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Food Research International 76 (2015) 787–795

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Microbiological and chemical parameters during cassava based-substrate fermentation using potential starter cultures of lactic acid bacteria and yeast

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A R T I C L E I N F O

Article history:
Received 29 May 2015
Received in revised form 19 July 2015
Accepted 24 July 2015
Available online 29 July 2015

Keywords:
Starter culture
Cassava
Bacteria
Yeasts
Indigenous fermented foods
Volatile compounds

A B S T R A C T

Traditional Brazilian indigenous fermented foods and beverages are potential sources of new food products that promote health, but they are still produced by natural fermentation. In the present work, *Lactobacillus fermentum* CCMA 0215 isolated from the indigenous fermented cassava beverage *yakupa* was used as single or mixed starter culture with five different yeast strains (*Torulaspora delbrueckii* CCMA 0234 and CCMA 0235, *Pichia caribbica* CCMA 0198, and *Saccharomyces cerevisiae* CCMA 0232 and CCMA 0233) to ferment cassava. Fermentations using each yeast as single starter culture were also performed. The microbial population and metabolites produced during cassava fermentation were investigated. In all assays, the inoculated microorganisms fermented cassava, judged by lowering the pH from 6.0 to 4.0–5.0 within 24 h. Lactic acid bacteria (LAB) and yeast population increased during fermentation. Lactic acid was the main organic acid produced, reaching a maximum value of 4.5 g/L at 24 h in the co-culture with *L. fermentum* CCMA 0215 and *T. delbrueckii* CCMA 0234. Other organic acids, such as malic, tartaric, and succinic acids, were detected in low concentrations (less than 0.5 g/L). Ethanol and glycerol were produced in all assays inoculated with yeasts (single and co-cultured with LAB), reaching the maximum concentration of approximately 2.3 g/L and 0.6 g/L, respectively. Twenty-two volatile compounds were detected after 48 h of fermentation, varying widely between single and co-cultures. The compounds 2-phenylethyl alcohol, 1-butanol, 3-methyl (isoamyl alcohol), and acetoin were detected in single and co-cultures. This study demonstrated co-cultures of yeasts and LAB had the ability to improve the aroma profile of the final product and the safety of the product by lowering the pH.

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1. Introduction

Brazilian indigenous people are traditional producers of fermented foods and beverages using different substrates like corn, rice, and cassava. The production is still homemade, and the empirical knowledge is transmitted from one generation to the other inside the tribes. *Cauim*, *caxiri*, *yakupa*, and *calugi* beverages are prepared by women of the tribe and then left to ferment by natural fermentation without any microbial or environmental conditions controlled (Almeida, Rachid, & Schwan, 2007; Freire, Ramos, Almeida, Duarte, & Schwan, 2014; Miguel, Santos, Santos, Duarte, & Schwan, 2014; Ramos et al., 2010; Santos, Almeida, Pereira, & Schwan, 2012). The indigenous beverage *yakupa* is an acid cassava-fermented product, in which lactic acid bacteria (LAB) are responsible for the acidity and the pH decrease (Freire et al., 2014). Yeasts, mainly *Saccharomyces cerevisiae*, are also present during the *yakupa* fermentation (Freire et al., 2014). Yeasts have been described in African and Brazilian fermented foods and are related to alcohol production and other aroma compounds, stimulation of LAB growth, and improvement of nutritional value (Jespersen, 2003; Kamda et al., 2015; Olasupo, Odunfa, & Obayori, 2010).

Nowadays, consumer awareness towards healthy diets and changing eating habits has created a huge market demand for new foods with health benefits. In this context fermented products, particularly non-dairy beverages, are gaining popularity and acceptance because of their functional benefits (De Dea Lindner et al., 2013). New products have been launched based on fruit and cereals, and the incorporation of lactic acid bacteria (LAB) in food and beverages is a global trend. It is well known that fermented foods have important healthy roles in the human diet, and studies have demonstrated that regular intake of fermented foods can reduce the incidence of chronic diseases (Keszei, Schouten, Goldbohm, & van den Brandt, 2010; Larsson, Andersson, Johansson, & Wolk, 2008; Sonestedt et al., 2011). Further, the challenge to overcome the disadvantages associated with fermented dairy products, like lactose intolerance, allergy, and the impact in cholesterol levels (Prado, Parada, Pandey, & Soccol, 2008), has stimulated the improvement of traditional fermented foods prepared with different substrates like cassava. Many advantages are related to the use of starter cultures in the fermentative process, as the rapid acidification of the product and, thus, the inhibition of spoilage and pathogenic bacteria growth

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http://dx.doi.org/10.1016/j.foodres.2015.07.041
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produce foods with consistent quality (Holzapfel, 2002). Amoa-awua and Jakobsen (1995) and Amoa-awua, Frisvad, Sefa-dedeh, and Jakobsen (1997) have shown some important activities that occur when traditional inoculum is used to ferment cassava: the breaking down of the coarse texture of cassava dough, the souring of cassava dough, the reduction in the level of cyanogenic glucosides, and the synthesis of volatile aroma compounds.

Djeni et al. (2015) performed biochemical and microbial characterizations of the traditional cassava-fermented food attieké in Côte d'Ivoire, Africa, and found that the dominating LAB strains can be potential starter cultures for cassava fermentations. The *Lactobacillus plantarum* BFE 6710 and *Lactobacillus fermentum* BFE 6620 were also used as starter cultures for cassava fermentation for *gari* (a cassava-fermented food in Africa) production in a pilot study under controlled conditions (Huch née Kostinek et al., 2008). Although the application of LAB in dairy foods has been widely explored, cassava is an innovative matrix for the application of this microbial group. In this study, a *L. fermentum* CCMA 0215 isolated from *yakupa* was selected on the basis of some technological properties and used as starter cultures in single and co-culture with different yeast species to ferment a cassava substrate. Microbial and metabolite compound analysis were performed to provide sufficient knowledge about the final biochemical characteristics of the beverage in order to control and to standardize the cassava fermentation process.

2. Materials and methods

2.1. Microorganisms and culture conditions

The yeast *S. cerevisiae* CCMA 0232, *S. cerevisiae* CCMA 0233, *Torulaspora delbrueckii* CCMA 0234, *T. delbrueckii* CCMA 0235, *Pichia caribbica* CCMA 0198, and the LAB *L. fermentum* CCMA 0215 belonging to the Culture Collection of Agriculture Microbiology (CCMA) of Federal University of Lavras (Brazil). These strains were isolated from natural cassava fermentations (Freire et al., 2014; Ramos et al., 2015) (except *P. caribbica* CCMA 0198 that was isolated from coffee fermentation) and used as potential starter cultures for the fermentation of new cassava substrate. These strains were previously selected based on technological characteristics such as α-amylase secretion, acid production and growth in a cassava substrate (data not shown). The yeasts and LAB isolates were stored at −80 °C in YPD broth 10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt, Germany) and Man Rogosa Sharpe broth (MRS, Merck, Darmstadt, Germany), respectively, with 20% (v/v) glycerol. The strains were reactivated by streaking them onto YPD agar (yeasts) and MRS agar (LAB), and incubated for 48 h at 30 °C (yeasts) and 37 °C (LAB).

2.2. Preparation of cassava substrate a nd starter cultures for fermentation assays

The cassava substrate for the fermentation assays was prepared in a similar way as for the *yakupa* beverage (Freire et al., 2014), with some modifications. The cassava roots used in all experiments were purchased from the local market in Lavras, Minas Gerais, Brazil. Peeled cassava roots (0.5 kg) were cooked with 1000 mL of sterile distilled water for approximately 40 min, and then 1000 mL of sterile distilled water was added. This mixture was sieved and pasteurized at 90 °C for 20 min.

The preparation of inoculum for single and co-culture fermentation was performed as described by Santos, Libeck, and Schwan (2014). The yeast and LAB strains were each successively sub-culturing on YPD broth (pH 3.5 at 30 °C for 24 h) and MRS broth (37 °C for 48 h), respectively. The cells were inoculated in the cassava substrate with a population of 5 log CFU/mL for yeasts and 7 log CFU/mL for bacteria in both single and co-culture fermentations.

2.3. Single fermentations

The washed cells of yeasts and LAB were separately inoculated into 250 mL flasks containing 100 mL of the pasteurized cassava substrate and incubated at 37 °C for 48 h. The experiments were performed in three independent assays. For each repetition, two samples were taken at each time (duplicate).

2.4. Co-culture fermentations

Five co-culture fermentations were performed: (1) *S. cerevisiae* (CCMA 0232) and *L. fermentum* (CCMA 0215); (2) *S. cerevisiae* (CCMA 0233) and *L. fermentum* (CCMA 0215); (3) *T. delbrueckii* (CCMA 0234) and *L. fermentum* (CCMA 0215); (4) *T. delbrueckii* (CCMA 0235) and *L. fermentum* (CCMA 0215); and (5) *P. caribbica* (CCMA 0198) and *L. fermentum* (CCMA 0215). Inoculum addition was the same as described in Section 2.3.

2.5. Enumeration of microorganisms

Samples (1 mL) were taken from each fermentation flask. Serial ten-fold dilutions were prepared in a solution of 0.9% NaCl (w/v) and 0.1% (w/v) bacto peptone (Difco). The total LAB, yeast, and Enterobacteriaceae populations were determined by plating in MRS (supplemented with 50 mg/L of nystatin), YPD (pH 3.5), and violet red bile agar (VRBG; Merck) media, respectively. The plates were incubated at 37 °C (LAB and Enterobacteriaceae) and 28 °C (yeasts) during 48 h, and the colony forming units (CFU) were enumerated in plates containing 30 to 300 colonies, and cell concentration was expressed as log CFU/mL. The analyses were performed in triplicate.

2.6. Analytical methods

2.6.1. Determination of pH

The pH levels of the fermenting cassava samples were measured on site with pH-Fix test strips (Macherey-Nagel GmbH and Co., Düren, Germany).

2.6.2. HPLC analysis

Organic acid (lactic acid, acetic acid, malic acid, succinic acid, and tartaric acid) and alcohol (ethanol and glycerol) analyses were performed as described by Duarte et al. (2010). A Shimadzu liquid chromatography system (Shimadzu Corp., Japan), equipped with a dual detection system consisting of a UV–Vis detector (SPD 10Ai) (for acid detection) and a refractive index detector (RID-10Ai) (for alcohol detection), was used. A Shimadzu ion exclusion column, Shim-pack SCR-101H (7.9 mm × 30 cm) was used at an operating temperature of 30 °C for ethanol and glycerol and 50 °C for acids. All samples were analyzed in duplicate.

2.6.3. GC–MS analysis

The volatile component profiles were determined at 0 and 48 h of fermentations according to the methods described by Oliveira, Faria, Sá, Barros, and Araújo (2006), with some modifications. First, samples were centrifuged twice, and then 7 mL was put into 10 mL culture tubes. A magnetic stir bar (22.2 mm × 4.8 mm) and 0.143 μg of 4-nonanol (internal standard) (Merck, Darmstadt, Germany) were added, and extraction was done by stirring the samples with 700 μL of chloroform (Merck, Darmstadt, Germany) for 15 min over agitation with a magnetic stirrer. After cooling at 0 °C for 10 min, the magnetic stir bar was removed and the organic phase was detached by centrifugation (RCF = 5118, 5 min, 4 °C). The extract was recovered into a vial using a Pasteur pipette. Then, the extract was dried with anhydrous sodium sulfate (Merck, Darmstadt, Germany) and picked up again into a new vial.

The extract analysis was performed using a gas chromatography quadrupole mass spectrometry (GC-Q/MS) GC-2010 Ultra (Shimadzu) and the mass spectrometer GCMS-QP2010 SE (Shimadzu), equipped with a split/splitless injector. A capillary column HP-FFAP (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies) was used for separation, and 1 μL was the injection volume. The temperature of the injector was set at 230 °C, and the oven temperature was programmed as follows: held at 50 °C for 5 min and then programmed to rise from 50 °C to 200 °C, at 3 °C/min. Held at 200 °C for 10 min and then programmed to go from 200 °C to 240 °C, at 10 °C/min. Finally, it was held at 240 °C for 20 min. The carrier gas was helium at 49.5 kPa, which corresponds to a linear speed of 15.5 cm/s. The detector was operated in the electron-impact mode (70 eV), and mass spectra were acquired by scanning over the mass/charge (*m/z*) range of 45–500. All samples were analyzed in duplicate. Volatile compounds were identified using the GC/MS Solution software (Version 2.6) by comparing mass spectra. The quantification of the volatile compounds was expressed as 4-nonanol (internal standard) equivalents, and the relative concentrations of the investigated compounds were calculated by relating the area of the internal standard to the area of the compound of interest.

2.7. Statistical analysis

The data were subject to analysis of variance (ANOVA), and differences in values were considered significant when the p value was less than 0.05. The statistical analyses were performed using the Sisvar 5.3 software. Principal component analyses (PCA) were performed with the software XLSTAT 7.5.2 (Addinsoft, New York, N.Y., USA) for group data from starter cultures and metabolites produced during cassava fermentation.

3. Results

3.1. LAB, yeasts, and Enterobacteriaceae populations during single and co-culture fermentations

The population of LAB and yeasts during single and co-culture fermentations in cassava media were evaluated. There was a significant ($p < 0.05$) increase of LAB population during the first 6 h for all fermentations, reaching around 8 log CFU/mL. However, after 12 h, there was a significant ($p < 0.05$) decrease of LAB when co-cultivated with the yeasts *S. cerevisiae* CCMA 0232 (7.6 log CFU/mL), *T. delbrueckii* CCMA 0234 (7.8 log CFU/mL), and *P. caribbica* CCMA 0198 (7.7 log CFU/mL). At 24 h of fermentation, the LAB population was recovered. It seems that there was a competition at 12 h in these assays, and at 24 h the LAB was able to adapt to this condition. After 48 h, the LAB population detected in the single culture of *L. fermentum* CCMA 0215 and co-culture with *S. cerevisiae* CCMA 0232 and *T. delbrueckii* CCMA 0234 remained high (around 8 log CFU/mL).

Regarding yeasts, their population in single and co-culture fermentations showed an increase during the first 12 h, reaching a population of around 6.6 to 7.3 log CFU/mL depending on the assay. As observed,

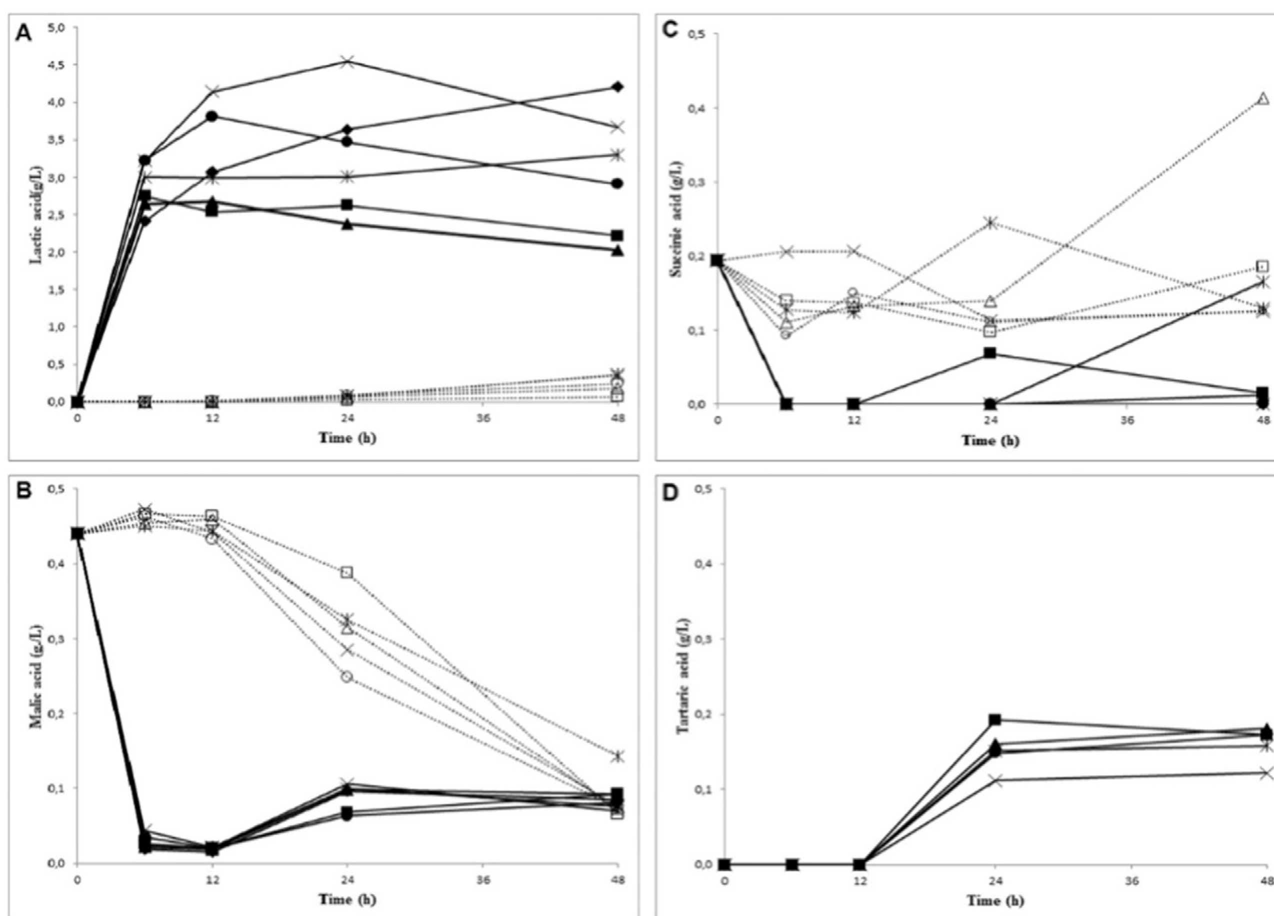

Fig. 1. Organic acids evaluated during 48 h of cassava fermentation. A) Lactic acid; B) malic acid; c) succinic acid; d) tartaric acid. Black symbols (continuous line) correspond to fermentations inoculated with LAB (single and co-culture) and white symbols (dotted lines) correspond to fermentations inoculated with single yeasts. ◆ = LAB; ■ = LAB + *S. cerevisiae* CCMA 0232; ▲ = LAB + *S. cerevisiae* CCMA 0233; × (continuous line) = LAB + *T. delbrueckii* CCMA 0234; * (continuous line) = LAB + *T. delbrueckii* CCMA 0235; ● = LAB + *P. caribbica* CCMA 0198; □ = *S. cerevisiae* CCMA 0232; △ = *S. cerevisiae* CCMA 0233; × (dotted line) = *T. delbrueckii* CCMA 0234; * (dotted line) = *T. delbrueckii* CCMA 0235; and ○ = *P. caribbica* CCMA 0198. Tartaric acid was not detected during fermentations performed with single cultures and was not plotted in the graph D. Standard deviation ranging from: 0.00–0.13 g/L (lactic acid), 0.00–0.21 g/L (malic acid), 0.00–0.23 g/L (succinic acid), and 0.00–0.17 g/L (tartaric acid).

the yeast population varied and was significantly different between the assays depending on the time of fermentation. The highest population was found for *P. caribbica* CCMA 0198 in the single culture at 24 h and in the co-culture with LAB at 48 h, reaching approximately 7.5 log CFU/mL.

The Enterobacteriaceae population was not detected in any of the assays (data not shown), neither in the beginning nor in the end of fermentations. The pasteurization process and the decreasing of pH by starter culture activity contributed to the growth inhibition of these bacteria.

3.2. Chemical parameters

The pH value changes during the fermentations were evaluated. At 6 h of fermentation, there was a drop of pH from 6.0 to 4.0 and 5.0 for assays containing LAB and single yeasts, respectively. The fermentation inoculated with only *T. delbrueckii* CCMA 0234 did not show this drop at the first 6 h, but it did drop at 12 h. In the assay co-cultured with LAB and *T. delbrueckii* CCMA 0235, the pH showed an increase after 24 h of fermentation, which may be due to the proteolytic activity of this yeast (data not shown). Maltose was the main carbohydrate detected (5.13 g/L), followed by a low concentration of glucose (0.016 g/L). Malic (0.44 g/L) and succinic (0.19 g/L) acids were also found in the cassava substrate. Lactic acid was the main organic acid produced in the fermentations containing *L. fermentum* (Fig. 1A). This acid was detected from 6 h of fermentation in the assays inoculated with LAB. The highest concentration was found in the assay co-cultured with *L. fermentum* CCMA 0215 and *T. delbrueckii* CCMA 0234, reaching a value of 4.5 g/L at 24 h. At the end, the highest concentration of lactic acid was detected

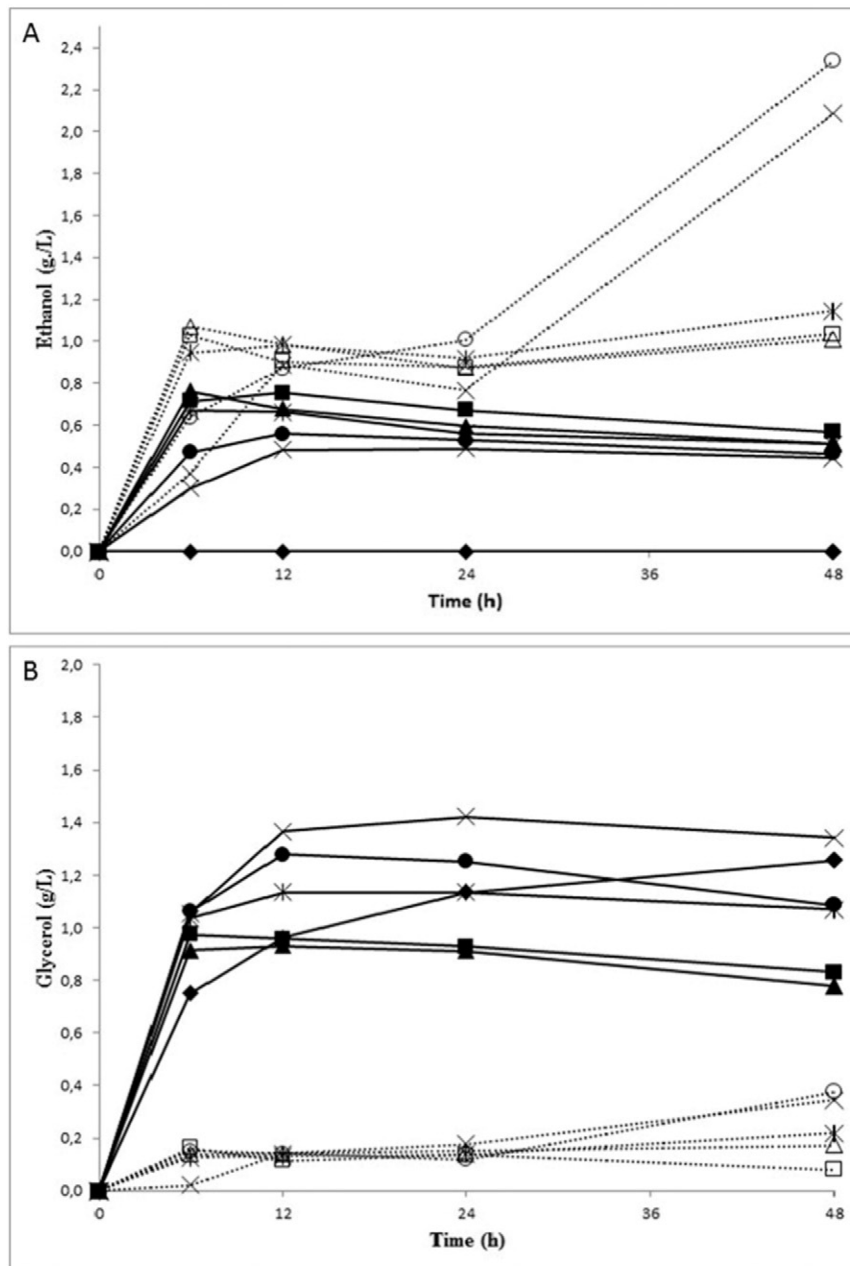

Fig. 2. Ethanol (A) and glycerol (B) evaluated during 48 h of cassava fermentation. Black symbols (continuous line) correspond to fermentations inoculated with LAB (single and co-culture) and white symbols (dotted lines) correspond to fermentations inoculated with single yeasts. ♦ = LAB; ■ = LAB + *S. cerevisiae* CCMA 0232; ▲ = LAB + *S. cerevisiae* CCMA 0233; × (continuous line) = LAB + *T. delbrueckii* CCMA 0234; * (continuous line) = LAB + *T. delbrueckii* CCMA 0235; ● = LAB + *P. caribbica* CCMA 0198; □ = *S. cerevisiae* CCMA 0232; △ = *S. cerevisiae* CCMA 0233; × (dotted line) = *T. delbrueckii* CCMA 0234; (dotted line) = *T. delbrueckii* CCMA 0235; and ○ = *P. caribbica* CCMA 0198. Standard deviation ranging from: 0.00–0.36 g/L (ethanol) and 0.00–0.12 g/L (glycerol).

in the assay containing only *L. fermentum* (4.2 g/L). Malic acid, detected at the beginning of the fermentation process (0.44 g/L for all assays), decreased during the first 6 h of fermentation in those assays containing LAB (single and co-culture), while it decreased slowly during the 48 h of fermentation in those assays containing the single yeasts (Fig. 1B). Succinic acid was detected in low concentrations in all assays (lower than 0.5 g/L). This acid decreased in the assays containing LAB during the first 6 h. In the fermentations with single yeasts, succinic acid levels were almost constant (around 0.15 g/L) except for those inoculated with *S. cerevisiae* CCMA 0233, in which succinic acid was slightly produced, reaching the highest concentration of 0.4 g/L (Fig. 1C). Tartaric acid was only detected in the assays inoculated with a co-culture of bacteria and yeasts from 24 h (around 0.15 g/L), remaining constant until 48 h of fermentation (Fig. 1D). Ethanol was produced in all assays inoculated with yeasts (single and co-cultured). The highest concentration of ethanol was detected at 48 h in the assays inoculated with single yeasts *P. caribbica* CCMA 0198 and *T. delbrueckii* CCMA 0234, reaching a value of 2.3 g/L and 2.1 g/L, respectively (Fig. 2A). When these yeasts were co-cultured with LAB, the ethanol production was around 0.5 g/L. Glycerol was particularly detected in the assays containing LAB, showing concentrations higher than 0.6 g/L, while for assays containing only yeast, the concentrations did not reach 0.4 g/L (Fig. 2B). Fig. 3 shows the PCA to correlate the metabolites (organic acids and alcohols) with the different fermentation assays. The first (PC1) and second (PC2) principal components explained 78.83% and 8.59%, respectively of the total variance (87.42%). On the positive side of PC1, all co-culture assays and the single culture with LAB were correlated with glycerol, tartaric acid and lactic acid. On the negative side of PC1 all assays containing single yeast were correlated with ethanol, succinic acid and malic acid.

Twenty-two volatile compounds were detected by GC–MS after 48 h of fermentation, and their concentrations are shown in Table 1. The compounds (i) 3-penten-2-ol; 2-butenal, 3-methyl; 1,1-dimethyl-3-chloropropanol; 2-hexanol; 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester; dibutyl phthalate; squalene; and bis(2-ethylhexyl) phthalate were detected in all the fermentation assays and in the substrate (control) with similar concentrations (Table 1). These results indicated that these compounds are related to the substrate and were not metabolized by the starter cultures. In order to correlate the volatile compounds with the different fermentation assays, a PCA analysis was performed (Fig. 4). The first (PC1) and second (PC2) principal components explain 33.31% and 23.73%, respectively, of the total variance (57.04%). On the positive side of PC2 and the positive and negative sides of PC1, all assays inoculated with a single culture of yeasts were correlated with the most number of compounds, except for the single culture with *S. cerevisiae* CCMA 0233. On the negative side of PC2 and the negative and positive sides of PC1, the assays containing LAB (all co-cultures and single cultures) and the single culture of the yeast *S. cerevisiae* CCMA 0233 were correlated with three volatile compounds: 2-propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester; acetoin; and 2-butenal, 3-methyl.

4. Discussion

The occurrence of yeasts together with LAB in naturally fermented foods have led to suggestions of possible interactions between these groups of microorganisms. Nout and Sarkar (1999) suggested that the growth of yeasts in fermented foods is favored by acidification of the environment created by LAB, and yeasts, which can provide growth factors such as vitamins and soluble nitrogen compounds, stimulate the growth of LAB. The association of LAB and yeast (*L. plantarum*, *Pediococcus acidilactici* and *T. delbrueckii)* showed to be appropriate for fermenting *Cucumeropsis mannii* cotyledons (Kamda et al., 2015). In the present study, the yeasts *S. cerevisiae* CCMA 0232, *S. cerevisiae* CCMA 0233,

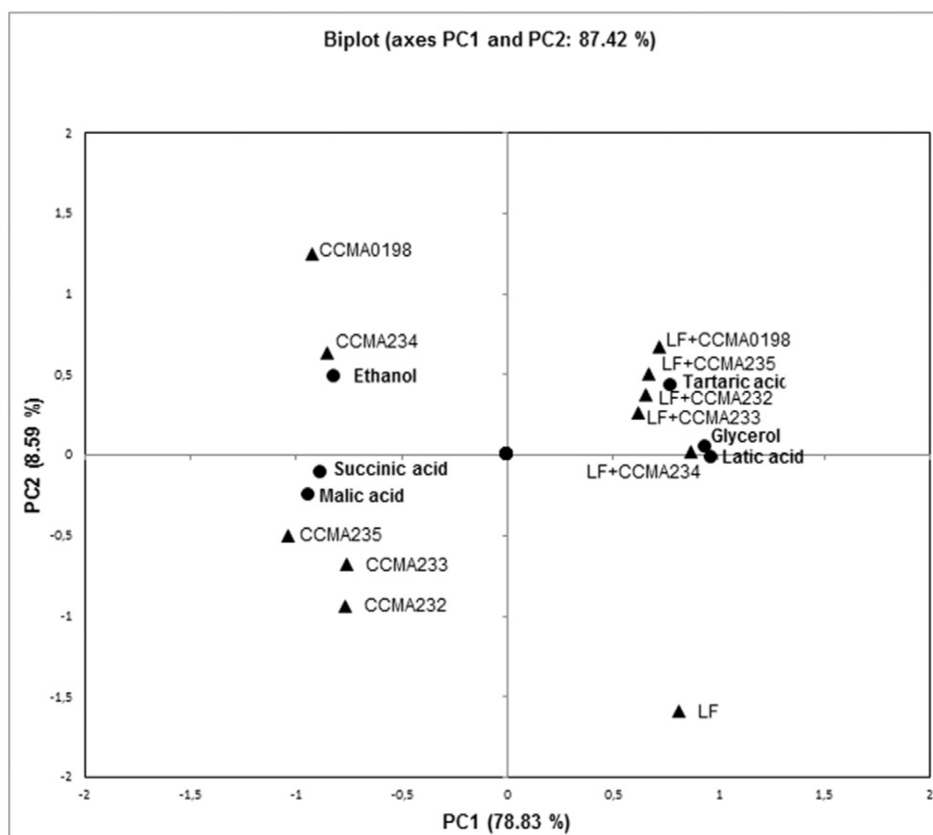

Fig. 3. Principal component analysis (PCA) of volatile compounds (GC–MS) obtained by single and co-cultive fermentation of cassava substrate after 48 h. LF = *L. fermentum*. The numbers from 1 to 22 represent the volatile compounds and are identified in Table 1.

Table 1
C\oncentration (μg/L) of volatile compounds obtained by GC–MS analysis after 48 h of single and co-culture fermentation assays.

	Compounds	Control	LAB	LAB + *S. cerevisiae* CCMA 0232	LAB + *S. cerevisiae* CCMA 0233	LAB + *T. delbrueckii* CCMA 0234	LAB + *T. delbrueckii* CCMA 0235	LAB + *P. caribbica* CCMA 0198	*S. cerevisiae* CCMA 0232	*S. cerevisiae* CCMA 0233	*T. delbrueckii* CCMA 0234	*T. delbrueckii* CCMA 0235	*P. caribbica* CCMA 0198
	Alcohols												
1	(i) 3-Penten-2-ol	0.129 ± 0.07b	0.082 ± 0.01b	0.101 ± 0.06c	0.058 ± 0.01b	0.101 ± 0.05c	0.112 ± 0.06c	0.165 ± 0.02c	0.097 ± 0.00b	0.073 ± 0.01a	0.098 ± 0.01a	0.117 ± 0.05a	0.104 ± 0.04a
3	1-Butanol, 3-methyl	Nd	0.003 ± 0.00a	0.082 ± 0.01b	0.077 ± 0.00b	0.085 ± 0.01b	0.070 ± 0.01b	0.067 ± 0.01b	0.147 ± 0.03c	0.174 ± 0.02b	0.209 ± 0.06b	0.144 ± 0.04a	0.202 ± 0.32a
4	1,1-Dimethyl-3-chloropropanol	0.086 ± 0.04a	0.065 ± 0.02b	0.077 ± 0.00b	0.042 ± 0.02b	0.068 ± 0.01b	0.074 ± 0.02b	0.114 ± 0.06c	0.064 ± 0.01a	0.051 ± 0.01a	0.073 ± 0.01a	0.072 ± 0.01b	0.076 ± 0.05a
5	2-Hexanol	0.297 ± 0.11c	0.350 ± 0.03d	0.403 ± 0.07e	0.221 ± 0.05d	0.340 ± 0.04d	0.422 ± 0.05d	0.618 ± 0.08d	0.346 ± 0.03d	0.302 ± 0.07c	0.392 ± 0.01b	0.367 ± 0.02b	0.427 ± 0.03a
7	Phenylethyl alcohol	Nd	Nd	0.079 ± 0.01b	0.052 ± 0.03b	0.125 ± 0.05c	0.038 ± 0.04b	0.149 ± 0.03c	0.157 ± 0.04c	0.145 ± 0.04b	0.151 ± 0.03b	0.093 ± 0.00a	0.203 ± 0.03a
	Esters												
8	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.221 ± 0.09b	0.113 ± 0.06c	0.216 ± 0.06d	0.137 ± 0.04c	0.120 ± 0.05c	0.120 ± 0.05c	0.173 ± 0.01c	0.168 ± 0.02c	0.093 ± 0.07b	0.233 ± 0.04b	0.106 ± 0.06a	0.238 ± 0.01a
14	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.023 ± 0.00a	0.015 ± 0.00a	Nd	Nd	Nd
17	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.023 ± 0.02a	Nd	Nd	0.072 ± 0.03a	Nd
19	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.009 ± 0.01a	Nd	0.016 ± 0.01a	Nd
20	Butyrolactone	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.036 ± 0.01a	Nd	0.049 ± 0.13a
	Acid												
6	Butanoic acid	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.085 ± 0.02b	Nd	10.533 ± 0.4c	Nd	19.864 ± 0.67b
10	n-Hexadecanoic acid	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.049 ± 0.1a	Nd	Nd	0.213 ± 0.04b	Nd
11	Oleic acid	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.061 ± 0.01a	Nd
13	Octadecanoic acid	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.024 ± 0.00a	Nd	Nd	0.075 ± 0.02a	Nd
18	n-Hexadecanoic acid	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.048 ± 0.00a	0.019 ± 0.01a	Nd	0.201 ± 0.05a	Nd
22	Beta-phenylethyl butyrate	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.056 ± 0.02a	Nd	0.083 ± 0.02a
	Aldehydes and ketones												
2	2-Butenal, 3-methyl	0.014 ± 0.03a	0.012 ± 0.02a	0.011 ± 0.01a	0.009 ± 0.00a	0.011 ± 0.01a	0.019 ± 0.00a	0.020 ± 0.01a	0.011 ± 0.00a	0.009 ± 0.00a	0.011 ± 0.01a	0.012 ± 0.01a	0.013 ± 0.01a
16	Acetoin	Nd	0.085 ± 0.03b	0.019 ± 0.00a	0.007 ± 0.00a	0.051 ± 0.01b	0.010 ± 0.00a	0.072 ± 0.00b	Nd	0.107 ± 0.1b	0.012 ± 0.01a	0.035 ± 0.01a	Nd
	Others												
9	Dibutyl phthalate	0.056 ± 0.01a	0.029 ± 0.00a	0.056 ± 0.01b	0.033 ± 0.01a	0.041 ± 0.00b	0.044 ± 0.02b	0.044 ± 0.00b	0.041 ± 0.00a	0.026 ± 0.00a	0.057 ± 0.01a	0.035 ± 0.01a	0.051 ± 0.00a
12	Squalene	0.044 ± 0.01a	0.066 ± 0.01b	0.043 ± 0.01a	0.541 ± 0.06e	0.082 ± 0.00b	0.045 ± 0.01b	0.063 ± 0.00b	0.495 ± 0.00c	0.019 ± 0.00a	0.027 ± 0.01a	0.237 ± 0.00b	0.031 ± 0.01a
15	Bis(2-ethylhexyl) phthalate	0.024 ± 0.02a	0.022 ± 0.01a	0.025 ± 0.01a	0.089 ± 0.03b	0.040 ± 0.01b	0.014 ± 0.01a	0.020 ± 0.02b	0.034 ± 0.01a	0.012 ± 0.01a	0.027 ± 0.00a	0.045 ± 0.06a	0.068 ± 0.08a
21	Tyramine, N-formyl	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.088 ± 0.01a	Nd	0.208 ± 0.10a

The values are the mean of two determinations ± standard deviation. Values with different letters (a–e) in the same row are significantly different ($p < 0.05$) as determined by Scott–Knott's test.
ND = not detected.

T. delbrueckii CCMA 0234, *T. delbrueckii* CCMA 0235, and *P. caribbica* CCMA 0198 were selected based on their fast growth on the cassava substrate (data not shown) and were tested in a co-culture with the amylolytic *L. fermentum* CCMA 0215. The introduction of starter cultures in traditional small-scale fermentations should incorporate considerations for improving processing conditions and product quality through: (i) rapid accelerated metabolic activities (acidification or alcohol production); (ii) improved and more predictable fermentation processes; (iii) desirable sensory attributes; and (iv) improved safety and reduced hygienic and toxicological risks (Holzapfel, 1997). A fast drop in the pH from 6.0 to 4.0 was observed in all the co-culture fermentations. This fact may be related to the lactic acid production which was higher (>2.0 g/L at 48 h) in the fermentations containing LAB (single and co-culture). Malic acid was rapidly consumed, which was probably converted to lactic acid by LAB in the malolactic fermentation (MLF). The essential role of MLF in winemaking is not only because it reduces the wine acidity, but also because it contributes to the microbial stability of the final product and its organoleptic quality (Moreno-Arribas & Polo, 2005). Interestingly, tartaric acid was detected only in the fermentations performed in a co-culture of LAB and yeast. In general, this acid is present in grapes and does not greatly vary during winemaking (Francesca et al., 2014). Our results suggested an interaction between LAB and yeast for tartaric acid production. This acid was detected in the cocoa pulp-based kefir beverages, which are composed of bacteria (including LAB) and yeast consortia (Puerari, Magalhães, & Schwan, 2012). Regarding succinic acid, the highest concentrations (>0.1 g/L) were observed in the assays containing single yeasts. This fact has also been described in other studies with LAB and yeast co-cultures (Álvarez-Martín, Flórez, Hernández-Barranco, & Mayo, 2008; Gadaga, Mtukumira, & Narvhus, 2001). The organic acids produced by yeast and bacterial species contribute to the refreshing flavor, unique aroma, and texture, while also controlling the growth of food spoilage microorganisms (Duarte et al., 2010).

Ethanol was detected (0.05 to 0.29% w/v) in all assays (single and co-culture), except in LAB single culture. Heterolactic LAB, like *L. fermentum*, produces besides lactic acid, ethanol, CO_2 and acetic acid via hexose pathway. In this study, acetic acid was detected only during the single LAB fermentation (less than 1 g/L) and this could explain the ethanol absence. Heterofermentative LAB can also convert acetyl phosphate to acetic acid in the presence of alternative electron acceptors, like the oxygen, instead of ethanol (Von Wright & Axelsson, 2012). Ethanol concentration was approximately three times higher (an average of 0.15% w/v of ethanol) in the single assays inoculated with the yeasts than in co-cultures. It may be due to competition by nutrients between yeast and LAB in co-cultivation and, consequently, the lack of fermentable sugars for ethanol's production by yeasts. However, ethanol concentrations were lower than 0.5% in all cultures, so the final product cannot be considered an alcoholic beverage in Brazil (Brasil, 2009). The non-*Saccharomyces* yeasts such as *T. delbrieckii* and *P. caribbica* showed ethanol production in low concentration. These yeasts are commonly associated with wine quality and are reported to have a positive effect on the taste and aroma of alcoholic beverages (Fleet, 2003; Lappe-Oliveras1, et al., 2008). Glycerol concentration was approximately five times higher (an average of 0.1% w/v of glycerol) in co-cultivation assays than in single-yeast cultivations. It is known that glycerol formation is coupled to yeast growth and is also formed in response to stressful conditions (Walker, 1998), which could be imposed by competition with LAB.

The production of volatile components varied widely between single and co-cultures. LAB and yeast interaction led to fluctuation in the volatile compounds produced, and this is in concurrence with other studies (Álvarez-Martín et al., 2008; Gadaga et al., 2001; Kamda et al., 2015). In general, the single cultures of yeasts were correlated to a high number of volatile compounds. Yeasts may produce a wide variety of volatile compounds, such as alcohols, esters, terpenes, lactones, aldehydes, and ketones, which contribute to the flavor of fermented foods and to the organoleptic properties (Romano, Suzzi, Domizio, & Fatichenti,

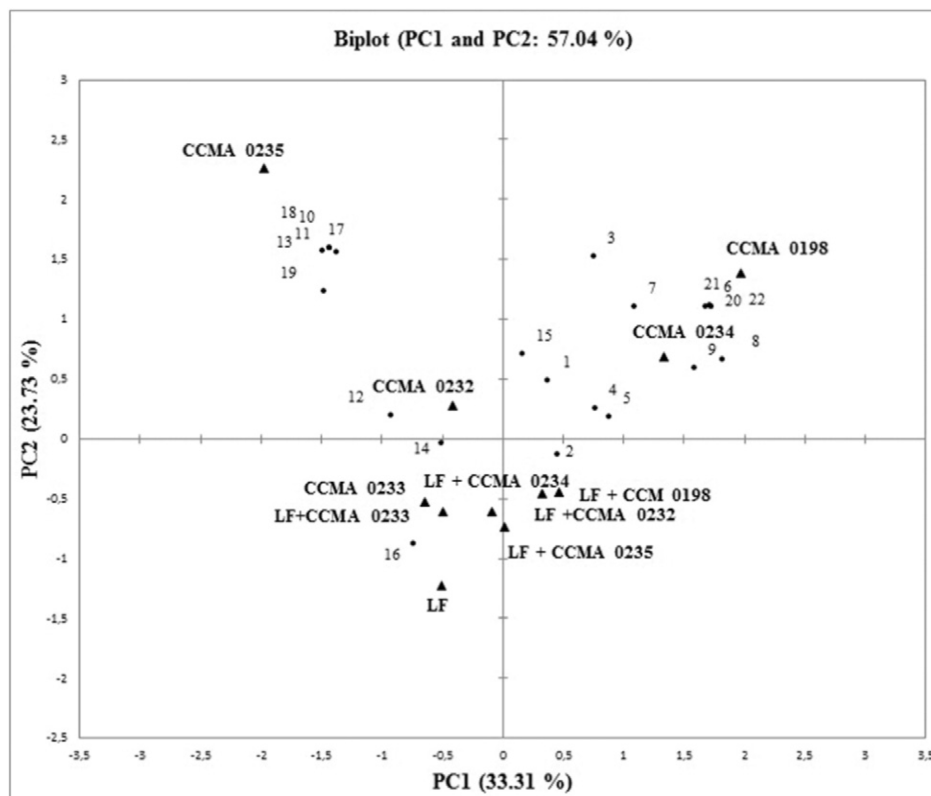

Fig. 4. Principal component analysis (PCA) of organic acids and alcohols (HPLC) obtained by single and co-cultive fermentations of cassava substrate after 24 h (organic acids) and 48 h (alcohols). LF = *L. fermentum*.

1997; Schwan & Ramos, 2014). The compounds 2-phenylethyl alcohol (odor of roses), 1-butanol, 3-methyl or isoamyl alcohol (banana and pear-like aroma), and acetoin (buttery odor) (Lyumugabe, Uyisenga, Songa, & Thonart, 2014; Samappito & Butkhup, 2010; Swiegers, Bartowsky, Henschke, & Pretorius, 2005) are important to the aromatic profile of many beverages, like wines, beers, and mezcal (Cárdenas, Ledesma, Ruiz Holgado, & Oliver, 1985; Katarína, Katarína, Katarína, Ivan, & Fedor, 2014; León-Rodríguez, González-Hernández, la Rosa, Escalante-Minakata, & López, 2006; Lyumugabe et al., 2014; Riu-Aumatell, Miró, Serra-Cayuela, Buxaderas, & López-Tamames, 2014). However, these compounds must be present in food in concentrations higher than the odor and/or taste thresholds (lowest concentration of a compound that is just enough for its recognition). The compound 1-butanol, 3-methyl was detected in concentrations above of its odor threshold (0.071 μg/mL) mainly on the single yeast cultures, while the 2-phenylethyl alcohol, also related to yeast's metabolism was detected in concentrations below odor threshold (0.479 μg/mL) (Tandon, Baldwin, & Shewfelt, 2000). Butanoic acid is another volatile compound produced during fermentation, and it was detected in its highest concentration in the single assays with the yeasts *T. delbrueckii* CCMA 0234 and *P. caribbica* CCMA 0198, both much higher than its odor threshold value (0.173 μg/mL) (Ferreira, López, & Cacho, 2000). This compound has an unpleasant smell and acrid taste, being responsible for rancid butter-like aromas, and therefore it is undesirable. Although, many volatile compounds are associated with microbial metabolism, some are related to vegetal origin and cannot be metabolized by microorganisms, e.g. 3-penten-2-ol; 2-butenal, 3-methyl; 1,1-dimethyl-3-chloropropanol; 2-hexanol; 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester; dibutyl phthalate; squalene; and bis(2-ethylhexyl) phthalate whose concentration did not vary between substrate and fermentation assays.

5. Conclusion

This study demonstrated the possibility of using the combination of LAB and yeast as starter cultures in the cassava fermentation process. The co-cultures of yeasts and LAB have the ability to improve the safety of the product by lowering pH and by producing organic acids (e.g. lactic and tartaric acids). Although most of the volatile compounds were correlated with the assays containing single yeasts, some pleasant odors were correlated with the co-culture fermentations. Therefore, pilot-scale fermentations with the co-cultures that focus on nutritional and sensory properties should be performed for future development of an innovative non-dairy functional cassava fermented beverage.

Acknowledgment

The authors thank the agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial and scholarship support.

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