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Characterization of lactobacilli strains derived from cocoa fermentation in the south of Bahia for the development of probiotic cultures

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A B S T R A C T

In the last years, several factors have contributed to the development of probiotic cultures from locally sourced strains. In this paper, we aimed to characterize *Lactobacillus plantarum* and *Lactobacillus fermentum* isolates derived from Brazilian cocoa fermentation for the development of new probiotic cultures. Isolates diversity was studied by RAPD and strains were further tested *in vitro* for their probiotic potential. Physiological traits such as heat tolerance, hydrophobicity, resistance to simulated gastrointestinal digestion and antibiotic susceptibility were studied. Besides, activity against food pathogens was tested through four different assays: deferred inhibition, co-aggregation, co-cultivation and antagonism of supernatants. Considering the resistance to simulated gastrointestinal digestion and the results from the antimicrobial and co-aggregation tests, *L. plantarum* 286 showed the most promising results, followed by *L. plantarum* 289, for further studies for their application as probiotics.

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1. Introduction

A huge variety of functional foods and pharmaceutical supplements containing probiotic bacteria were developed around the world with only few internationally known strains such as *Lactobacillus rhamnosus* GG, *Lactobacillus casei* (Shirota and DN-114001), *Lactobacillus acidophilus* La5 or *Bifidobacterium animalis* subsp. *lactis* (BB12 and DN-173010). These strains were helpful in paving the way for the growing awareness by consumers about the fact that some friendly microorganisms can contribute to enhance the intestinal health by boosting the immune systems or by inhibiting pathogenic bacteria in the gut. Although probiotic products are globally commercialized and their benefits are generally claimed for people of different genders, race, age, geographic location and health status, access to products containing the referred strains is sometimes limited. Usually, only people from developed countries or belonging to higher social classes can afford the premium probiotic products (Sybesma, Kort, & Lee, 2015). At the same time, there are some factors that contribute to the development of a new generation of so-called locally sourced probiotics. For instance, differences in composition of gut microbiota of people from different geographical regions, specific nutritional and health requirements in different geographic regions, survival capacity in local food matrices, valorization of probiotic strains isolated locally and the available technological tools for the development of industrial cultures (Sybesma et al., 2015) are key factors for the development of probiotic cultures derived from locally isolated and characterized strains. The search for more isolation sources of potentially probiotic microorganisms including traditional

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fermented foods of different cultures and geographical regions is a current trend in different fields of microbial biotechnology (Mahasneh & Abbs, 2010). Brazil is world-widely known for its biological diversity and there is a need for further exploring it in order to know it and to protect it (Pimm et al., 2014). In relation to this work, it has been shown a great variety of lactic acid bacteria with probiotic potential to be isolated from artisanal fermented foods in different parts of Brazil (Puerari, Magalhães-Guedes, & Schwan, 2015; Ramos et al., 2015; Ramos, Thorsen, Schwan, & Jespersen, 2013; Saito et al., 2014; Santos, Ávila, & Schwan, 2015). Isolation and characterization of new strains of lactobacilli from uninvestigated sources can have the dual advantage of revealing taxonomic characteristics and obtaining strains with interesting functional traits that may be useful for biotechnological and/or probiotic applications (Ortu et al., 2007). Cocoa is the most important agricultural product of southern Bahia in Brazil (Schroth et al., 2011). Cocoa beans (Theobroma cacao L.) are the raw material for chocolate production. Spontaneous fermentation of cocoa pulp by indigenous microorganisms (yeasts, lactic and acetic acid bacteria) is crucial for developing chocolate flavor precursors. Among lactic acid bacteria, *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus fermentum* are the major species found in cocoa fermentation (Camu et al., 2007; Nielsen, Hønholt, Tano-Debrah, & Jespersen, 2005). In previous studies, particular attention was put on: 1) the dynamics of cocoa fermentation; 2) the role of lactic acid bacteria naturally present in the fermented beans; 3) the impact on the quality of fermented cocoa (Camu et al., 2007; Lagunes Gálvez, Loiseau, Paredes, Barel, & Guiraud, 2007; Schwan & Wheals, 2004). Considering the economic importance of Cocoa for the development of our region and the facts recently reported as motivational for the manufacture of new probiotic cultures from locally sourced strains (Sybesma et al., 2015), the aim of this study was to characterize *L. plantarum* and *L. fermentum* strains, isolated from cocoa fermentation, in order to select a strain for potential use as probiotic.

2. Materials and methods

2.1. Strains and culture conditions

The strains of *Lactobacillus* used in this study were isolated from the regular cocoa fermentation process for chocolate production carried out at Mars Center for Cocoa Science (Barro Preto, Bahia, Brazil). Five isolates of *L. plantarum* (277, 281, 286, 289 291) and five isolates of *L. fermentum* (244, 260, 263, 265, 266) were randomly selected. These isolates had been kept in the collection of Mars Center for Cocoa Science.

The isolates were previously identified by 16S rDNA amplification with the primers 27f and 1512R and sequenced with primer 27f. Strains were kept at −80 °C in MRS (Fluka, Sigma-Aldrich, St, Louis, USA) with 15% glycerol and reactivated in MRS broth at 37 °C, overnight (16 h) in aerobiosis.

2.2. RAPD analysis

The study of the isolates diversity was kindly carried out by Dr. Svetoslav Todorov at Dr. Augusto Nero's laboratory (Federal University of Viçosa, MG, Brazil). The isolates were cultured in MRS broth for 20 h at 37 °C. Total DNA was extracted using the GenElute® Bacterial Genomic DNA kit (Sigma, St Louis,MO, USA) according to the manufacturer's instructions and quantified on NanoDrop (Thermo Fisher, San Pablo, Brazil). Concentration of DNA was adjusted to 20 ng/μl. RAPD-PCR was executed with primers OPL01 (GGCATGACCT), OPL02 (TGGGCGTCAA), OPL04 (GACTGCACAC), OPL05 (ACGCAGGCAC), OPL14 (GTGACAGGCT) and OPL20 (TGGTGGACCA) (Kit L of the RAPD® lomer kits, Operon Biotechnologies, Cologne, Germany), according to Todorov, Ho, Vaz-Velho, and Dicks (2010). The marker was 1 kb DNA ladder of Thermo Fisher (Fermentas).

2.3. Heat tolerance assay

Heat resistance was evaluated according to Paéz et al. (2012). Overnight cultures in MRS broth were harvested (5000 × g, 15 min, 5 °C) They were washed twice with phosphate buffered saline (PBS) solution (pH 7.5) and resuspended in 10% skim milk (Nestlè, Brazil). Cell suspensions were placed in a water bath at 60 °C for 5 min and then immediately cooled in an ice bath. Viable cells were counted (MRS agar, 48 h, 37 °C, aerobic incubation) immediately before and just after exposure to heat.

2.4. Hydrophobicity

The adhesion ability of the strains was estimated as a measure of their hydrophobicity, which was determined according to Burns et al. (2008). Cultures of the strains were harvested in the stationary phase (5000 × g, 15 min, 5 °C), washed twice in PBS solution (pH 7.5) and resuspended in the same buffer. The cell suspension was adjusted to an OD_{600nm} of approximately 1.0 in the buffer and 3 ml of the bacterial suspensions were mixed with 0.6 ml of *n*-hexadecane (Merck, Darmstadt, Germany) and vortexed for 120 s. The two phases were allowed to stand for 1 h at 37 °C. The aqueous (bottom) phase was carefully removed and the OD_{600nm} was measured. The decrease in the absorbance of the aqueous phase was considered as a measure of the cell surface hydrophobicity (H%). The hydrophobicity was calculated with the formula $H\% = [(OD_0 - OD)/OD_0]/100$, in which OD_0 and OD were the optical density before and after extraction with *n*-hexadecane, respectively.

2.5. Resistance to simulated gastrointestinal digestion

The resistance to simulated gastrointestinal digestion conditions was determined according to Saito et al. (2014). The strains were reactivated in MRS broth for 24 h at 37 °C, centrifuged (4000×*g*, 15 min, 5 °C) and washed with PBS buffer (pH 7.5). Subsequently, the pellet was resuspended in 20 ml of Chocolate milk (Nestlè, Brazil) and mixed with the same volume of a simulated saliva-gastric solution. Saliva-gastric solution contained $CaCl_2$ (0.22 g/l), NaCl (16.2 g/l), KCl (2.2 g/l), $NaHCO_3$ (1.2 g/l), and 0.3% (w/v) bovine pepsin (Sigma-Aldrich, St. Louis, MO, U.S.A.). For the assessment of viable cells, we removed 1 ml sample for cell counts immediately after mixture and quickly lowered pH to 2.5, with 5 N and 0.1 N HCl. Samples were brought to 37 °C in a water bath and maintained for 90 min. Aliquots of 1 ml were taken periodically (30 min) and serial dilutions were plated for cell counts. After 90 min of simulating saliva-gastric digestion, samples were centrifuged (4000×*g*, 15 min, 5 °C). The supernatant was removed, the pellet was washed twice with PBS buffer (pH 7.5) and resuspended to the original volume in 1% (w/v) bovine bile (Sigma-Aldrich) at pH 8.0. An aliquot was removed for cell viability assessment and the remaining cell suspension was incubated in a water bath for 10 min at 37 °C. After incubation, a sample was collected for cell viability assessment. Again, samples were centrifuged (4000 × *g*, 15 min, 5 °C), the supernatant was removed and the pellet was washed twice with PBS buffer (pH 7.5) and resuspended to the original volume in 0.3% (w/v) bovine bile (Sigma-Aldrich) plus 0.1% (w/v) pancreatin (Sigma-Aldrich) at pH 8.0. Aliquots of 1 ml were taken before and after an incubation period of 180 min at 37 °C to assess cell viability. The test was performed in

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