça.

2. Materials and methods

2.1. Moringa seed extract preparation

The Moringa (MO) seed extract *Moringa oleifera* Lamarck was prepared according to Okuda et al. (2001). Briefly, the MO seeds were collected in Taquaritinga, São Paulo, Brazil in a 0.1 mol/L $CaCl_2$ solution. at the 1 g of seed to 100 g solution ratio.

2.2. Juice extraction and clarification and fermentation broth preparation

The experiment was carried out in 2015/2016 harvest at the Sugar and Alcohol Technology and Fermentation Microbiology Laboratory of the Technology Department of the FCAV/UNESP in Jaboticabal, São Paulo, Brazil. Sugarcane variety CTC8 was grown for 12 months in the Sertãozinho region in São Paulo, Brazil and harvested manually, with previous de-topping and no-burn straw removal. Whole stalks were separated into two groups for juice extraction and clarification: (1) freshly cut stalks and promptly processed; and (2) stalks stored in a greenhouse at controlled temperatures (24–36 °C) for 24 h and then processed.

The juices were extracted by milling the stalks through a 3-roll mill and the juice filtered through a 60-mesh porosity nylon membrane filter. The filtered juice was analyzed for Brix, Reducing Sugars (RA), Total Reducing Sugars (TRS), Total Acidity, Polarization (Pol), Purity (CTC, 2005) and pH were determined.

The soluble solids and pH of the filtered juices were adjusted to 16° Brix with water and pH 6.0 with calcium hydroxide (6° Bé), respectively. Sugarcane juices were clarified with lime (calcium hydroxide) or with lime more MO extract. After liming, the juice was heated to a boiling point, cooled to 80 °C, and transferred to decanters, remaining for 2 h. The decantation occurred in 20 min for the moringa extract treatment at the dosage of 1300 mg/L. Total acidity, TRS (CTC, 2005) and pH of the clarified juices were determined as previously described.

Fermentation broths were prepared by adjusting the Brix of clarified sugarcane juices to 16° Brix with distilled water and by heating the broth to 32 °C, The pH of the broths were kept at 6. The total acidity, total reducing sugars (TRS) (CTC, 2005), and pH of the fermentation broths were determined as well.

2.3. Inoculum preparation

Saccharomyces cerevisiae CA-11 was obtained from Latin American LNF. It was prepared by hydrating the lyophilized cells, adapting to the broth and multiplying, according to the producer recommendation. Microbiological evaluations were carried out to monitor yeast viability and multiplication. Cells were centrifuged at 2500g/30 °C for 5 min and maintained in 0.75% (w/v) saline solution and stored at refrigerator until the beginning of the fermentation process.

2.4. Fermentation process

The fermentation process was carried out in a 6-L stainless steel tank with conical bottom containing 1.5 L of inoculum (cell concentration at 10^7 CFY/mL) and 4.5 l of fermentation broth. All fermentations were carried out for no > 12 h or until fermentable sugars concentrations reached ≤1°Brix.

2.5. Microbiological evaluation

The microbiological analyses were performed by the direct method during the fermentation process (inoculum, beginning, and end) to evaluate viability of yeast cells, bud rate and viability (Ceccato-Antonini, 2010).

Microbial counts were carried out on various media by the plating technique on the extracted sugarcane juice, fermentation broths and fermentation process. MRS was used for the quantification of lactic acid bacteria, WLN (with added ampicillin and nalidixic acid at 50 mg/L each) for yeasts and total yeasts determination, and PCA for total microbial counts (Ceccato-Antonini, 2010).

2.6. Broth after fermentation analysis

The Total Residual Reducing Sugars (TRRS), glycerol (CTC, 2005) and alcohol (digital densimeter) contents of the broths post fermentations were determined at the end of the fermentation process.

2.7. Distillation

The broths post fermentations siphoned from the side of the tanks without the need for centrifugation. At the end of fermentation, between 3.5 and 4 L of these broths after fermentation were added to a 10-L copper alembic and distilled by direct fire. The distilled products were separated into the 10% head, 80% heart, and 10% tail fractions. A composite sample from the heart fractions (distillates) was prepared and its chemical composition analyzed by high performance liquids chromatography (HPLC) and gas chromatography (GC) as described below.

2.8. Determination and characterization of distillates

The components of cachaça samples were identified and quantified in a gas chromatograph GC Varian 3900 coupled with a Galaxie Chromatography software and a Varian Capillary Column CP - Wax 52 CB (L = 30 m, Di = 0.53 mm, Ef = 1 μm CP8738. A 1 μL aliquot from each sample was injected at 175 °C injector temperature and at 210 °C detector temperature, with synthetic air as the carrier gas for the determination and quantification of acrolein, aldehyde, esters, methanol, and higher alcohols (propyl, isobutyl, and isoamyl).

Ethyl carbamate was determined by HPLC according to Anjos et al. (2011) using a Shimadzu chromatograph, equipped with two high-pressure pumps (model SPD-M20A), degasser (model DGU-20A3), interface (model CBM-20A), and automatic injector (model SIL-10AF).

The alcohol content, volatile acidity (BRASIL, 2005), conductivity, turbidity (INMETRO, 2005), copper levels (Instituto Adolfo Lutz, 2005), and pH of the distillate were determined as well.

2.9. Experimental design

The experimental design consisted of 3 main treatments (juice "in natura" (without clarification), liming clarified juice, and juice clarified using the seed extract of *Moringa oleifera* Lamarck as flocculant) and three replicates. The secondary treatment consisted of two raw materials, freshly harvested stalks and stalks stored for 24 h.

2.10. Statistic analysis

The results were submitted to analysis of variance by F test and the means were compared by Tukey test (5%) using the AgroStat software as described by Barbosa and Maldonado Junior (2015).

2.11. Key resources table

Resource	Source	Identifier
Acids		
pH	N/A	N/A
Total acidity	N/A	N/A
Alcohol		
Alcohol content	N/A	N/A
Chemical		
acrolein		
ampicillin		
CaCl2		
calcium hydroxide		
copper		
Ethyl carbamate		
glycerol		
lactic acid		
methanol		
nalidixic acid		
propyl		
Coefficient of congeners		
Alcohol sec. butyl	N/A	N/A
Furfural	N/A	N/A
Higher Alcohols	N/A	N/A
Hydroxymethyl	N/A	N/A
N-butanol	N/A	N/A
N-propanol	N/A	N/A
Total aldehydes	N/A	N/A
Total esters	N/A	N/A
Volatile acidity	N/A	N/A
Inoculum		
CA-11	N/A	N/A
Statistic Analysis		
AgroStat	N/A	N/A
Sugars		
Polarization (POL)	N/A	N/A
Reducing sugars	N/A	N/A
Total reducing sugars	N/A	N/A
Total residual reducing sugars	N/A	N/A

3. Results and discussion

3.1. Extracted juice chemical characterization

Brix, pH, TRS, RS, total acidity, Pol and Purity results of the juices from the two sugarcane materials (freshly processed stalks and 24 h stored stalks) are shown in Appendix Table A.1. No significant differences ($p < 0.05$) were observed from the extracted juices with average results of 19.5°Brix, pH 5.22, 16.4% TRS, 0.22% RS, 1.02 g/L H_2SO_4 Total Acidity, 16.78% Pol, and 85.82% Purity.

The results allow to conclude that both extracted sugarcane juices were suitable for processing due to their high values of 16° Brix, 14% Pol, 15% TRS, Purity above 85%, and low values of 0.8% RS and 0.8 g H_2SO_4/L total acidity (Ripoli and Ripoli, 2009).

Oliveira Filho et al. (2016) reported a significant Brix increase in sugarcane stalks after a 24 h storage at 30 °C while Krishnakumar et al. (2013) observed a pH reduction from 5.72 to 3.77. The effect of the increase of the Brix and reduction of the pH is result of the deterioration of the sugarcane, the evaporation of the water increase the Brix and the multiplication of the microbial contamination produce acids and reduced the pH of the extracted juice (Alcarde, 2014).

Unlike the present study, in which TRS decreased by 11.38% and purity by 3.54%, although not significantly. This behavior may be explained by storage at higher temperatures in both studies. Lower storage temperatures result in an environment less favorable to the multiplication and action of the contaminants on the raw material.

3.2. Effect of clarification on microbial counts

The microbiota of the extracted juice (Table 1) was significantly different ($p < 0.05$) after 24 h storage with counts of lactic bacteria increasing by 17.56%. Although, total microbial counts and yeast counts did not differ significantly, but they increased by 18.10%; whereas, yeast counts decreased by 5.54%.

The changes observed in the microbial population resulted from the interaction of the microorganisms already present in the plant ecosystem (Madigan et al., 2016) with the sugars present in the stalks, causing the stalks and the fermentable sugars to deteriorate (Cardoso, 2013).

This interaction becomes detrimental to the quality of the raw material since the increasing contaminants in the stalks affect the fermentation process and, consequently, the quality of the final product because these microorganisms metabolize the sugars to produce compounds that harm the chemical-sensory characteristics of the distillate (Alcarde, 2014).

Microbial counts for the fermentation broths are summarized in Tables 2 and 3. The comparison between 'in natura' and clarified juices indicated a significant mean decrease of 33.96% of the contaminants in all cultivated media. This behavior is attributed to the clarification process since the heating and precipitation of impurities removed the microbiological contaminants present in the juice, benefiting the process.

The moringa seed extract used to clarify the juice (clarified juice + MO) significantly reduced the total microbial counts (41.66%) compared to liming, proving its potential antimicrobial activity, as reported by Ullah et al. (2015), and resulting in a more efficient fermentation process.

The results from the processed raw material (Table 2) show that the contaminants increased significantly in the stored stalks (Table 1), 23.06% on average, for all evaluated microorganisms.

Table 1
– Total microbial counts, lactic acid bacteria and yeast counts of extracted sugarcane juice from stalks stored for 0 h or 24 h at 24–36 °C. Analysis of variance and comparison of means by Tukey test.

	Total microorganisms (CFU/mL × 10^5)	Lactic acid bacteria (CFU/mL × 10^5)	Yeast (CFU/mL × 10^4)
0 h	5.58A	5.58B	4.87A
24 h	6.59A	6.56A	4.60A
F test	5.69 ns	7.40	0.68 ns
MSD	1.03	0.87	0.80
CV	9.83	8.34	9.76

Significant at 1% ($p < 0.01$); Significant at 5% ($0.01 < p < 0.05$); ns = non-significant; CV = Coefficient of variation; MSD = Minimum significant difference.

Table 2
– Total microbial counts, lactic acid bacteria and yeast of the fermentation broths showing analysis of variance and comparison of means by Tukey test.

	Total microorganisms (CFU/mL × 10^5)	Lactic acid bacteria (CFU/mL × 10^5)	Yeast (CFU/mL × 10^4)
Juice treatment (T)			
"in natura" juice	6.00A	5.99A	5.04A
Clarified juice	4.25B	3.89B	3.12B
Clarified juice + MO	3.50C	3.70B	2.84B
F test	54.46**	87.00**	8.31**
MSD	0.68	0.53	1.63
CV	10.72	8.50	31.99
Raw material (M)			
0 h	4.27B	4.09B	3.16B
24 h	4.89A	4.97A	4.18A
F test	17.25**	15.54**	8.92*
MSD	0.33	0.50	0.77
CV	8.00	11.99	22.80
Inter. TxM	3.30 ns	4.33*	0.30 ns

** Significant at 1% ($p < 0.01$).
* Significant at 5% ($0.01 < p < 0.05$); ns = non-significant; CV = Coefficient of variation; MSD = Minimum significant difference.

Table 3
– Lactic acid bacteria present in the fermentation broth for the interaction of juice x raw material treatment.

	0 h	24 h	
Juice "in natura"	5.98aA	6.01aA	0,87
Clarified juice	3.40bB	4.39bA	
Clarified juice + MO	2.90bB	4.51bA	
	0.86		MSD

Columns classified with lowercase letters; rows classified with uppercase letters.

The interaction results of the treatments (Table 3) for both raw materials used show that the clarification significantly removed the lactic acid bacteria; however, even with the treatment, the stored raw material entails a greater contamination by these bacteria.

The viable alternative for treating a poor-quality raw material is to use moringa seed extract as a flocculant in the clarification process for providing a greater reduction of the microorganisms and a shorter decantation time; contributing to produce organic cachaça. Proving the reducing of the contaminations, another research can be done with different concentrations and correlation the clarification effect with antimicrobial activity to find the better concentration for both effect.

3.3. Juice clarification

The pH, total acidity and TRS results for the clarified juices are shown in Appendix Table A.2. The pH and total acidity results show that moringa seed extract did not affect acid removal from the juice since these values were similar in the treatments, with or without moringa extract.

The TRS results were not significantly different for clarification with or without the moringa seed extract. The results were 14.25% for clarification without the coagulant and 14.26% for clarification with moringa seed extract, this indicating no loss of sugars in the sedimentation of the sludge. A very important step of the process, decantation should not remove the sugars to be metabolized, this process needs remove the compounds that affect negatively the process. Likewise, Costa et al. (2014) reported that the use of the moringa seed extract did not reduce the TRS in the clarification process used limed juice at pH 7 and moringa seed extract at the concentration of 5 mg/L.

The used raw materials (Appendix Table A.2) were not significantly different regarding the presence of acids and did not affect significantly the pH and total acidity results. However, total reducing sugars (TRS) of the stored raw material was significantly lower 12% after the 24-hour storage.

This result impacted the process yield negatively since the sugars lost could be metabolized into alcohol for cachaça, besides the production of undesirable compounds by the increasing contaminating microorganisms, as shown in Table 1, the increase of lactic bacteria with the raw material after 24-hour storage can be the fact of this decay of sugars.

Similarly, Krishnakumar et al. (2013) reported a reduction of 23.63% of the total reducing sugars. This result impacts the next stages of the process, such as the total reducing sugars of the broth, the alcoholic content of the broths post fermentations, and the secondary compounds of cachaça.

3.4. Chemical composition of fermentation broths

The pH and total acidity results of fermentation broths (Table 4) were not significantly different ($p < 0.05$) for the juice treatments and the raw materials used, which is a recommended result for the clarification process (Amorim, 2005). However, Krishnakumar et al. (2013) reported a pH reduction from 5.72 to 3.77 for stalks stored for 24 h at 30 °C.

The total reducing sugars (TRS) results show that the juice clarification process did not remove sugars, but the storage of cane stalks for 24 h decreased the TRS by 12.38%. Krishnakumar et al. (2013) under similar conditions reported a 23.63% decrease of the total reducing sugars. This reduction results from the metabolization of these sugars by the higher number of contaminating microorganisms present in the stalks, as shown in Table 1, making storage undesirable.

Table 4
– pH, total acidity and TRS of the fermentation broths showing analysis of variance and comparison of means by Tukey test.

	pH	Total acidity (g/L H_2SO_4)	TRS (%)
Juice treatment (T)			
Juice "in natura"	5.28A	0.91A	13.97A
Clarified juice	5.58A	0.64A	13.38A
Clarified juice + MO	5.61A	0.75A	13.77A
F test	4.42 ns	4.20 ns	0.17 ns
MSD	0.37	0.28	3.08
CV	3.84	20.72	12.70
Raw material (M)			
0 h	5.62A	0.70A	14.61A
24 h	5.35A	0.83A	12.80B
F test	3.72 ns	1.55 ns	7.81
MSD	0.33	0.26	1.59
CV	5.33	30.04	10.06
Inter. TxM	0.69 ns	0.66 ns	0.44 ns

Significant at 1% ($p < 0.01$); Significant at 5% ($0.01 < p < 0.05$); ns = non-significant; CV = Coefficient of variation; MSD = Minimum significant difference.

3.5. Fermentation process

The results obtained for the employed juice treatments were not significantly different in all evaluation stages by the direct fermentation process method. The inoculum presented mean cell viability of 86%, bud rate of 21%, and bud viability of 83%. The concentrations of total microorganisms, lactic acid bacteria, and yeasts (Fig. 1) were determined as 10^7 CFU/mL, showing no significant difference among treatments. These values are among those recommended to initiate the fermentation process in an adequate manner, with viability > 85% and contamination lower than 10^8 CFU/mL (Alcarde, 2014; Amorim, 2005).

The average results obtained for the quality of the raw material in the inoculum (Fig. 2) were not significantly different regarding viability of the cells, bud rate and viability, this means that both raw materials utilized did not affect the inoculum. Total microbial counts and lactic acid bacteria counts were not significantly different (Fig. 3); however, stored cane had 7.89 CFU/mL × 10^7, 3.82% more colony forming units than the yeast. The high presence of yeast can be attributed for presence of the yeast in the sugarcane in deterioration.

At the beginning of the fermentation process, cell viability and bud rate decreased in the inoculated yeast compared to the inoculum. However, no significant difference was observed between juice treatments, with 78% average viability, 15% bud rate, 78% bud viability, and regarding total microorganism counts (Fig. 1). However, the lactic bacteria and yeast were significantly lower compared to the "in natura" juice, especially the lactic bacteria in the presence of the moringa extract that due to its antibacterial activity resulted in an initial fermentation of a clarified juice with 4.65% lower contamination.

The cell viability and bud rate of raw materials recently processed and stored for 24 h, were not significantly different ($p < 0.05$), but bud viability reduced significantly at the start of the fermentation (Fig. 2). This drop can be justified by the higher number (5.09%) of total microorganisms in the fermentation process (Fig. 3).

At the end of the fermentation, the stored stalks (Fig. 3) had significantly higher total microorganism counts 4.09% (7.63 CFU/mL × 10^7), lactic acid bacteria counts 5.13% (7.78 CFU/mL × 10^7), and yeasts counts 14.83% (7.51 CFU/mL × 10^7). This behavior can be explained by the development of contaminants due the deterioration of the stalks and their presence throughout the whole production process,

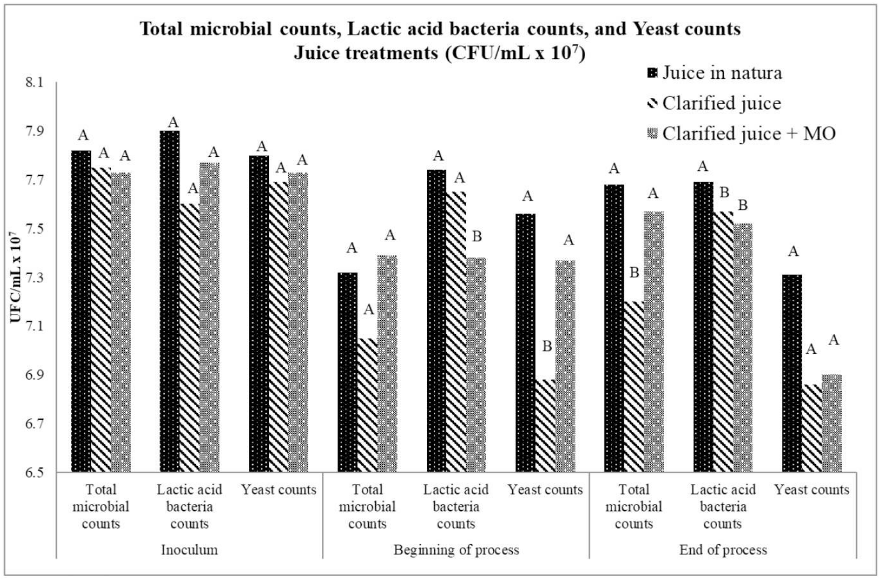

Fig. 1. - Total microbial counts, lactic acid bacteria and yeast of the juice treatments showing analysis of variance and comparison of means by Tukey test.

impacting cell viability with 17.65% and bud rate with 52.41% less than the raw material without being stored (Fig. 2).

The employed clarification processes (Fig. 1) showed no significant difference for yeast but resulted in lower bacterial contamination (1.82%) in the fermentation process, besides a significant reduction of 6.25% in the total microbiota of the limed clarified juice. The antimicrobial activity of the moringa seed extract was not maintained until the end of the fermentation since bacterial contamination was the same as in the clarified juice without flocculant.

The yeast maintained the initial 72% average cell viability, but low 9% bud rate and 58% bud viability. This behavior was not expected since at the end of the fermentation when sugar concentrations are low, the yeast uses its aerobic metabolic route, destined to the production of ATP for multiplication (Madigan et al., 2016).

During the fermentation process, the viability of yeast CA-11 remained higher than 85%, unlike that obtained by Montijo et al. (2014). The result was lower than the recommended only in the fifth fermentation cycle, both doing the recycle of the yeast (Amorim, 2005). It is noteworthy that the microorganisms were, as recommended, $< 10^8$ (Alcarde, 2014).

3.6. Chemical composition of broths post fermentations

At the end of the fermentation, the pH, total acidity, TRRS, alcohol and glycerol contents of broths post fermentations were determined (Table 5). The pH and total acidity results were not significantly different ($p < 0.05$) for the different treatments of the juice (with and without moringa extract) and fresh and stored raw materials. Likewise, Oliveira Filho et al. (2016) under similar storage conditions did not observe a significant pH reduction, and reported a close average value of 4.28, whereas total acidity increased significantly from 3.19 to 3.30 g H_2SO_4/L, close to the result found in this study.

The contents of total residual reducing sugars (TRRS) were not significantly different for the raw materials used. On the other hand, Oliveira Filho et al. (2016) reported an increase in the average TRRS for stalks stored up to 96 h. The results of the clarification process of the juice "in natura" were lower compared to the other treatments used, which may be explained by higher number of microorganisms, especially lactic acid bacteria, as shown in Fig. 2.

The alcoholic contents were not significantly different for the studied juice treatments and used raw materials, which was not expected since the increase of the contaminating microorganisms in the

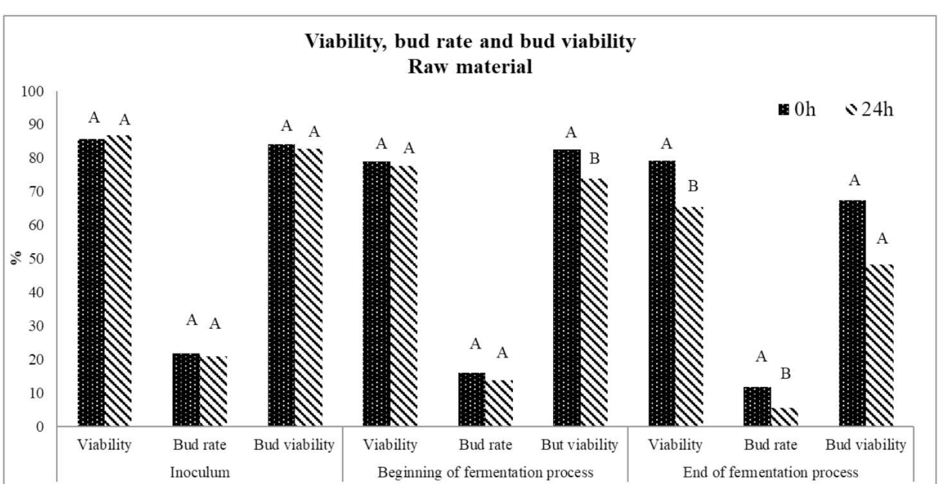

Fig. 2. - Cell viability, bud rate and bud viability for the raw material treatments (fresh and 24 h storage) showing analysis of variance and comparison of means by Tukey test.

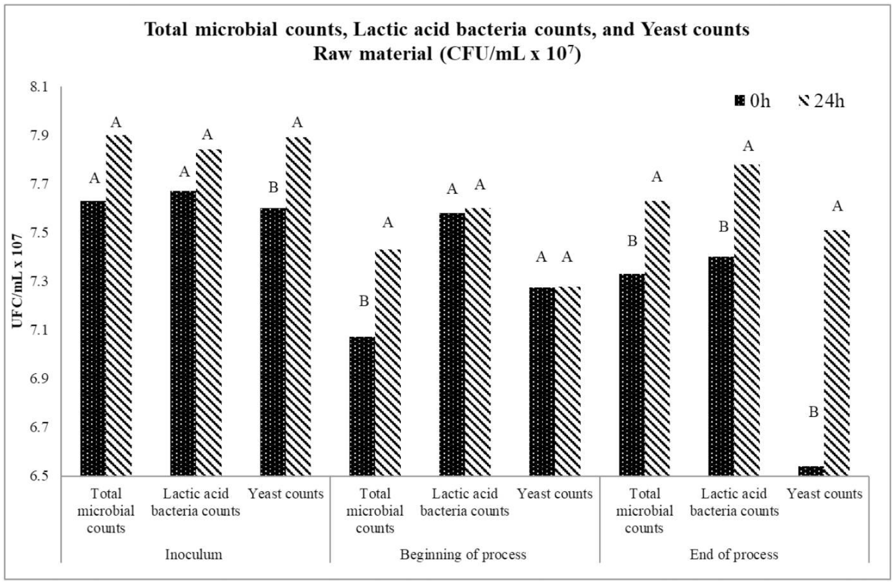

Fig. 3. - Total microbial counts, lactic acid bacteria and yeast of the raw material treatments showing analysis of variance and comparison of means by Tukey test.

Table 5
– Alcoholic content and glycerol content of broths post fermentations showing analysis of variance and comparison of means by Tukey test.

	pH	Total acidity (g/L H_2SO_4)	TRRS (%)	Alcohol content (% v/v)	Glycerol (%)
Juice treatment (T)					
Juice "in natura"	3.62A	3.56A	0.29B	8.01A	0.86A
Clarified juice	3.72A	3.32A	0.58A	8.00A	1.00A
Clarified juice + MO	3.72A	3.71A	0.53A	7.93A	0.95A
F test	2.25 ns	0.86 ns	21.04**	0.05 ns	2.12 ns
MSD	0.09	0.75	0.12	0.62	0.17
CV	3.17	25.75	30.93	9.44	22.00
Raw material (M)					
0 h	3.70A	3.46A	0.50A	8.18A	0.90B
24 h	3.69A	3.60A	0.44A	7.78A	0.98A
F test	0.24 ns	1.57 ns	1.73 ns	2.32 ns	7.79*
MSD	0.04	0.22	0.09	0.55	0.06
CV	2.21	11.29	37.52	12.33	11.34
Inter. TxM	0.34 ns	1.03 ns	1.59 ns	0.28 ns	0.44 ns

** Significant at 1% ($p < 0.01$).
* Significant at 5% ($0.01 < p < 0.05$); ns = non-significant; CV = Coefficient of variation; MSD = Minimum significant difference.

fermentation process affects the inoculum performance negatively. Although not significant, the alcohol content of stored stalks decreased by 4.9%, however, this effect can be cumulative when performing consecutive fermentative cycles. These results were higher than those of Montijo et al. (2014) and Bergamo & Uribe (2013), who obtained alcohol content between 6 and 7% using yeast CA-11.

Glycerol is a compound produced in the metabolic pathway of ethanol fermentation, at inversely proportional ratios. The results were not significantly different for the clarification treatments, with an average of 0.94% glycerol content, but higher than the 0.59% reported by Montijo et al. (2014).

The newly processed raw material and the lower amount of lactic acid bacteria and yeasts present in the fermentation (Fig. 3) process resulted in less glycerol, 0.90%, with an increase of 8.8% in this content due to the metabolization of sugars present in compounds undesirable. The sugars used for the glycerol production can be used for the ethanol production and had more productivity and did not have a compound would impair the quality of the cachaca (Amorim, 2005).

3.7. Chemical characterization of distillates

Cachaças were characterized regarding their components using gas chromatography (aldehydes, acroleins, esters, methanol, 2-butanol, 1-propanol, isobutanol, 1-butanol, isoamyl, and furfural) and liquid chromatography (ethyl carbamate). In addition to the alcoholic content, volatile acidity, conductivity, turbidity, pH, and copper are shown in Table 6.

The alcoholic graduation results of the cachaças varied slightly due to the distillation mechanism and the objective of obtaining cachaça with the highest alcohol content. However, results were close to the average of 42.18% v/v, within the 38 and 48% v/v alcoholic graduation range required by the legislation (BRASIL, 2005). In addition, electrical conductivity was 15.59 µg/L on average and pH 4.5 on average.

The turbidity of both raw materials (fresh and stored) increased slightly and so did the turbidity of the cachaça, indicating an increase of compounds that disperse the light.

Table 6 shows that the sum of the coefficient of congeners (volatile acidity, total aldehydes, total esters, furfural + hydroxymethyl, higher alcohols) for the cachaça produced from "in natura" juice of non-stored stalks was 212.65 mg/100 mL of anhydrous alcohol. The coefficient sum was 6.62% lower for clarified juice and 9.59% lower for juice clarified using the moringa seed extract.

For the cachaça produced from the stalks stored for 24 h, the sum for the "in natura" juice was 221.57 mg/100 mL of anhydrous alcohol, whereas the congeners decreased 3.92% for the clarified juice and 10.39% for the juice clarified with moringa seed extract. The values of the sum of congeners were within the recommended interval, between 200 and 650 mg/100 mL of anhydrous alcohol.

Likewise, Ribeiro et al. (2017) also observed a reduction of 23.59% (using CA-11 inoculum) and 0.88% (using native yeast) of the congener coefficient using juice clarification for producing organic cachaça. This reduction of the sum of the congeners observed for both raw materials is due to the lower formation of higher alcohols.

The reduction of the congeners was similar for the sum of the higher alcohols, and isoamyl and isobutanol compounds of the sum. It should be noted that this is expected, since the coefficient of congeners is a result of the sums of the compounds found in cachaça, such as the alcohols mentioned with the same reduction behavior. The cachaça produced from fresh stalks (no storage) compared to that produced with the clarified "in natura" juice, the sum of the alcohols was of 19.17%, whereas the reduction was 27.03% when using the moringa seed

Table 6
- Components and characterization of the obtained cachaças.

Analyzed components	Legislation		0 h			24 h		
	Min.	Máx.	Juice "in natura"	Clarified juice	Clarified juice + MO	Juice "in natura"	Clarified juice	Clarified juice + MO
Secondary components								
Volatile acidity[a]		150	53.03	61.49	67.3	60.51	66.54	62.41
Total aldehydes[a]		30	6.12	5.73	5.31	4.04	3.94	4.78
Total esters[a]		200	5.06	4.55	5.44	3.91	3.28	4.06
Furfural + hydroxymethyl[a]		5	9.86	14.79	13.08	14.32	17.34	12.57
Higher alcohols[a]		360	138.58	112.01	101.12	138.79	121.79	114.72
Coefficient of congeners[a]	200	650	212.65	198.57	192.25	221.57	212.89	198.54
Organic contaminants								
Methanol[a]		20	0.00	0.00	0.00	1.23	0.00	0.00
Alcohol sec. butyl[a]		10	0.00	0.00	0.00	0.00	0.00	0.00
n-Butanol[a]		3	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl carbamate[b]		210	< 6.23	< 6.23	< 6.23	< 6.23	< 6.23	< 6.23
Acrolein[a]		5	0.00	0.00	0.00	0.00	0.00	0.00
Alcohol content[c]	38	48	42.43	41.96	41.90	42.64	42.38	41.82
Electrical conductivity[d]			14.92	16.18	16.05	15.71	15.84	14.84
Turbidity[e]			0.10	0.15	0.22	0.10	0.18	0.22
pH			4.62	4.42	4.53	4.62	4.50	4.47
Higher alcohols								
n-Propanol[a]			13.30	12.90	14.30	13.13	13.71	13.74
Isobutanol[a]			41.54	32.12	28.72	39.66	33.38	31.78
Isoamyl[a]			83.74	66.99	58.10	86.00	74.70	69.20
Copper[f]		5	4.80	4.00	3.80	3.80	4.70	4.40

[a] mg/100 mL anhydrous alcohol
[b] μg/L.
[c] % V/V.
[d] μs at 25 °C.
[e] NTU.
[f] mg/L.

extract. For the stalks stored for 24 h, the reduction was 12.24% when using the liming process and 17.34% for the natural flocculant.

The results were satisfactory as a solution for producing a quality cachaça using raw materials with high content of higher alcohols. This fact occurs due to the removal of amino acids through the clarification process since they are the precursors for the formation of these compounds during the fermentation process, with the characteristic odor of distilled beverages, isoamyl has a floral aroma characteristic of the "bouquet" of beverages (Alcarde, 2014).

Other factors that influence the formation of higher alcohols are high temperatures, low pH of the broth, low activity yeasts and stored sugarcanes (Cardoso, 2013). This percent reduction is higher than the 5.59% reduction in the sum of the higher alcohols reported by Ribeiro et al. (2017) using CA-11 yeast and clarification process.

Among the other compounds, the esters had similar results for the treatments used, however, the low average content of anhydrous alcohol (4.38 mg/100 mL) is detrimental to the drink, since the esters make up the largest class of aromatic compounds, which give the characteristic fruity aroma/flavor to the drink.

Furfural, a secondary compound with a peculiar odor that exhibits color when exposed to air and light (Azevedo et al., 2007), contents were higher than those recommended in both treatments, with results between 9.89 until 17.34 mg/100 mL, the for the raw material were 12.59 mg/100 mL to freshly harvested stalks and 14.74 mg/100 mL to stalks stored for 24 h. Its production can be justified by the residual pentoses and hexoses (TRRS, Table 5) from the fermentation that became furfural during the distillation (Alcarde, 2014).

The other secondary compounds did not vary significantly according to treatments, with average volatile acidity and total aldehydes of 61.88 and 4.99 mg/100 mL of anhydrous alcohol, respectively, the results are within the ideal range, according to the Normative Instructions n° 13/2005.

The contaminants (organic and inorganic) must be below the maximum values adopted in the legislation to ensure higher quality beverage while providing for consumer health. The organic contaminants showed (Table 6) no production of secondary alcohols, butyl, n-butanol, and acrolein. The presence of methanol was only found in the cachaça produced with stalks stored for 24 h with the "in natura" juice.

The determination used for ethyl carbamate (Anjos et al., 2011) have a minimum limit for quantification, with the results showed in the Table 6, it was not quantified in the evaluated cachaças since the analysis allows results just higher than 6.23 μg/L, satisfactory results, since the ethyl carbamate is being widely investigated due to its genotoxic and carcinogenic potential, with half of the spirits and cachaças produced in Brazil having concentrations above the limit of 210 μg/L (RIACHI et al., 2014; BRASIL, 2014). Masson et al. (2014) reported contents ranging from 23 to 930 μg/L for cachaças produced in Minas Gerais.

Regarding the inorganic contaminants, the copper contents of the produced cachaças (Table 6) were 4.25 mg/L on average and within the recommended range, and apparently not influenced by the treatments used.

4. Conclusions

The processing of stored raw material entails a fermentative process with more contaminants and higher production of undesirable compounds. The clarification process using the *Moringa oleifera* Lamarck seed extract shows potential for controlling contaminating microorganisms during the fermentation process, and lower formation of higher alcohols in the cachaça as well.

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Appendix A

Table A.1
– Results for Brix, pH, TRS, RS, total acidity, pol and purity of juices extracted from fresh and 24 h stored stalks, showing analysis of variance and comparison of means by Tukey test.

	Brix (°)	pH	TRS (%)	RS (%)	Total acidity (g/L H_2SO_4)	Pol (%)	Purity (%)
0 h	19.4A	5.23A	17.39A	0.23A	0.96A	17.05A	87.59A
24 h	19.6A	5.21A	15.41A	0.24A	1.08A	16.51A	84.05A
F test	0.38 ns	0.53 ns	0.57 ns	1.12 ns	1.48 ns	0.16 ns	0.33 ns
MSD	0.74	0.08	7.29	0.02	0.27	3.69	17.05
CV	1.68	0.75	19.60	4.78	11.61	9.69	8.76

Significant at 1% ($p < 0.01$); Significant at 5% ($0.01 < p < 0.05$); ns = non-significant; CV = Coefficient of variation; MSD = Minimum Significant Difference.

Table A.2
– Results for pH, total acidity and TRS of clarified juices showing analysis of variance and comparison of means by Tukey test.

	pH	Total acidity (g/L H_2SO_4)	TRS (%)
Juice treatment (T)			
Clarified juice	5.72A	0.73A	14.25A
Clarified juice + MO	5.74A	0.78A	14.26A
F Test	0.01 ns	0.23 ns	0.0002 ns
MSD	0.47	0.30	2.12
CV	5.17	25.15	9.27
Raw material (M)			
0 h	5.83A	0.65A	15.17A
24 h	5.63A	0.86A	13.35B
F Test	1.72 ns	3.90 ns	9.15
MSD	0.43	0.29	1.67
CV	4.71	24.15	7.30
Inter. TxM	0.005 ns	0.19 ns	0.75 ns

Significant at 1% ($p < 0.01$); Significant at 5% ($0.01 < p < 0.05$); ns = non-significant; CV = Coefficient of variation; MSD = Minimum Significant Difference; MO = moringa seed extract.

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