International Journal of Food Microbiology 248 (2017) 39–46

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Nondairy beverage produced by controlled fermentation with potential probiotic starter cultures of lactic acid bacteria and yeast

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ARTICLE INFO

Article history:
Received 21 September 2016
Received in revised form 22 November 2016
Accepted 20 February 2017
Available online 21 February 2017

Keywords:
Cassava
Rice
Lactobacillus plantarum
Lactobacillus acidophilus
Torulaspora delbrueckii
Functional food

ABSTRACT

This work aimed to develop a nondairy fermented beverage from a blend of cassava and rice based on Brazilian indigenous beverage *cauim* using probiotic lactic acid bacteria (LAB) and yeast. The indigenous strains *Lactobacillus plantarum* CCMA 0743 (from *cauim*) and *Torulaspora delbrueckii* CCMA 0235 (from *tarubá*), and the commercial probiotic, *L. acidophilus* LAC-04, were used as starter cultures in single and co-cultivations. The bacteria populations were around 8.0 log (CFU/mL) at the end of all fermentations as recommended for probiotic products. Higher residual starch contents were noted in the single LAB cultures (10.6% [w/w]) than in co-cultures (<6% [w/w]), showing that co-culture may help the digestibility. For all different assays (single and co-culture), lactic acid was the main organic acid detected (>1.6 g/L) and ethanol was lower than 0.5% (w/v) consisting in a non-alcoholic beverage. The assays containing yeast showed the highest antioxidant activity (around 10% by DPPH and ABTS methods). Therefore, a nondairy fermented beverage was successfully obtained, and the co-culture of LAB and *T. delbrueckii* could increase the product's functional properties.

1. Introduction

Consumers worldwide are becoming increasingly aware of the relationship between diet and health, and the market for so-called functional foods has been growing in recent years (Carrillo et al., 2013). Experts estimate that among functional foods, probiotic foods comprise 60–70% of the total market (Tripathi and Giri, 2014). Probiotics are live microorganisms, which when administered in adequate amounts in the diet, deliver health benefits to the host (FAO/WHO, 2002).

Lactic acid bacteria (LAB), including the genera *Lactobacillus* and *Bifidobacterium*, are commonly used in probiotic preparations. Naturally found in traditional fermented foods, *Lactobacillus* species are technologically suitable for food applications, since they are more resistant to low pH and have adaptation to milk and other food substrates (Tripathi and Giri, 2014). Although the LAB are commonly associated with dairy products, this group of microorganisms also plays a role in other food systems, and this versatility incites scientists to search for new applications to obtain novel products for a continuously growing functional food market (Molina et al., 2012).

Traditional fermented foods are rich sources of microorganisms with probiotic characteristics and have been tested as starter cultures for production of many functional beverages, for example traditionally fermented millet alcoholic beverage in Korea (Oh and Jung, 2015), fermented cereal beverages like oat, barley, and malt (Salmerón et al., 2015), and a rice-based fermented beverage (Ghosh et al., 2015). In addition, vegetable-derived substrates are potential sources for the development of new functional foods and beverages, such as a fermented coconut water beverage (Prado et al., 2015), extracts of soy and quinoa (Bianchi et al., 2014), peanut-soy milk (Santos et al., 2014), soybean (Molina et al., 2012), and cassava (Freire et al., 2015), among others.

Cassava (*Manihot esculenta* Crantz) plays an important role in the global food diet, mainly in developing countries. Over 100 countries are producing cassava, and Brazil is the second-largest producer in the world, with a production amount of 21.4 million tons in 2013 (IBGE, 2013). Brazilian indigenous people are traditional producers of cassava fermented foods and beverages, such as *cauim*, *caxiri*, *yakupa*, and *tarubá*, and the microbiota present during the natural substrate fermentation has been already studied (Almeida et al., 2007; Freire et al., 2014; Ramos et al., 2010, 2015; Santos et al., 2012). These studies have shown the co-existence of bacteria, mainly LAB, and yeast during the fermentations. Furthermore, rice (*Oryza sativa*) is a cereal produced and consumed worldwide; it plays important roles in dietary health, containing bioactive compounds such as phenolic compounds, tocopherols, tocotrienols, and others (Iqbal et al., 2005). In this context, cassava and rice blend may be important substrates to be fermented by potential probiotics LAB strains and yeast to developing functional fermented foods. In addition, cassava is a raw material, free from cholesterol, lactose, and gluten, safe for vegetarians and people who are lactose-intolerant or who have celiac disease.

Cauim is a non-alcoholic beverage produced by Brazilian Indians using several substrates such as rice, cassava, corn, peanut, cotton

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.02.011
0168-1605/

seed, banana, and pumpkin separately or combinations of them. This beverage is a staple food for adults and children (Almeida et al., 2007; Ramos et al., 2010, 2011). The purpose of this work was to develop a nondairy beverage based on Brazilian indigenous beverage, *cauim*, by selecting a potential probiotic LAB strain isolated from different Brazilian indigenous foods (*cauim*, *calugi*, *caxiri*, *yakupa* and *chicha*) to be used as starter culture in a blend of cassava and rice. The fermentative performance of starter cultures was monitored by the microbial dynamics and metabolites evaluations during the fermentation. Antioxidant activity, minerals, and acceptance of the beverage were evaluated. This knowledge will enable future pilot-scale fermentations with appropriate starter cultures for the development of a nondairy functional cassava and rice fermented beverage.

2. Materials and methods

2.1. Screening of LAB strains for starter culture selection

2.1.1. Microorganisms

Eighty-seven LAB strains, which were previously isolated from different Brazilian indigenous foods and identified by phenotypic and molecular methods (Almeida et al., 2007; Freire et al., 2014; Miguel et al., 2012, 2014; Puerari et al., 2015; Ramos et al., 2010, 2011; Santos et al., 2012) were initially employed in this study and belong to the Culture Collection of Agriculture Microbiology (CCMA) of the Federal University of Lavras, Brazil.

The yeast strain *Torulaspora delbrueckii* CCMA 0235 isolated from tarubá, a Brazilian indigenous cassava food and belonging to CCMA; and the commercial probiotic culture *L. acidophilus* LAC-04 acquired from Danisco (Yo Mix, Deutschland) were also employed in the cassava and rice blend fermentation. All strains were stored at −80 °C with 20% (v/v) glycerol and cultured in Man Rogosa Sharpe (MRS, Merck, Darmstadt, Germany) broth at 37 °C for 48 h for LAB, and YPD [10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L soy peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt, Germany), and 20 g/L agar (Merck, Darmstadt, Germany)] at 28 °C for 48 h for yeast.

The 87 indigenous LAB strains were tested for technological and probiotic characteristics.

2.1.2. α-Amylase secretion assay

For this assay, modified MRS agar plates containing 0.2% (w/v) soluble starch as the only carbon source were spotted with the LAB strains grown for 48 h at 37 °C in MRS broth. After incubation, the plates were flooded with iodine solution (1% iodine [w/v]; 2% potassium iodide [w/v]); the appearance of a clear zone around the colonies (Kostinek et al., 2005) indicated enzyme secretion. The assay was performed in triplicate. The positive strains were selected and used for further tests.

2.1.3. Acid production assay

Acid production test was carried out according to Kostinek et al. (2005). LAB strains were inoculated in MRS broth adjusted to pH 6.5, and the culture pH was measured with pH-Fix test strips (Macherey-Nagel GmbH and Co, Düren, Germany) after 6, 24, and 48 h of incubation. The assay was performed in triplicate.

2.1.4. Survival at pH 2.0

The LAB strains were subjected to a pH 2.0 tolerance assay to select the resistant isolates according to methodology described by Ramos et al. (2013). Cell cultures at optical density of 0.2 at 600 nm and corresponding to approximately 10^8 cell/mL were centrifuged and resuspended in MRS broth with pH adjusted to 2.0 using 1 N HCl and incubated for 3 h at 37 °C. The strains were subsequently inoculated on neutral pH MRS agar plates for 48 h at 37 °C to observe their growth. The assay was performed in triplicate.

2.1.5. Bile tolerance assay

The method described by Guo et al. (2009) was utilized to study the effect of bile on the growth rate of acid-tolerant LAB isolates. Tolerance to bile was evaluated based on the time required to increase the absorbance at 620 nm by 0.3 units in MRS broth with and without 0.3% oxgall. The difference in time (hours) to obtain 0.3 units between the measurements of the culture media with and without bile was considered as the adaptation time (AT) of the cells to adapt to media containing bile. The experiments were performed in triplicate.

2.2. Cassava and rice substrate preparation

The substrate for controlled fermentation assays was prepared based on the Brazilian indigenous beverage, *cauim* (Almeida et al., 2007), with some modifications. The cassava roots and the rice were purchased from the local market in Lavras, Minas Gerais, Brazil. Peeled cassava roots (4 kg) were ground in stainless steel blender (Cemaf, São Paulo, Brazil) to obtain cassava dough. After that, the dough was left to dry for 30 min at 60 °C to obtain cassava flour. Approximately 0.6 kg of cassava flour and 0.3 kg of rice were cooked together in 9 L of sterile distilled water for approximately 15 min after boiling. The mixture was sieved and the heat treatment was carried out by heating at 90 °C/20 min, followed by immediate cooling to 4 °C (Santos et al., 2014).

2.3. Starter cultures for cassava and rice blend fermentation

Three different starter cultures were tested for the development of the beverage—the selected strain *Lactobacillus plantarum* CCMA 0743, which demonstrated probiotic and technological potentials during screening tests; the yeast strain *Torulaspora delbrueckii* CCMA 0235; and the commercial probiotic culture *L. acidophilus* LAC-04.

The inoculum preparation for single and co-culture fermentations was performed as described by Santos et al. (2014). The LAB and yeast cells were washed twice with sterile peptone water (0.1% w/v, Himedia, Mumbai, India) by centrifuging (6000*g*) for 5 min at 20 °C. Microbial cells were inoculated in the substrate with a population of 5 log CFU/mL for yeast and 7 log CFU/mL for bacteria, in both single and co-culture fermentations. These initial populations were based on those found in previous study about indigenous beverages (Almeida et al., 2007; Freire et al., 2014; Ramos et al., 2010, 2011).

2.4. Single and co-culture fermentations

The washed cells of the LAB strains and yeast were inoculated into 500 mL Erlenmeyer flasks containing 400 mL of cassava and rice substrate, and incubated at 37 °C for 48 h. For the single fermentation assays, each microorganism was inoculated in the substrate separately, while co-culture fermentations were performed as follows: (1) *L. plantarum* CCMA 0743 (7 log CFU/mL) and *T. delbrueckii* CCMA 0235 (5 log CFU/mL); (2) *L. acidophilus* LAC-04 (7 log CFU/mL) and *T. delbrueckii* CCMA 0235 (5 log CFU/mL). The experiments were performed in three independent assays.

2.5. Enumeration of microorganisms, pH, and starchy content determinations

Samples (1 mL) were taken from each fermentation flask. The total LAB, yeast, and Enterobacteriaceae populations were determined at 0, 6, 12, 24, 36 and 48 h of fermentation by plating in MRS agar (supplemented with 50 mg/L of nystatin), Dichloran Rose Bengal Chloramphenicol (DRBC) agar, and violet red bile agar (VRBG; Merck) media, respectively. Plates were incubated at 37 °C (LAB and Enterobacteriaceae) and 28 °C (yeast) for 48 h, and the colony-forming units (CFU) were enumerated. The pH levels of the fermenting cassava and rice samples were measured on site with pH-Fix test strips (Macherey-Nagel