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ORIGINAL ARTICLE

Antagonistic lactic acid bacteria in association with *Saccharomyces cerevisiae* as starter cultures for standardization of sour cassava starch production

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Abstract In order to improve cassava's palatability and reduce its toxicity, this root is fermented and applied in foods, such as sour cassava starch used to prepare cheese bread and biscuits. This fermentation occurs spontaneously with lactic acid bacteria (LAB) and yeasts. However, it remains an empirical process, with long duration and lack of product quality homogeneity. This work aims to use starter cultures in a pilot-scale fermentation process for the production of sour cassava starch. After differentiation of strains, *Lactobacillus plantarum Lp*3, which exhibited great total titratable acidity (TTA) (5.01 ± 0.05%) and antagonistic activity against *Bacillus cereus*, *Escherichia coli* and *Salmonella* Typhimurium, together with *Lactobacillus brevis Lb*9 (with lesser TTA values: 2.71 ± 0.10%, but amylolytic activity: 2.75 ± 0.61 mm) were tested as single and co-cultures with *Saccharomyces cerevisiae* UFMG-A1007. LAB and yeasts were inoculated at counts of 8 and 7 $\log_{10}$ CFU/g, respectively, and they remained until the 28th day only in co-culture, highlighting the importance of the yeast for the LAB viability. Although single cultures lead to higher acidity during fermentation, the final product acidity obtained with single cultures did not differ from the acidity obtained with *L. plantarum Lp*3 in association with *S. cerevisiae* UFMG-A1007. Therefore, this co-culture exhibited higher potential to be tested as a starter culture in industrial-scale fermentation studies because both microorganisms were in high counts until the end of fermentation and contributed to a final product safe for human consumption, with satisfactory acidity, expansion capacity, and physicochemical properties.

Keywords Starter cultures · Lactic acid bacteria · *Saccharomyces cerevisiae* · Fermentation · Sour cassava starch

Introduction

Cassava (*Manihot esculenta* Crantz) is extensively cultivated in tropical and subtropical countries and it is considered a primary staple with a world per capita consumption of 21.0 kg/year (FAO 2016). Brazil is responsible for 10% of the root produced worldwide, with a production of 21,082,867 tons of cassava in 2016 (FAOSTAT 2016).

Cassava fermentation aims to increase the durability of the root and its nutritional value, decrease toxicity, and give the final product characteristic properties and flavor (Padonou et al. 2010). Cassava traditional fermentation originates several products, including sour cassava starch, which is widely consumed in Brazil in the form of biscuits and cheese bread. Nowadays, the demand for sour cassava starch in international markets has increased due to its use in gluten-free baked products (Granza et al. 2018). Sour

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cassava starch fermentation occurs from the microbiota present in the water, the root or the fermentation tanks and is generally composed of lactic acid bacteria (LAB), which are the microorganisms responsible for reducing the pH and conferring innocuity to the final product (Freire et al. 2015; Lacerda et al. 2005, 2011).

Besides the LAB, yeasts are also present in cassava fermentation. The yeast *Saccharomyces cerevisiae* is commonly used in several fermentation processes to obtain beverages, such as beer and wine, and to obtain foods, such as steamed bread (Albertin et al. 2011; Zhang et al. 2018). Its incorporation into the process of traditional cassava fermentation contributes to LAB growth, increases the quality (aroma, flavor, softness, and nutritional value improvements) of the final product, besides significant fermentation time reduction (Freire et al. 2014; Schwan et al. 2007).

However, the lack of standardization, due to the complex microbiota responsible for the fermentation, causes the sour cassava starches produced to have varied physicochemical characteristics. Consequently, the implementation of starter cultures could generate processes that are less rudimentary, agiler, safer, and standardized (Penido et al. 2018). Thus, the objective of this study was to use the LAB, *Lactobacillus plantarum* and *Lactobacillus brevis*, in association with *Saccharomyces cerevisiae* UFMG-A1007 as starter cultures for the production of sour cassava starch on a pilot-scale fermentation standardized process.

Materials and methods

Material

All the chemicals (analytical grade) were from Merck® (Darmstadt, Germany), the culture media were from Acumedia (Lansing, MI, USA), and the primers and enzymes were from Invitrogen (Carlsbad, CA, USA).

Microorganisms

Ten isolates of *L. plantarum* (*Lp*1–*Lp*10) and twelve isolates of *L. brevis* (*Lb*1–*Lb*12) identified in a previous study from a cassava flour manufacturer located in Formiga (MG, Brazil) (Penido et al. 2018) were included in the present study. The LAB isolates were numbered chronologically according to the successive sample collections made in the cassava flour manufacturer and the letters *Lb* were used for the isolates of *L. brevis* and *Lp* for the isolates of *L. plantarum*. *S. cerevisiae* UFMG-A1007 was supplied by the Laboratory of Taxonomy, Biodiversity, and Biotechnology of Fungi from the Department of Microbiology, Universidade Federal de Minas Gerais, Brazil. Standard strains were obtained from culture collections: *Bacillus cereus* American Type Culture Collection (ATCC) 11778, *Escherichia coli* ATCC 25922 and *Salmonella* Typhimurium CCD S004.

Differentiation of *Lactobacillus plantarum* strains

The isolates from *L. plantarum* were subjected to a repetitive element sequence-based polymerase chain reaction (rep-PCR) fingerprinting technique using the primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) adapted from Gevers et al. (2001) for the verification of the occurrence of different strains among the isolates. The bacterial DNA was extracted using the lytic method. Pure colonies of each LAB were resuspended in 1 mL of lithium chloride and vortexed. The suspension was washed twice with 1 mL of phosphate-buffered saline and centrifuged (MiniSpin® Eppendorf, Hamburg, Germany) (8000 rpm, 5 min). Five hundred microliters of lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), and 25 mM saccharose containing 10 mg mL^{-1} lysozyme) were added and the suspension taken to the shaker incubator CT712 (Cientec, Viçosa, MG, Brazil) (150 rpm, 1 h, 37 °C). Ten microliters of proteinase K were added and the tubes incubated (1 h, 55 °C). After the addition of 0.3 g of zirconia beads, the suspension was stirred (30 min) and centrifuged (12,000 rpm, 10 min). A volume of 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged (12,000 rpm, 10 min). About 1/10 volume of 3 M sodium acetate and 2 volumes of ice-cold absolute ethanol were added to the aqueous phase and the tubes were stored at − 20 °C overnight. The mixture was centrifuged (14,000 rpm, 12 min), oven dried (10 min, 75 °C), and resuspended with 50 µL of Tris–EDTA.

Purification of the DNA was performed with the Wizard SV Genomic DNA Purification System kit (Promega Corporation, Madison, Wisconsin, USA). Lysis buffer (200 µL) and silica were added to the tubes, which were centrifuged (13,000 rpm, 3 min). Six hundred and fifty microliters of ethanol were added and new centrifugation performed. The contents were eluted with 100 µL of nuclease-free water heated to 70 °C and centrifuged (13,000 rpm, 3 min).

For the amplification reaction by rep-PCR fingerprinting, the reagents used were 10 µL of Pre-Mix IV-B 2X, 20 µM of the primer $(GTG)_5$ 10 u/µL, 1 µM $MgCl_2$, and 50 ng DNA, in a final volume of 20 µL. Amplification occurred by initial denaturation at 94 °C for 7 min, 30 cycles containing the denaturation steps at 94 °C for 1 min, annealing at 40 °C for 1 min and extension at 65 °C for 8 min, final extension at 65 °C for 16 min. The PCR

products were separated by 1.5% agarose gel electrophoresis for 16 h at a voltage of 1.55 V/cm.

Selection of lactic acid bacteria for use as starter cultures

The LAB isolates were tested for antagonistic activity against *B. cereus* ATCC 11778, *E. coli* ATCC 25922 and *S.* Typhimurium CCD S004 strains by the spot-on-lawn method to verify direct inhibitory activity (Oliveira et al. 2008). The isolates were cultured on de Man, Rogosa and Sharpe (MRS) agar with the aid of sterilized toothpicks and incubated in 2.5 L anaerobic jars (Permution, Curitiba, PR, Brazil) at 37 °C for 48 h. After this period, the plates were placed upside down and 1 mL of chloroform was introduced into their lids. The supernatant with the revealing cultures consisted of *B. cereus*, *E. coli*, and *S.* Typhimurium strains, which were previously cultured in brain heart infusion (BHI) broth, *Escherichia coli* broth, and selenite cystine broth, respectively. The developing cultures were incubated at 37 °C for 48 h. In the preparation of *B. cereus*, *E. coli* and *S.* Typhimurium supernatants, 500, 300, and 500 µL of the incubated broth were inoculated in 15 mL of 0.9% semisolid BHI, nutrient, and nutrient agar, respectively. The LAB colonies grown on MRS agar were sealed and a 15 mL overlay of semi-solid agar containing each of the pathogenic cultures was added. After solidification, the plate was incubated at 37 °C for 48 h. Inhibition halos formed around the LAB inoculum site, which consisted of a clear zone, indicated the inhibition of pathogenic bacteria. Diameters of these halos were measured using a universal pachymeter (Series 530, Mitutoyo, Takatsu-ku, Kanagawa, Japan).

All LAB isolates were tested for degradation of starch on MRS agar plates plus 2% soluble starch incubated at 37 °C for 48 h in anaerobic jars. After growth, the revelation was performed with Lugol's solution for the visualization of starch hydrolysis halos, measured with the aid of a universal pachymeter.

The production of total acids was also evaluated. Aliquots of 10 mL of the broth were used for titration of total acidity (AOAC 2016).

Pilot-scale fermentation

For the production of sour cassava starch, four starter cultures were tested: *L. plantarum* (starter culture A), *L. brevis* (starter culture B), *L. plantarum* in co-culture with *S. cerevisiae* UFMG-A1007 (starter culture C); and *L. brevis* in co-culture with *S. cerevisiae* UFMG-A1007 (starter culture D). The experiments were conducted in four batches, each of which contained the four treatments ordered randomly. In total, there were four replicates for each treatment tested.

Single and co-cultures submerged fermentation processes were conducted by inoculating the starter cultures into 100 mL of a sterilized culture medium (20 g L^{-1} non-fermented cassava starch, 10 g L^{-1} glucose and 5 g L^{-1} beef extract) incubated at room temperature (24–48 h), which was used to inoculate 500 mL of the same medium for the same period. The resulting broth was used to inoculate a 5-L bioreactor containing 10% w/v of commercial cassava starch (non-fermented and non-sterilized) and distilled water, for 28 days (Penido et al. 2018). Samples of 10 mL of several points of the bioreactor were collected at 0, 7, 14, 21 and 28 days of fermentation to determine the pH, TTA (AOAC 2016) and viability of starter cultures. After the end of the fermentation, the material was sun-dried for approximately 8 h and stored under refrigeration temperature.

Monitoring of starter cultures during pilot-scale fermentation

LAB and yeast monitoring were performed by isolating one representative of each starter culture (on MRS or yeast extract-malt extract agar, respectively) per week and per block to be purified and stored at − 86 °C. The DNA of LAB was extracted and submitted to PCR amplification of the 16S rRNA gene and analysis of the molecular profiles obtained by Restriction Fragment Length Polymorphism (RFLP) (Penido et al. 2018). The molecular profiles obtained between the isolates at different fermentation times 0 (pure starter culture), 7, 14, 21 and 28 days were compared. These molecular profiles were also compared with the molecular profiles of *L. brevis* and *L. plantarum* identified in a previous study (Penido et al. 2018). The yeast mitochondrial DNA (mtDNA) was extracted according to the methodology described by Querol and Barrio (1990) and Querol et al. (1992) with some modifications. The cells were grown in 1.2 mL of YPD (1% yeast extract, 2% peptone and 1% glucose) medium at 26–28 °C with overnight incubation and centrifuged (12,000 rpm, 3 min). The pellet was washed with sterile distilled water and centrifuged under the same conditions as above. The pellet was resuspended in 500 µL of Solution I (0.9 M Sorbitol and 0.1 M EDTA), and 30 µL of lytic enzyme Zimoliase 20 T (1 µg/µL dissolved in Solution I) were added. The tubes were homogenized and incubated (30 min, 37 °C).

After that, the samples were centrifuged (12,000 rpm, 1 min), the pellet was resuspended in 500 µL of Solution II (50 mM Tris–HCl and 20 mM EDTA, pH 7.4), and 13 µL of 10% SDS were added. The tubes were homogenized and incubated (5 min, 65 °C). 200 µL of 3 M potassium

acetate was added to the samples, which were kept at − 20 °C for 1 h. After this procedure, the cells were centrifuged (14,000 rpm, 15 min, 4 °C).

Seven hundred microliters of isopropanol were added to the supernatant and centrifuged (12,000 rpm, 10 min). Five hundred microliters of 70% ethanol were added to the tubes, which were centrifuged again (12,000 rpm, 5 min). The DNA left after elimination of the ethanol was hydrated with 20 µL of diethyl pyrocarbonate (DEPC) treated water and stored at − 20 °C.

DNA digestion was performed using 20 µL of the total DNA plus 10 µL of a mixture containing 4.0 µL of DEPC-treated water, 3 µL of 10× *Hinf*I enzyme buffer, 1.5 µL RNase (20 mg/mL), 1.5 µL restriction enzyme *Hinf*I. The tubes were incubated at 37 °C overnight. Digestion products (plus GelRed ™ and 6× run buffer) were separated and analyzed by 1.0% agarose gel electrophoresis (80 V for 150 min) in 0.5× TBE. The generated mtDNA restriction profiles were visualized under UV light and photographed using an image capture system. The molecular profiles obtained between the isolates at different fermentation times 0 (pure starter culture), 7, 14, 21 and 28 days were compared.

Physicochemical and microbiological evaluation

The physicochemical characteristics of the different sour cassava starches produced in a pilot-scale fermentation process were used as criteria to evaluate the starter cultures tested. TTA was determined by titration with 0.1 M sodium hydroxide using phenolphthalein as an indicator and pH was determined by potentiometry. The expansion capacity was calculated by the ratio of the sour cassava starch cookie diameter after baking for a period of 20 min at 220 °C and the initial diameter (Maeda and Cereda 2001). The moisture content was obtained by the oven drying method at 105 °C and the ash content was determined by incineration of the samples in a muffle furnace at 550 °C (AOAC 2016). The starch content was obtained through the energetic hydrolysis of the starch in a strongly acid medium (Cereda et al. 2004).

These different sour cassava starches produced were submitted to microbiological analyses to search for *Bacillus cereus* (by the plate count method for *B. cereus* in foods), fecal coliforms (by the most probable number method for total coliforms, thermotolerant coliforms and *E. coli* in foods), and *Salmonella* spp. (by the presence/absence method for *Salmonella* in foods) (Da Silva et al. 2012), and compared to microbiological specification from Brazilian legislation (Brazil 2001).

Statistical analysis

All analyses were performed in triplicate. Data were subjected to analysis of variance (ANOVA) and significant differences between means were determined by Duncan's test (0.05 significance level). Minitab® 18 (Minitab Incorporated, State College, PA, USA) was used for the statistical analysis.

Results and discussion

Lactobacillus plantarum strains

Rep-PCR fingerprinting was used to distinguish the genetic relationships among the ten *L. plantarum* isolates. The dendrogram revealed a low genetic heterogeneity among the isolates (Fig. 1). All the isolates showed a similarity higher than 70% and were considered to be of the same species (De Vuyst et al. 2008). According to Gevers et al. (2001), the similarity index between bands patterns from the same strains can vary from 91 to 97%. Most isolates of *L. plantarum* showed similarity higher than 91% and were considered as belonging to the same strain, with some exceptions: the isolates *Lp*6, *Lp*9, and *Lp*10. In addition, some isolates appear to be indistinguishable, such as *Lp*1, *Lp*3, and *Lp*4, besides *Lp*7 and *Lp*8.

The differentiation of strains should be one of the first steps in the selection of starter cultures because the technological, probiotic, antagonistic and sensorial capacities are specific to each strain (De Las Rivas et al. 2006). This differentiation may contribute to the selection of a strain with the desirable characteristics for the fermentation process. The technique of Gevers et al. (2001) used in the present study was already used to verify the predominant LAB and yeast (*Lactobacillus fermentum* and *Pichia kudriavzevii*, respectively), at strain level, during the production of *mawè*, a mass produced by the spontaneous fermentation of cereals in West African countries (Houngbédji et al. 2018). The differentiation of *L. plantarum* is important due to the high similarity between the sequences of this species and homologous species.

Selection of lactic acid bacteria for use as starter cultures

Among the important characteristics taken into consideration while selecting a starter culture, the antagonistic activity against food pathogens is crucial because it may contribute to the obtainment of an innocuous final product. Moreover, this antagonistic activity plays an important role in competitiveness since the inoculated microorganism must be able to predominate during fermentation. At least

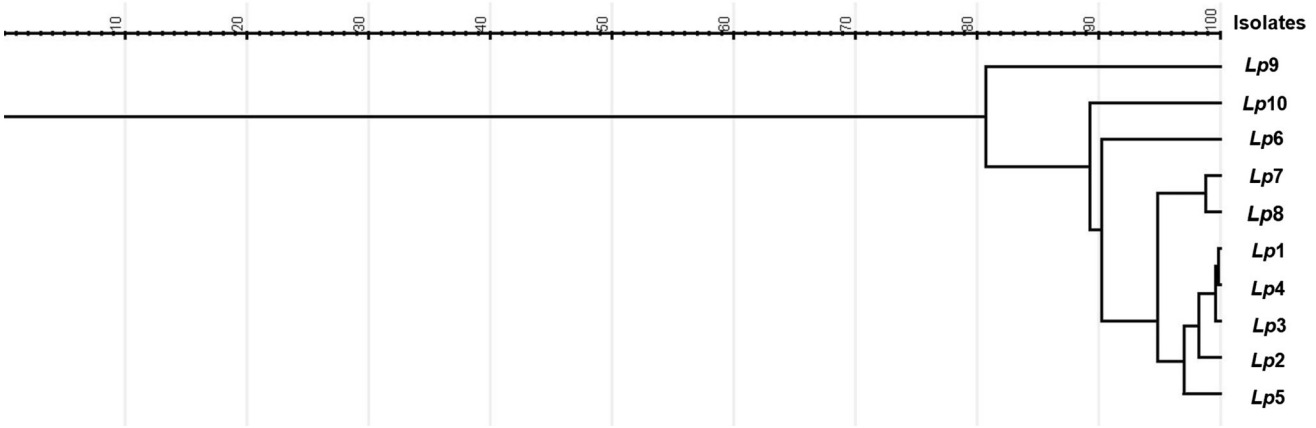

Fig. 1 Dendrogram obtained after cluster analysis of the digitized $(GTG)_5$-PCR fingerprints of *Lactobacillus plantarum* strains isolated from the fermentation process of sour cassava starch. Similarity (%) between patterns was calculated using the Pearson correlation coefficient

one isolate of *L. plantarum* and *L. brevis* was able to inhibit all the pathogens tested. The largest inhibition halo was shown against *S*. Typhimurium, followed by *E. coli* and *B. cereus*, in that exact order for all *L. plantarum* isolates. Regarding *L. brevis* isolates, inhibition halos against *S*. Typhimurium were larger than inhibition halos against *B. cereus*, and, for some isolates (*Lb*2, *Lb*3, *Lb*8, *Lb*9, *Lb*11, and *Lb*12), the inhibition of *E. coli* was even greater than the inhibition of *B. cereus* (Fig. 2).

The isolates of LAB exhibited a varied degree of inhibition against the different pathogens. Among the 10 isolates of *L. plantarum*, 7 exhibited antagonistic activity against *B. cereus* and only 1 out of 12 isolates of *L. brevis* exhibited antagonistic activity against *B. cereus* (Figs. 3, 4). All isolates of *L. plantarum* and *L. brevis* exhibited inhibition halos against *E. coli* and *S*. Typhimurium. As a result, it is possible to suggest that most *L. plantarum* isolates evaluated in the present work (*Lp*1, *Lp*2, *Lp*3, *Lp*4, *Lp*5, *Lp*6, and *Lp*8) and one isolate of *L. brevis* (*Lb*4) had satisfactory antagonistic effects and are capable of inhibiting the main pathogens frequently investigated in the sour cassava starch, which are responsible for foodborne diseases when the matrix is a starchy flour.

Adebayo et al. (2013) investigated the antimicrobial properties of the LAB (*Leuconostoc lactis*, *L. plantarum*, *L. fermentum*, *Leuconostoc carnosum* and *L. brevis*) isolated during cassava fermentation for the production of *fufu* using the agar well assay method, against the following bacteria: *Shigella flexi*, *Bacillus subtilis*, and *E. coli*. The results showed that, except for *L. fermentum*, all LAB exhibited inhibitory activity against the enteropathogenic bacteria tested. The authors attributed this inhibitory effect

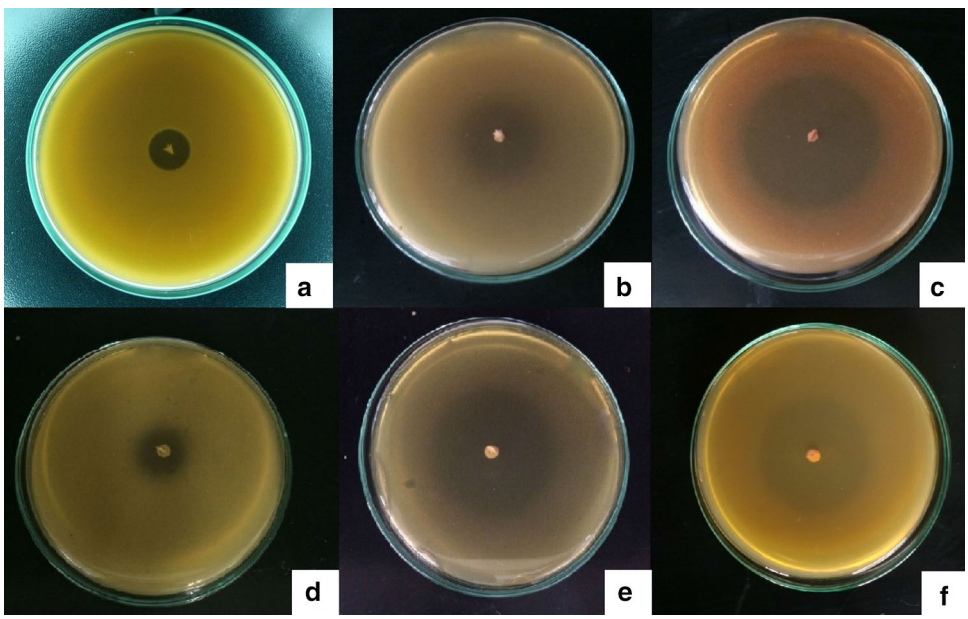

Fig. 2 Inhibition halos of *Lactobacillus plantarum* against *Bacillus cereus* (**a**), *Escherichia coli* (**b**) and *Salmonella* Typhimurium (**c**); inhibition halos of *Lactobacillus brevis* against *Bacillus cereus* (**d**), *Escherichia coli* (**e**) and *Salmonella* Typhimurium (**f**) by the spot-on-lawn method

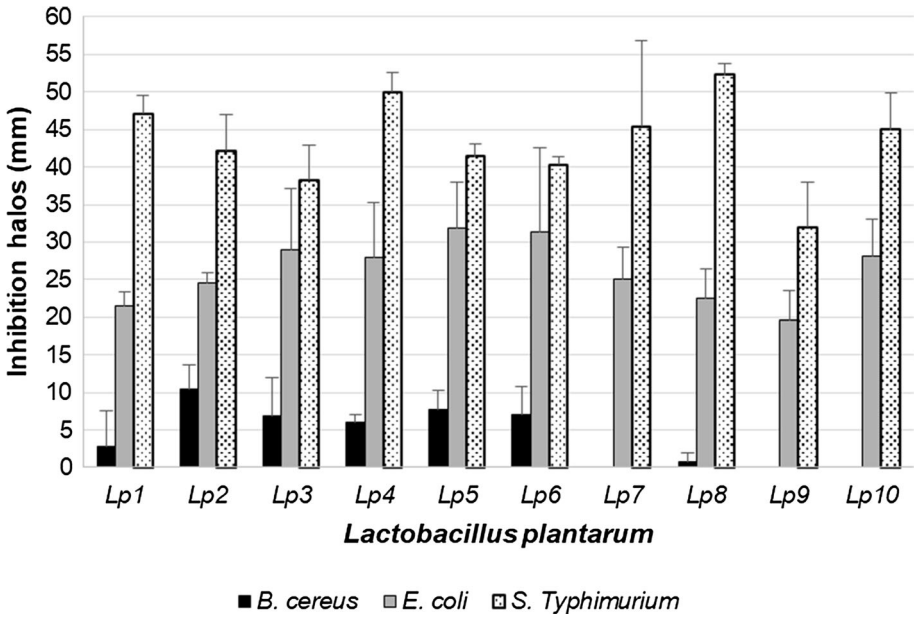

Fig. 3 The diameter of inhibition halos (mm) of *Lactobacillus plantarum* isolates (*Lp*1–*Lp*10) against *Bacillus cereus*, *Escherichia coli*, and *Salmonella* Typhimurium by the spot-on-lawn method

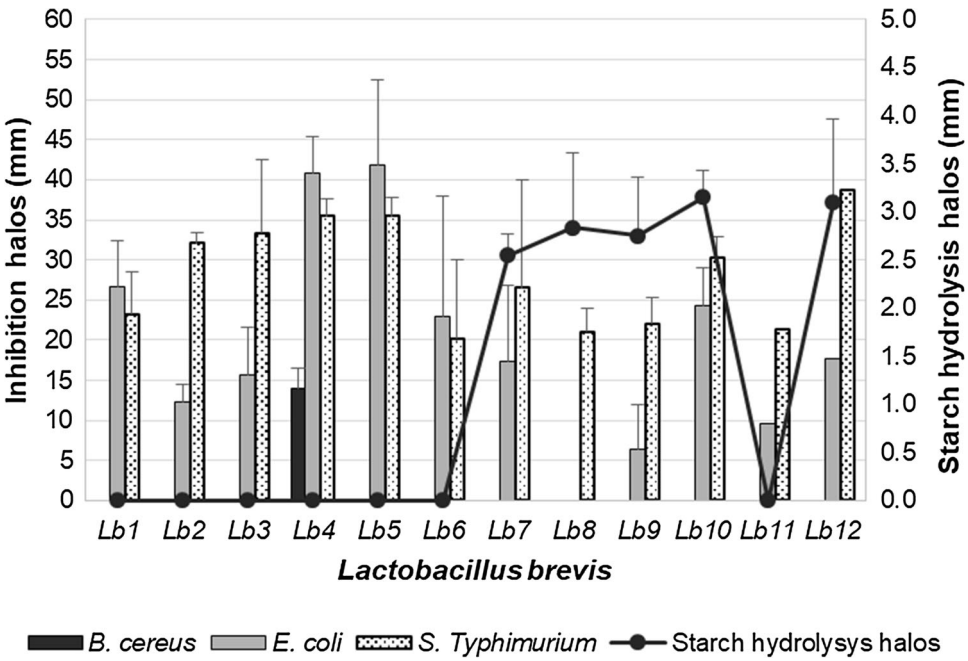

Fig. 4 The diameter of starch hydrolysis halos (mm) and inhibition halos (mm) of *Lactobacillus brevis* isolates (*Lb*1–*Lb*12) against *Bacillus cereus*, *Escherichia coli*, and *Salmonella* Typhimurium by the spot-on-lawn method

of the LAB to the production of bacteriocins, which probably were produced because of stimulation by the presence of competing microorganisms.

Anyogu et al. (2014) evaluated the antimicrobial activity, by the agar diffusion method, of the LAB isolated and identified from the submerged cassava fermentation. Four isolates of *L. plantarum*, the predominant species, exhibited inhibitory activity classified as strong, with zones of inhibition of 2–4 mm, in relation to *B. cereus*. For *E. coli* and *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium), the activity was weak for two isolates and strong for the other two. Only two isolates exhibited antimicrobial activity against *S. aureus*. The antimicrobial effect was attributed to the production of organic acids because it was no longer observed after neutralization.

It is already known that the LAB, commonly predominant in cassava fermentation, are able to inhibit the growth of pathogenic microorganisms frequently found in starchy products. As a result, the action of these bacteria does not allow the proliferation of pathogens during the fermentation process, contributing to the innocuity of the final product. This is mainly due to the acidification power of

LAB, which can decrease the pH of the medium to values low enough to inhibit the growth of most pathogenic and deteriorating microorganisms. However, some authors report that some strains of *L. plantarum* can be bacteriocinogenic (Botthoulath et al. 2018).

Another technologically interesting feature in a starter culture for cassava fermentation is amylolytic capacity once it makes it possible to hydrolyze root starch releasing simple sugars for other microorganisms to use. The LAB convert these simple sugars into organic acids (Oyewole 2001). Unfortunately, none of *L. plantarum* isolates exhibited amylolytic activity. However, *L. brevis* isolates *Lb*7, *Lb*8, *Lb*9, *Lb*10 and *Lb*12 showed starch degradation halos ranging from 2.55 ± 0.22 mm to 3.15 ± 0.28 mm (Fig. 4). There are reports in the literature of amylolytic lactic acid bacterial strains isolated from traditional cassava fermentation, such as *Lactobacillus acidophilus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *L. brevis*, *L. fermentum*, *Lactobacillus manihotivorans* and *L. plantarum* (Freire et al. 2017; Ramos et al. 2015; Reddy et al. 2008).

In the evaluation of the acidification capacity, the TTA values were higher with 48 h than with 24 h and 0 h, in this order, for all isolates of *L. plantarum* and *L. brevis*. At the initial time, the TTA values did not differ between the different isolates of both species (0.95–1.22% for *L. plantarum* and 0.59–0.71% for *L. brevis*). However, this situation changed over time. For *L. plantarum*, at 24 h, the *Lp*1 isolate exhibited the highest TTA value (3.21 ± 0.09%), followed by the isolates *Lp*6 and *Lp*3; and, at 48 h, the isolate *Lp*1 (5.62 ± 0.65%), followed by the isolate *Lp*3. For *L. brevis*, both at 24 h and 48 h, the *Lb*12 isolate (3.50 ± 0.03% and 5.34 ± 0.03%, respectively) had the highest acidity value, followed by the isolates *Lb*4 and *Lb*5. One of the most relevant characteristics for potential starter cultures is their acidification ability because acid production and consequent lowering of pH allow the development of aromatic compounds and prolong the lag phase of sensitive organisms, including pathogens frequently found in foods (Kostinek et al. 2007).

For the production of sour cassava starch in a pilot-scale fermentation process, an isolate from each LAB was selected. The isolate of *L. plantarum* selected was *Lp*3. This isolate exhibited antagonistic activity against all pathogens tested, being in the group with the largest halos of inhibition against *B. cereus* and *E. coli*; and good acidification ability with both 24 h (2.69 ± 0.05%) and 48 h (5.01 ± 0.05%). The selected isolate of *L. brevis* was *Lb*9. This isolate exhibited antagonistic activity against *E. coli* and *S.* Typhimurium; starch degradation halo (2.75 ± 0.61 mm); and a satisfactory acidification capacity for both 24 h (1.42 ± 0.05%) and 48 h (2.71 ± 0.10%). The isolate *Lb*4, which was the only isolate of *L. brevis* able to inhibit *B. cereus*, was not taken into consideration because it did not exhibit amylolytic activity.

Pilot-scale fermentation

For the production of sour cassava starch in a pilot-scale fermentation process, the following starter cultures were tested: *L. plantarum Lp*3 (starter culture A), *L. brevis Lb*9 (starter culture B), *L. plantarum Lp*3 in association with *S. cerevisiae* UFMG-A1007 (starter culture C), and *L. brevis Lb*9 in association with *S. cerevisiae* UFMG-A1007 (starter culture D).

The pH and TTA values of sour cassava starch samples were measured weekly during cassava starch fermentation (Table 1). In relation to the pH, the different starter cultures did not differ over time, but differed from each other. From the seventh day of fermentation, the single starter cultures, A and B, had the lowest pH values ($p < 0.05$) when compared to the pH values of the mixed starter cultures, C and D. In regard to TTA, for the single starter cultures, the acidity increased ($p < 0.05$) gradually during the 28 days of fermentation. While the starter culture of *L. plantarum* (A) exhibited the highest values throughout the time, the co-cultures (C and D) slightly increased ($p < 0.05$) the acidity after the fourteenth day of fermentation. The values of pH and TTA obtained by Penido et al. (2018), during the 28 days of the pilot-scale fermentation process, did not differ among the different starter cultures but changed over time. The highest acidity value and the lowest pH value happened on the 7th day of fermentation.

The organic acids produced by the LAB species and, to a lesser extent, by the yeasts, contribute to the characteristic flavor and aroma of sour cassava starch and help to control the growth of deteriorating microorganisms. Cadena et al. (2006) studied the sour cassava starch agroindustry in Colombia. The pH of the supernatant water decrease while the acidity increased for most of the samples analyzed.

L. plantarum, *L. brevis*, and *S. cerevisiae* UFMG-A1007 were inoculated in the bioreactors as starter cultures in the concentration of approximately 8 $\log_{10}$ CFU/g, 8 $\log_{10}$ CFU/g, and 7 $\log_{10}$ CFU/g, respectively. The counts of the different microorganisms used as starter cultures decreased over time, and, after 21 days of fermentation, *L. brevis* (B) was no longer isolated as a single culture. The counts of microorganisms differed only at days 21 and 28 (Table 1).

Saccharomyces cerevisiae UFMG-A1007 (C2 and D2) was present until the end of the fermentation. This may suggest that this strain contributed to the multiplication and maintenance of the LAB used as starter cultures in the present work, especially *L. brevis*, which as a single culture (B) did not survive until the end of fermentation, but in

Table 1 Monitoring of the pH, total titratable acidity and counts of starter cultures during the pilot-scale fermentation process

Starter culture	Time (days)				
	0	7	14	21	28
pH					
A	3.36 ± 0.31^{b}	2.96 ± 0.22^{b}	2.89 ± 0.12^{b}	2.92 ± 0.16^{c}	3.05 ± 0.35^{b}
B	4.10 ± 0.17^{a}	$3.50 + 0.56^{ab}$	2.76 ± 0.23^{b}	2.77 ± 0.39^{c}	2.80 ± 0.57^{b}
C	3.33 ± 0.31^{b}	3.95 ± 0.42^{a}	4.23 ± 0.79^{a}	3.78 ± 0.57^{b}	3.99 ± 0.82^{a}
D	4.09 ± 0.16^{a}	3.89 ± 0.29^{a}	4.41 ± 0.29^{a}	4.55 ± 0.12^{a}	4.25 ± 0.72^{a}
TTA					
A	$0.60 \pm 0.09^{a,z}$	$1.21 \pm 0.10^{a,y}$	$1.74 \pm 0.23^{a,x}$	$1.96 \pm 0.71^{a,x}$	$1.75 \pm 1.00^{a,x}$
B	$0.34 \pm 0.06^{a,y}$	$0.49 \pm 0.15^{b,y}$	$1.12 \pm 0.23^{b,x}$	$1.51 \pm 0.32^{a,x}$	$1.60 \pm 0.53^{a,x}$
C	$0.65 \pm 0.12^{ax,y}$	$0.34 \pm 0.19^{b,y}$	$0.40 \pm 0.27^{c,xy}$	$0.65 \pm 0.24^{b,xy}$	$0.91 \pm 0.46^{b,x}$
D	$0.31 \pm 0.09^{a,x}$	$0.40 \pm 0.05^{b,x}$	$0.36 \pm 0.10^{c,xy}$	$0.44 \pm 0.06^{b,x}$	$0.48 \pm 0.19^{b,x}$
Counts (log_{10} CFU/g)					
A	$7.83 \pm 0.52^{a,w}$	$7.61 \pm 0.25^{a,wx}$	$7.62 \pm 0.61^{a,wx}$	$7.07 \pm 0.22^{a,x}$	$4.64 \pm 3.21^{c,y}$
B	$7.84 \pm 0.74^{a,w}$	$6.95 \pm 0.39^{a,x}$	$6.47 \pm 0.89^{a,x}$	$0.00 \pm 0.00^{b,y}$	$0.00 \pm 0.00^{d,y}$
C1	$7.74 \pm 0.50^{a,w}$	$7.28 \pm 0.25^{a,w}$	$7.41 \pm 0.25^{a,w}$	$7.41 \pm 0.27^{a,w}$	$6.62 \pm 0.60^{a,x}$
C2	$6.82 \pm 1.05^{a,w}$	$7.30 \pm 0.22^{a,wx}$	$6.70 \pm 0.56^{a,x}$	$6.07 \pm 1.15^{a,y}$	$4.80 \pm 2.78^{bc,z}$
D1	$7.81 \pm 0.53^{a,w}$	$7.27 \pm 0.47^{a,x}$	$7.06 \pm 0.63^{a,x}$	$7.02 \pm 0.14^{a,x}$	$5.85 \pm 0.88^{abc,y}$
D2	$6.94 \pm 0.70^{a,w}$	$7.09 \pm 0.28^{a,w}$	$6.73 \pm 0.16^{a,w}$	$6.57 \pm 0.32^{a,wx}$	$6.09 \pm 0.62^{ab,x}$

Mean values ± standard deviation followed by different superscript letters (a, b, c in the same column and w, x, y, z in the same row) for the same parameter evaluated are significantly different ($p < 0.05$). Starter cultures: A: *L. plantarum*; B: *L. brevis*; C: *L. plantarum* (C1) + *S. cerevisiae* (C2); D: *L. brevis* (D1) + *S. cerevisiae* (D2)

association with this strain (D1) was able to remain for 28 days. *L. plantarum* was found in higher counts in co-culture (C1) when compared to the single culture (A). These observations highlight the importance of the yeast for the LAB viability. In addition, *S. cerevisiae* is capable of producing organoleptic compounds that might be present in the final product (Freire et al. 2014). This occurs because the yeast was able to remain until the end of the cassava fermentation process, which was not commonly observed for other species of yeasts found in the production of sour cassava starch (Lacerda et al. 2005; Penido et al. 2018). Padonou et al. (2010) tested different single and mixed starter cultures to obtain *lafun*, an African cassava fermented food, and observed that the product obtained using *S. cerevisiae* presented better sensorial quality and efficiency in the softening process of cassava roots. This yeast was selected as the most suitable starter culture for the production of *lafun*.

Molecular monitoring of the two LAB species inoculated as starter cultures, *L. plantarum* and *L. brevis*, in the pilot-scale fermentation process using the RFLP molecular technique was efficient. These microorganisms were detected in all samples during the entire fermentation, which confirms their presence after being inoculated as starter cultures at the beginning of fermentation and reinforces their potential to be used as starter cultures in the production of sour cassava starch.

Molecular monitoring of the yeast by mtDNA restriction analysis technique was also proven effective. The forty isolates of *S. cerevisiae* exhibited a dominant molecular profile (bands pattern), which corresponds to the molecular profile expected of the strain *S. cerevisiae* UFMG-A1007. It was verified that the restriction enzymes, which cleave the mtDNA in specific regions, were able to create a characteristic number of 72 fragments with various lengths in the lanes for this strain. Consequently, it was possible to observe that the yeast strain used as co-culture in the cassava starch fermentation was present during the entire process.

Physicochemical and microbiological analyses of the sour cassava starches produced

In Table 2, it is possible to observe the results of the physicochemical characterization performed in the four samples of the sour cassava starch obtained from the use of the single starter cultures and co-cultures. The TTA of sour cassava starch samples ranged from 1.03 to 1.92% and single starter cultures exhibited higher TTA values. These results are similar to those presented by Edward et al. (2011), that found titratable acidity of 1.3–1.6% when using lyophilized strains of *L. plantarum*, *L. fermentum*, *Weissella paramesenteroides*, and *Leuconostoc mesenteroides* as starter cultures for *gari* production through

Table 2 TTA, expansion capacity, moisture, starch and ash content of the sour cassava starch samples obtained with the different starter cultures

Starter culture	TTA (%)	Expansion capacity	Moisture content (%)	Starch content (%)	Ash content (%)
A	1.92 ± 0.70[a]	1.14 ± 0.09[a]	13.14 ± 0.68[a]	80.23 ± 8.34[a]	0.07 ± 0.03[a]
B	1.92 ± 0.28[a]	1.11 ± 0.05[a]	12.28 ± 1.67[a]	86.88 ± 3.60[a]	0.07 ± 0.02[a]
C	1.46 ± 0.55[ab]	1.10 ± 0.08[a]	12.66 ± 0.81[a]	83.36 ± 4.09[a]	0.07 ± 0.02[a]
D	1.03 ± 0.15[b]	1.17 ± 0.08[a]	13.24 ± 0.76[a]	83.10 ± 4.89[a]	0.04 ± 0.02[b]

Mean values ± standard deviation followed by different superscript letters in the same column are significantly different ($p < 0.05$). Starter cultures: A: *L. plantarum*; B: *L. brevis*; C: *L. plantarum* + *S. cerevisiae*; D: *L. brevis* + *S. cerevisiae*

cassava solid state fermentation. Although single starter cultures (A and B) lead to higher acidity during fermentation, the final product acidity obtained with these single cultures did not differ from the acidity obtained with starter culture C, *L. plantarum Lp*3 in association with *S. cerevisiae* UFMG-A1007.

It was possible to obtain sour cassava starch with all the four starter cultures tested because, according to the values established by the legislation, it can be considered sour cassava starch when the acidity is between 1.0 and 5.0% (Brazil 1978), and all the samples exhibited TTA among this range. When the acidity is lower than 1.0%, sweet cassava starch is obtained. Therefore, all the four starter cultures tested in this study were able to ferment cassava starch leading to sour cassava starch after 28 days of fermentation.

No significant differences were found between the expansion capacities for the sour cassava starches obtained with the different starter cultures. However, this expansion capacity was higher than 1.00 for all treatments, indicating that the products obtained were able to expand, one of the main technological characteristics responsible for the baking properties of sour cassava starch. Penido et al. (2018) analyzed samples of sour cassava starch obtained after 28 days in a pilot-scale fermentation process and verified expansion capacities (0.98–1.10) similar to the ones in the present study. This indicated that the fermentative process was efficient and capable of promoting a suitable chemical modification with consequent expansion of the final product.

Brazilian legislation defines the physicochemical parameters to determine the quality of the sour cassava starch: maximum moisture of 14%, minimum of 80% of starch and maximum of 0.5% of ash (Brazil 1978). No significant differences were found between moisture and starch content for the sour cassava starch obtained with the different starter cultures. The sour cassava starch obtained with *L. brevis* in co-culture with *S. cerevisiae* UFMG-A1007 presented the lower ash content. Nevertheless, all the samples were within the parameters established by legislation for moisture, acidity, starch and ash content. Marcon et al. (2009) found that commercial samples of fermented and sun-dried sour cassava starch contained a moisture content of approximately 14.19%, higher than the values found in the present study. Additionally, the ash content was around 0.19%.

The sour cassava starches produced in a pilot-scale fermentation process with the aid of selected starter cultures were evaluated for the microbiological parameters and the results, for all samples analyzed, were the absence of *Salmonella* spp. in 25 g and fecal or thermotolerant coliforms < 3 MPN (Most Probable Number)/g. The sour cassava starch obtained with *L. brevis* and *S. cerevisiae* UFMG-A1007 exhibited a count of 1.90×10^1 CFU/g of suggestive colonies of *B. cereus*. Although theses suggestive colonies were found, their count was lower than the tolerance allowed by Brazilian law, which is 3.00×10^3 CFU/g of food (Brazil 2001). This means that all sour cassava starches produced in a pilot-scale fermentation process were suitable for human consumption. It is possible to suggest that the production of antimicrobial metabolites by *L. plantarum* and *L. brevis* associated with a fermentation performed with adequate hygiene procedures can provide control of the pathogenic microorganisms.

Conclusion

From the results obtained, the most suitable isolates to be tested in a pilot-scale fermentation process as starter cultures were *L. plantarum Lp*3, which exhibited great total titratable acidity and antagonistic activity against all pathogens tested, and *L. brevis Lb*9, which exhibited moderate acidity but had amylolytic activity. All four starter cultures tested led to the production of sour cassava starches in accordance with the legislation in both physicochemical and microbiological parameters. Therefore, these products were characterized as suitable for human consumption. The use of single cultures of LAB led to a slightly better TTA for the final product, but their use in co-culture with yeasts is advantageous because the LAB remained viable until the last day of fermentation in higher counts. *L. plantarum* in association with the strain *S. cerevisiae* UFMG-A1007 showed higher potential as a

starter culture in further fermentation studies in an industrial-scale fermentation process, since this culture led to a satisfactory acidity of the final product, assured innocuity, and contributed to the standardization of the sour cassava starch production in a pilot-scale fermentation process.

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