Food Microbiology 36 (2013) 22–29

Contents lists available at SciVerse ScienceDirect

Food Microbiology

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

Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products

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ARTICLE INFO

Article history:
Received 10 November 2012
Received in revised form
15 February 2013
Accepted 27 March 2013
Available online 15 April 2013

Keywords:
LAB
Probiotic
Functional properties
Brazilian food products
Adhesion
TEER

ABSTRACT

A total of 234 LAB isolates from Brazilian food products were initially screened for their ability to survive at pH 2.0. Fifty one of the isolates survived and were selected. They were characterized by phenotypic methods, rep-PCR and identified using 16S rRNA gene sequencing as *Lactobacillus fermentum* (34 isolates), *Lactobacillus plantarum* (10) and *Lactobacillus brevis* (7). Based on being either highly tolerant to bile, showing an ability for auto-aggregation and/or hydrophobic properties, one *L. fermentum* (CH58), three *L. plantarum* (CH3, CH41 and SAU96) and two *L. brevis* (SAU105 and FFC199) were selected. The highest co-aggregation ability with *Escherichia coli* was observed to *L. plantarum* CH41. *L. brevis* SAU105 and FFC199 and *L. fermentum* CH58 exhibited antagonistic activity towards the pathogens *Listeria monocytogenes* and *Staphylococcus aureus*. *L. plantarum* CH3 and CH41 and *L. brevis* FFC199 showed adhesion ability to Caco-2 cells (1.6, 1.1 and 0.9%, respectively) similar to the commercial probiotic, *Lactobacillus rhamnosus* GG (1.5%). They were able to increase the transepithelial electrical resistance (TEER) of Caco-2 cells over 24 h ($p < 0.05$). The present work showed that the probiotic characteristics were strain-specific and that the isolates *L. plantarum* CH3 and CH41 (cocoa) and *L. brevis* FFC199 (cauim) exhibited potential probiotics properties.

1. Introduction

Lactic acid bacteria (LAB) have been used as food supplements, and are highly valued for their probiotic properties. Many probiotic isolates, such as *Lactobacillus acidophilus* LA1, *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* Lp01, *Lactobacillus fermentum* RC-14 are already widely used and produced on an industrial scale (Giraffa et al., 2010). However, a large number of potentially probiotic microorganisms present in different kinds of food products continue unknown. Spontaneously fermented foods may constitute a reservoir for new LAB spp. strains with potential probiotic characteristics (Mathara et al., 2008; Taked et al., 2011). Several naturally fermented products from Brazil harbour lactic acid bacteria. These include Cauim (kawi) which is a non-alcoholic beverage from various substrates produced by Brazilian Indians, with *Lactobacillus* spp. being the predominant bacteria found in this product (Almeida et al., 2007; Ramos et al., 2010, 2011). Another traditional process in Brazil involving LAB is the cocoa fermentation, which is a key step in the technological transformation of cocoa into chocolate (Pereira et al., 2012). Finally, an industrial product consumed in Brazil and worldwide is fresh sausage. It is freshly prepared and not fermented. However, it constitutes a reservoir of LAB (Ammor and Mayo, 2007). Although the LAB microbiota of the above mentioned products have previously been identified (Almeida et al., 2007; Pereira et al., 2012; Ramos et al., 2010, 2011), their probiotic potential has not been characterized.

Giraffa et al. (2010) described that in order for a probiotic to be of benefit to human health, it must survive passage through the upper GIT (gastro intestinal tract) and be able to function in the gut environment. The functional requirements of probiotics include tolerance to acid and bile, adherence to epithelial surfaces and antagonistic activity towards intestinal pathogens. Auto-aggregation of probiotic strains is thought to influence the adhesion capacity of the bacteria cells to intestinal epithelial cells, and co-aggregation with pathogens may prevent colonization of the latter in the gut (Del Re et al., 2000). Bacterial adhesion is initially based on non-specific physical–chemical interactions between two surfaces and the physical and chemical characteristics of the bacterial cell surface depend on surface hydrophobicity (Kotzamanidis et al., 2010; Kos et al., 2003).

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http://dx.doi.org/10.1016/j.fm.2013.03.010

Mammalian epithelial cells have been used as *in vitro* models for studying the adhesion of probiotic bacteria to the GIT (Anderson et al., 2010; Klingberg et al., 2005a) and to determine the effect on the strength of the epithelium barrier by measurements of transepithelial electrical resistance (TEER) (Klingberg et al., 2005b). Treatment with probiotic bacteria may prevent or reverse increased permeability of the epithelium and act antagonistically towards pathogens (Klingberg et al., 2005b). Measurements of the TEER of polarized monolayers of epithelial cells are therefore seen as a useful method of providing information about potential probiotic effect of microorganisms (Anderson et al., 2010; Klingberg et al., 2005b).

The objective of this study was to identify to species level and evaluate the probiotic properties of LAB isolated from different naturally fermented Brazilian products. They were characterized by phenotypic tests, rep-PCR and identified by 16S rRNA gene sequencing. Probiotic properties were evaluated by tolerance to low pH and bile, surfaces properties (aggregation, co-aggregation and hydrophobicity), antagonistic activity towards pathogenic bacteria and by assays employing the human Caco-2 cell line (adhesion and TEER).

2. Material and methods

2.1. Bacterial isolates and culture conditions

A total of 234 LAB isolates belonging to the microbial collection of the Microbial Physiology Laboratory/Department of Biology/Federal University of Lavras (UFLA), Brazil, and isolated from cocoa fermentation (99 isolates) (Pereira et al., 2012), industrial sausage (91 isolates) (data not published) and fermented food cauim (44 isolates) (Ramos et al., 2010) were initially employed in this study. The samples were previously processed (Pereira et al., 2012; Ramos et al., 2010). However, the particular isolates employed in the present work had not been fully identified. They were presumptively identified as LAB by culturing on Man Rogosa Sharpe (MRS, Merck, Darmstadt, Germany) agar and examining for colony and cell appearance, catalase activity, Gram stain, motility and the production of CO_2 from glucose in MRS broth with a Durham tube.

Among the 234 isolates, 51 were selected for further studies according to their tolerance to pH 2.0 (described in Section 2.3.1), and they are presented in Fig. 1. The LAB isolates were cultured in MRS broth at 37 °C for 24 h and stored at −80 °C with 20% (v/v) glycerol. The pathogenic strains *Escherichia coli* JM109, *Salmonella* Typhimurium 224/87, *Staphylococcus aureus*, *Listeria monocytogenes* EGDe, *L. monocytogenes* LO28 and *L. monocytogenes* 4446 obtained from the culture collection of the Food Science Department, Faculty of Science, Copenhagen University, were employed in the antagonistic assay (Section 2.3.5). They were grown in Brain Heart Infusion (BHI; Oxoid, Roskilde, Denmark) broth, 37 °C for 24 h and stored as described above.

2.2. Characterization and identification

2.2.1. Rep-PCR

The 51 selected isolates were genotypically characterized by rep-PCR. Genomic DNAs were extracted using Instagene (Bio-Rad, Deutschland) following the instructions of the manufacturer. Fingerprints of genomic DNA were obtained with PCR amplification of repetitive bacterial DNA elements (rep-PCR) using the $(GTG)_5$ primer as described by Nielsen et al. (2007). Cluster analysis was performed using BioNumerics software (version 4.50), similarities were calculated using DICE correlation coefficient and UPGMA algorithm as previously described (Parkouda et al., 2010).

2.2.2. Identification to species level by 16S rRNA gene sequencing and phenotypic tests

A total of 14 representatives (4, 3 and 7 of each group obtained by rep-PCR) were selected for16S rRNA gene sequence analysis using the primers 7F (5′ AGA GTT TGA TYM TGG CTC AG 3′) and 1510R (5′ ACG GYT ACC TTG TTA CGA CTT 3′) according to Padonou et al. (2009). Amplified PCR products were sent to sequencing at Macrogen (Netherlands). Searches in EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007) were performed to determine the closest known relatives of the partial 16S rRNA gene sequences that were obtained. Phenotypic assays were performed to confirm the identification. Fermentations of cellobiose, melezitose, gluconate, glycerol and xylose, and growth at 42 °C were evaluated as recommended in The Prokaryotes (Hammes and Hertel, 2006).

2.3. Screening for probiotic properties

2.3.1. Tolerance to pH 2.0

The 234 isolates were subjected to a pH 2.0 tolerance assay in order to select the resistant isolates for further studies. LAB cells cultivated in MRS broth (Merck, Darmstadt, Germany) at 37 °C for 24 h were centrifuged (5000 rpm for 5 min at 24 °C) and washed two times in 0.1% w/v peptone water pH 7.0. The cell cultures (optical density of 0.2 at 600 nm) in peptone water corresponding to approximately 10^8 cell/mL were centrifuged and re-suspended in MRS broth (Merck, Darmstadt, Germany) with pH adjusted to 2.0 using 1N HCl and incubated for 3 h at 37 °C. Samples (10 μL) were obtained at time 0 and after 3 h and inoculated in MRS agar plates. Tolerance to pH 2.0 was indicated by subsequent growth on MRS agar plates after 48 h of incubation at 37 °C.

2.3.2. Determination of bile tolerance

To study the effect of bile on the growth rate of acid tolerant LAB isolates (Section 2.1), a method described by Guo et al. (2009) was utilized. Tolerance to bile was evaluated based on the time required to increase the absorbance at 600 nm by 0.3 units in MRS broth with and without 0.3% oxgall. The difference in time (h) 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 experiment was performed in duplicate.

2.3.3. Cell surface hydrophobicity

Bacterial cell surface hydrophobicity was assessed for the 51 acid tolerant isolates (Section 2.1) by measuring microbial adhesion to hydrocarbons (MATH) as described by Kotzamanidis et al. (2010). Cells cultivated at 37 °C for 24 h were washed twice in phosphate buffered saline (PBS; 1.54 mM KH_2PO_4, 0.1 μM NaCl, 2.71 mM Na_2HPO_4 $7H_2O$) and finally re-suspended in 3 mL of 0.1 M KNO_3 containing about 10^8 CFU/mL (optical density of 0.2 at 600 nm) of bacteria and the absorbance was measured at 600 nm (A0). One millilitre of xylene was then added to the cell suspension to form a two-phase system. After a 10-min pre-incubation at room temperature, the two phase system was mixed by vortexing for 2 min. Then, the water and xylene phases were separated through incubation for 20 min at room temperature (approximately 23 °C). The aqueous phase was carefully removed and its absorbance at 600 nm (A_1) was measured. The percentage of cell surface hydrophobicity ($H\%$) was calculated using the following formula: $H\% = (1 - A_1/A_0) \times 100$. The assay was performed in duplicate and repeated twice.

2.3.4. Auto-aggregation and co-aggregation assay

The capacity of the cell to form aggregates (floccules) with cells of the same or different strains (e.g. pathogenic) is denominated

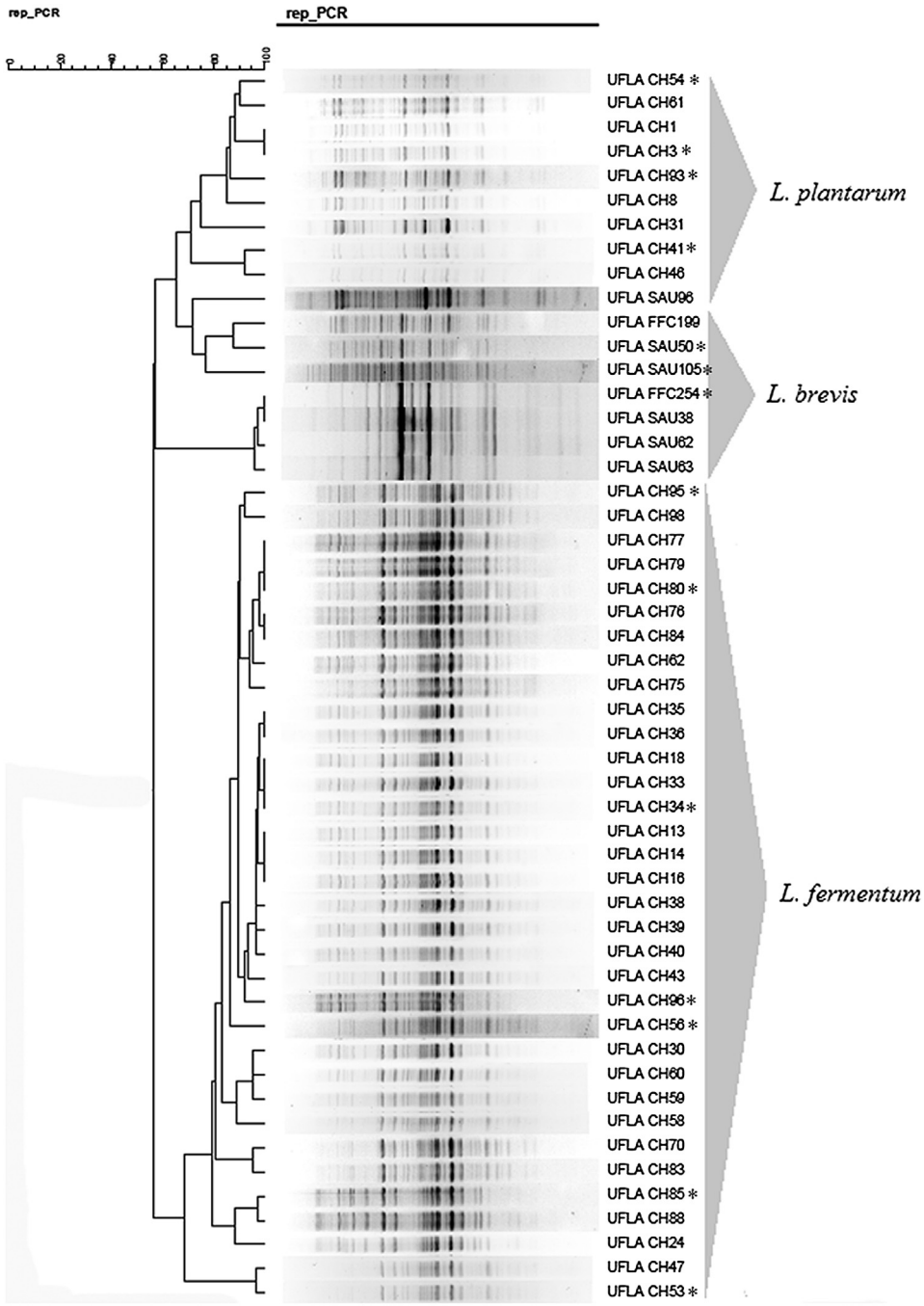

Fig. 1. Dendrogram generated after cluster analysis of rep-PCR fingerprints of the *Lactobacillus* strains. The reference codes indicate the source of LAB: CH = cocoa fermentation, SAU = sausage and FFC = fermented food cauim. Stars indicate isolates selected to sequence analysis.

auto-aggregation and co-aggregation, respectively (Del Re et al., 2000). Auto-aggregation and co-aggregation assays were performed according to Kos et al. (2003). Auto-aggregation and co-aggregation were determined after 5 h of incubation at 37 °C. The auto-aggregation percentage is expressed as: $1 - (A_5/A_0) \times 100$, where A_5 represents the absorbance at time $t = 5$ h and A_0 the absorbance at $t = 0$.

The percentage of co-aggregation was calculated using the equation (Kos et al., 2003):

$$\text{Co} - \text{aggregation } (\%) = (((Ax + Ay)/2) - A(x+y)/(Ax + Ay)/2) \times 100$$

where x and y represent each of the two isolates in the control tubes, and $(x + y)$ the mixture. The aggregation and co-aggregation assays were performed in duplicate and repeated twice.

2.3.5. Antagonism towards E. coli, Salmonella Typhimurium, S. aureus and L. monocytogenes strains

The antagonism towards various bacterial pathogens was evaluated according to Zago et al. (2011) with some modifications. *E. coli* JM109, *Salmonella* Typhimurium 224/87, *S. aureus*, *L. monocytogenes* EGDe, *L. monocytogenes* LO28 and *L. monocytogenes* 4446, were included as pathogens in inhibition assays. Overnight cultures grown in BHI broth were inoculated

(2% v/v) in BHI agar (melted and then cooled down to temperature between 45 and 50 °C). After vigorous homogenization, the inoculated agar media were poured into Petri dishes. Wells (5 mm) were made on each agar plate. Overnight cultures of the 6 selected LAB isolates were centrifuged and the supernatants were recovered. The supernatant was filter-sterilized (0.45 mm, Millipore, Cork, Ireland) and pH was adjusted to 7.0, aliquots of 30 μl of each supernatant were added to the wells. Petri dishes were incubated for 24 h at 37 °C and the diameters of the halos of inhibition were recorded in mm. The assay was performed in triplicate.

2.3.6. Growth and maintenance of Caco-2 cells

The human colon adenocarcinoma cell line Caco-2 (DSMZ, Braunschweig, Germany) was grown in Modified Eagles Medium (MEM) supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS; Lonza, Basel, Switzerland), 1× Non Essential Amino Acids (NEAA), and 0.1 mg/mL gentamicin. All solutions were obtained from Invitrogen, Gibco (Naerum, Denmark). The cells were propagated at 37 °C in a humidified atmosphere of 5% CO_2.

2.3.7. Adhesion capacity to Caco-2 cell line

Bacterial adhesion capacity to the Caco-2 cell line was investigated for the 6 selected LAB isolates. The Caco-2 cells were subcultivated (2×10^5 cell/mL) in 24 well tissue culture plates (Nunc, Roskilde, Denmark) and grown at 37 °C in a humidified atmosphere of 5% CO_2 for 21 days to obtain differentiation (Riedel et al., 2006) in cell media without antibiotics. The adhesion assay was performed as previously described (Klingberg et al., 2005a). Experiments were performed with duplicate determinations and repeated three times. The probiotic strain *L. rhamnosus* GG (LGG; Valio Ltd, Helsinki, Finland) was employed as a reference strain.

2.3.8. Transepithelial electrical resistance as measurements of epithelial barrier function

The bacterial effect on the epithelial barrier was evaluated by measurement of TEER using the Millicell Electrical Resistance System (Millipore, Bedford, MA) as previously described (Klingberg et al., 2005b). To obtain polarized monolayers, Caco-2 cells were seeded onto Transwell filter inserts (0.4 μm pore size, 12 mm inside diameter, polycarbonate; Corning Incorporated, Corning, NY) at a concentration of 2×10^5 cells/mL and cultivated for 21 days. Overnight cultures of bacteria were suspended in cell growth medium without antibiotics. The bacterial suspension (500 μl) was added to the apical compartment at 10^6 CFU/ml and incubated at 37 °C in a humidified atmosphere of 5% CO_2. TEER was measured before the addition of the bacteria (time zero) and then at various time intervals and expressed as the ratio of TEER at time t in relation to the initial value (at time zero) for each series. The net value of the TEER was corrected for background resistance by subtracting the contribution of cell free filter and the medium (150 Ω). The TEER of monolayers without added bacteria represented the control for each experiment. Experiments were performed with triplicate determinations and repeated twice. The probiotic strain LGG was also employed as a reference strain.

2.4. Statistical analyses

Analyses of the variance and the Scott–Knott test were performed with SISVAR 5.1 software (Ferreira, 2008). Principal component analyses (PCA) were performed using the XLSTAT 7.5.2 software (Addinsoft's, New York, NY, USA).

2.5. Nucleotide accession numbers

The nucleotide sequences determined in this study have been assigned to GenBank Accession numbers JX845637–JX845650.

3. Results

3.1. Selection of isolates for further characterization

Tolerance to pH 2.0 in MRS broth was chosen as selection criteria for isolates to be included in further experiments. Amongst the 234 LAB investigated, a total of 51 isolates were able to grow after 3 h exposed to pH 2.0: 43 from cocoa fermentation, 6 from industrial sausage and 2 from cauim.

3.2. Identification of LAB to species level

The 51 acid tolerant LAB isolates were clustered according to their rep-PCR profiles into 3 groups at a similarity level of 60% (Fig. 1). According to the grouping by rep-PCR, 14 strains were selected and sequenced. Based on 16S rRNA gene sequence analysis against sequences in the EzTaxon server, rep-PCR grouping and fermentation of some carbohydrates, the isolates of group 1, 2 and 3 were identified as *L. fermentum* (34 isolates), *L. plantarum* (10) and *Lactobacillus brevis* (7), respectively. The similarity values of the sequences ranged from 99.42 to 100% for *L. fermentum*, 99.92 to 100% for *L. plantarum* and 99.92 to 100% for *L. brevis*. The isolates identified as *L. plantarum* by 16S rRNA gene analysis were able to ferment cellobiose, gluconate and melezitose, but not xylose and glycerol. In comparison, isolates identified as *L. fermentum* were able to ferment xylose and gluconate but not cellobiose, melezitose and glycerol. The isolates identified as *L. brevis* were able to ferment gluconate and xylose, but not cellobiose, melezitose and glycerol. All isolates grew at 45 °C, except the *L. fermentum* isolates. The phenotypic results confirm the identity obtained by 16S rRNA gene sequencing.

3.3. Bile tolerance, hydrophobicity and auto-aggregation properties

The 51 LAB isolates selected by tolerance to pH 2.0 and identified to species level were evaluated according to their bile tolerance and surface properties (hydrophobicity and aggregation assays). The results obtained from these assays were subject to PCA analysis (Fig. 2). The first (PC1) and the second (PC2) principal components explain 41.9 and 22.2% of the total variance, respectively.

As indicated by the groupings in the PCA plot (Fig. 2), all of the 51 LAB isolates were able to tolerate bile salts at 0.3% (w/v) (oxgall) for 3 h. However, the growth and time of adaptation in the bile media was strain dependent. Isolates *L. plantarum* CH3, CH41 and *L. brevis* FFC199 had the shortest adaptation time ($p < 0.05$) of approximately one hour, while two *L. brevis* isolates from sausages, had the longest adaptation time of 4 h. Regarding surface properties, excepted for *L. brevis* SAU105 which was highly hydrophobic (hydrophobicity value of 61.0%) all other isolates showed to be non-hydrophobic ($H \leq 1.5\%$). Furthermore, *L. plantarum* SAU96 and *L. fermentum* CH58 showed the highest ability for auto-aggregation (61.9 and 55.1%, respectively, $p < 0.05$, Table 1) as compared to the other isolates showing only moderate auto-aggregation (12.1–21.0%). There were no correlations between product type or species and grouping according to the investigated properties.

3.4. LAB pathogen interactions

Based on bile tolerance, auto-aggregation properties and grouping in the PCA plot (Fig. 2) the following isolates: *L. plantarum*

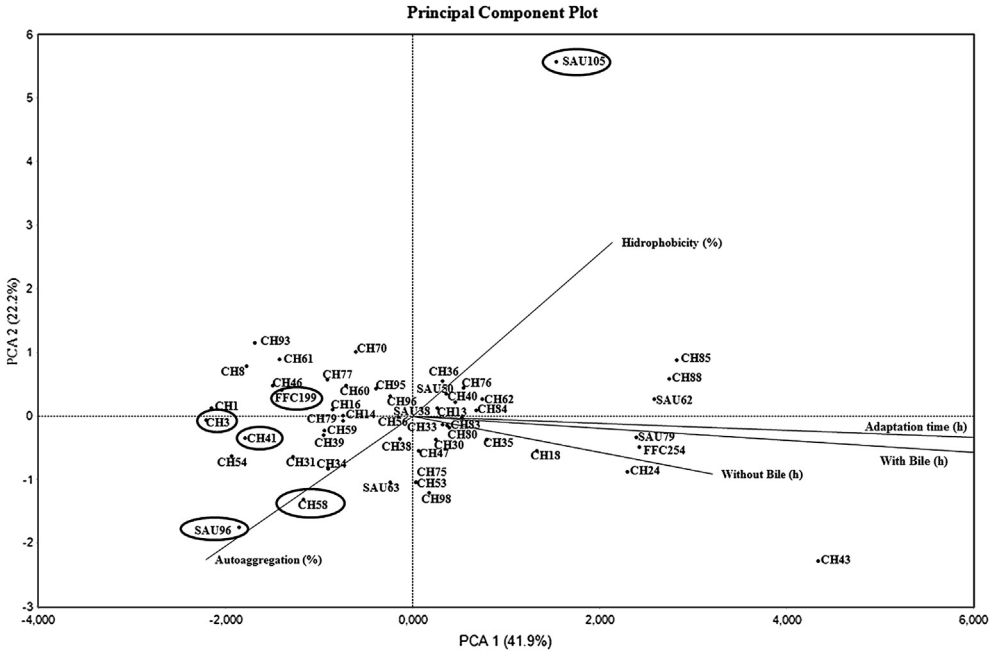

Fig. 2. Principal Component Analysis (PCA) of functional proprieties as tolerance to bile (growth in presence and absence of bile, as well the time of adaptation) and surface pattern (hydrophobicity and auto-aggregation) for the 51 *Lactobacillus* isolates. Isolates highlighted were the selected strains.

CH3, CH41 and SAU96, *L. fermentum* CH58 and *L. brevis* SAU105 and FFC199 were selected for co-aggregation experiments with *E. coli* JM109 and screened for antagonistic activity against relevant pathogens (Table 2). Co-aggregation values ranged between 1.1% (*L. brevis* FFC199) and 30.7% (*L. plantarum* CH41). The results for antagonistic activity of the LAB against pathogenic bacteria are shown in Table 2. The *L. plantarum* SAU96, *L. fermentum* CH58 and *L. brevis* SAU105 and FFC199 isolates showed antagonistic activity towards *S. aureus*. The highest activity towards *S. aureus* was obtained by the species *L. brevis* (FFC199 and SAU105 isolates). They showed halo of inhibitions greater than 3 mm (Table 2). The isolates *L. plantarum* CH3, *L. fermentum* CH58 and *L. brevis* SAU105 and FFC199 had activity towards *L. monocytogenes* 4446 (halo of inhibitions of 1–2 mm).

3.5. Adhesion to Caco-2 cells

The adhesion ability to Caco-2 cells was evaluated for the 6 selected LAB and the results are presented in Fig. 3. All 6 isolates showed a very low percentage of adhesion ability. However, the isolates *L. plantarum* SAU96 and CH3 showed higher percentages (1.8 and 1.6% of adhesion, respectively) of adhesion to Caco-2 cells compared to the positive control *L. rhamnosus* GG (1.5%). The isolates *L. plantarum* CH41, *L. brevis* FFC199 and *L. fermentum* CH58 showed moderated (1.1, 0.9 and 0.8%, respectively) adhesion ability, while the *L. brevis* SAU105 (0.3%) isolate showed low percentage of adhesion to Caco-2 cells.

3.6. TEER measurements

Changes in the TEER of polarized Caco-2 cells were used as an indicator of the effect of selected LAB strains on the intestinal epithelial barrier function (Fig. 4). All isolates maintained the TEER almost constant for the first 6 h, except for *L. plantarum* SAU96 isolate, which caused a slight increase in the first hour and a decrease in the TEER until 6 h. After 24 h, all of the tested isolates showed a tendency to increase the ratio of TEER. Compared to the control (Caco-2 cells without bacteria), the *L. plantarum* CH3 and CH41 and *L. brevis* FFC199 isolates induced a significant ($p < 0.05$) increase in TEER after 24 h. The increase was similar to that caused by the reference probiotic strain *L. rhamnosus* GG.

4. Discussion

Among 234 LAB previously isolated from 3 different products, 51 were selected based on their acid tolerance. Most of the isolates (43 out of 51) selected for further studies in this work originated from Brazilian cocoa fermentations. This is perhaps not surprising, as cocoa fermentations are characterized by low pH (approximately 2.0) (Nielsen et al., 2007; Pereira et al., 2012). The LAB isolated from

Table 1
Tolerance of selected *Lactobacillus* strains to bile salts, auto-aggregation, and hydrophobicity. AT = adaptation time. The reference codes indicate the source of LAB: CH = cocoa fermentation, SAU = sausage and FFC = fermented food cauim.

Isolate	Time required to increase A620 nm 0.3 units			Auto-aggregation (%)	Hydrophobicity (%)
	MRS (h)	MRS + bile (h)	AT (h)		
L. plantarum CH3	2.94 ± 0.04^{a}	3.71 ± 0.08^{a}	0.76 ± 0.12^{a}	20.94 ± 1.65^{b}	0.83 ± 0.05^{a}
L. plantarum CH41	4.01 ± 0.03^{c}	4.45 ± 0.10^{b}	0.43 ± 0.06^{a}	18.08 ± 0.34^{b}	0.00 ± 0.00^{a}
L. fermentum CH58	4.46 ± 0.07^{d}	6.07 ± 0.17^{d}	1.61 ± 0.24^{b}	55.61 ± 1.82^{c}	1.48 ± 0.64^{a}
L. brevis FFC199	3.71 ± 0.10^{b}	4.74 ± 0.01^{b}	1.03 ± 0.09^{a}	16.31 ± 0.61^{b}	0.26 ± 0.03^{a}
L. plantarum SAU96	3.54 ± 0.15^{b}	5.23 ± 0.26^{c}	1.69 ± 0.11^{b}	61.89 ± 0.44^{c}	0.00 ± 0.00^{a}
L. brevis SAU105	4.56 ± 0.26^{d}	7.41 ± 0.08^{e}	2.85 ± 0.35^{c}	12.17 ± 0.24^{a}	60.98 ± 2.84^{b}

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (±standard deviation) within the same column followed by different superscript letters differ significantly ($p < 0.05$) by Scott–Knott test.

Table 2
Percentage of co-aggregation of selected *Lactobacillus* isolates with *E. coli* JM109. The reference codes indicate the source of LAB: CH = cocoa fermentation, SAU = sausage and FFC = fermented food cauim.

Strain	% Co-aggregation with *E. coli* JM109 after 5 h	Antagonism activity	
		L. monocytogenes 4446	*S. aureus*
L. plantarum CH3	7.442 ± 0.22[b]	+	−
L. plantarum CH41	30.667 ± 1.02[e]	−	−
L. fermentum CH58	21.00 ± 1.11[c]	+	+
L. brevis FFC199	1.093 ± 0.12[a]	+	++
L. plantarum SAU96	21.082 ± 0.28[c]	−	+
L. brevis SAU105	28.067 ± 0.43[d]	+	+++

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (±standard deviation) within the same column followed by different superscript letters differ significantly ($p < 0.05$) by Scott–Knott test. (+) = 1–2 mm; (++) = 3–4 mm; (+++) = >4 mm. No antagonism activity was observed towards others strains *E. coli* JM109, *S.* Typhimurium 224/87, *L. monocytogenes* EGDe and *L. monocytogenes* LO28.

cocoa are inherently able to survive the harsh fermentation conditions, and are, therefore, also likely to be able to survive the passage through the GIT.

Three different species were identified in the present study: *L. fermentum* (37 isolates), *L. plantarum* (10 isolates) and *L. brevis* (7 isolates). All the *L. fermentum* strains (34) and most of the *L. plantarum* isolates (9) identified in this work were isolated from cocoa fermentation. The results are in agreement with observations of Nielsen et al. (2007) and Pereira et al. (2012), showing that *L. fermentum* and *L. plantarum* species are the dominant LAB in cocoa fermentations. Only one *L. plantarum* strain was isolated from sausage, while the *L. brevis* strains were isolated from both sausage (5 isolates) and cauim (2 isolates). These species have already been reported in these products (Almeida et al., 2007; Ammor and Mayo, 2007; Ramos et al., 2010, 2011).

According to Fuller (1992), bile, even at low concentrations, can inhibit the *in vitro* growth of microorganisms. Gilliland et al. (1984) reported that 0.3% is considered to be a critical concentration for screening for resistant strains. In the present study, all of the strains were able to grow in 0.3% (w/v) bile, although the time required to grow with and without bile was different between the isolates. Thus *L. plantarum* CH3, *L. plantarum* CH41 and *L. brevis* FFC199 had the highest bile tolerance, while isolates belonging to *L. fermentum* species and to *L. brevis* species had the lowest tolerance. Our results confirm previous reports showing that the tolerance is not necessarily related to the species of LAB, but may also be strain-specific (Maldonado et al., 2012).

Analyses of surface properties, including auto-aggregation and hydrophobicity showed that *L. plantarum* SAU96 and *L. fermentum* CH58 exhibited the highest auto-aggregation activity, while *L. plantarum* CH41 obtained the highest value for co-aggregation with *E. coli*. Regarding hydrophobicity, none of the isolates presented high capacity except for the *L. brevis* SAU105 isolate. Some authors have suggested that these surface properties correlate with their adhesive capacity (Kos et al., 2003; Kotzamanidis et al., 2010). However, here it seems that there is no correlation between hydrophobicity and adhesion ability, since *L. plantarum* CH41 and *L. plantarum* SAU96 had the highest rates of adhesion to Caco-2 cells and at the same time they did not showed hydrophobicity capacity.

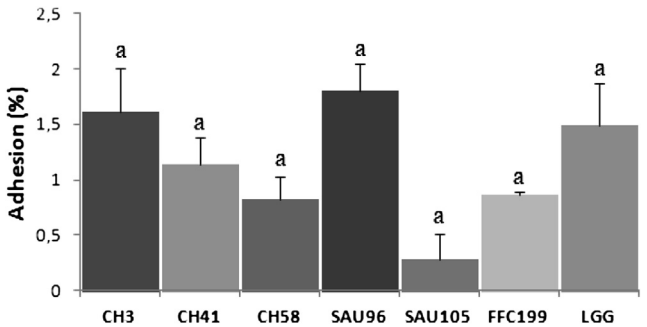

Fig. 3. Percentage of adhesion of six selected LAB strains to Caco-2 cells. LGG was used as a reference strain. Bars indicate standard deviations from the mean. The letter **a** over the bars indicate that the mean values did not differ significantly ($p < 0.05$) by Scott–Knott test.

The *L. plantarum* CH3 and CH41 and *L. brevis* FFC199 isolates, as well as the *L. rhamnosus* GG positive control were able to increase the TEER ratio over 24 h of incubation. Jensen et al. (2012) demonstrated a tendency to increase the TEER polarized monolayers of Caco-2 cells from 6 to 24 h incubation for four *L. reuteri* isolates, whereas *L. rhamnosus* GG revealed a tendency to decrease the TEER over the same time period. Contrary to these observations, it was observed that several of the investigated LAB including *L. rhamnosus* GG caused a significant increase in the TEER of the polarized Caco-2 cell monolayer as compared to the control. Jensen et al. (2012) described that *L. plantarum* MF1298 decreased the TEER, whereas Klingberg et al. (2005b) showed that this strain caused the highest increase in the TEER of polarized Caco-2 monolayers. In concurrence with the observations by Klingberg et al. (2005b) our study related the highest TEER over 24 h to *L. plantarum* isolates. According to Anderson et al. (2010) induction of enhanced expression of genes involved in tight junction signalling is a possible mechanism by which *L. plantarum* MB452 improves intestinal barrier function.

Strains of *L. plantarum* have previously been proven to be able to survive gastric transit and colonize the intestinal tract of humans and other mammals (Georgieva et al., 2008; Mathara et al., 2008) and several strains have been used for the development of functional and therapeutic foods (Shah, 2007). Based on the results reported here *L. plantarum* CH3 and CH41 from cocoa seemed to be suitable candidates to be used as probiotics. This specie has been also reported in fermented olives possessing desirable *in vitro* probiotic properties (Argyri et al., 2013; Peres et al., 2012). Similar findings for an increase in TEER of Caco-2 cells caused by a *L. plantarum* isolate from silage were reported by Anderson et al. (2010), showing that different kinds of products can be a reservoir of potential probiotic strains.

The high hydrophobicity of *L. brevis* SAU105 did not correlate with the adhesion capability to Caco-2 cell. This isolate presented low rates of adhesion to Caco-2 cells, and low increase in the TEER of Caco-2 monolayers. Similar results were found by other authors (Mathara et al., 2008; Zago et al., 2011), confirming that hydrophobicity values do not correlate with adhesion properties. *L. brevis* FFC199 exhibited a short time of adaptation to the media containing bile, moderate auto-aggregation property, adhesion to Caco-2 cells, and causing a relatively high TEER of Caco-2 cells. Kos et al. (2003) found a relationship between auto-aggregation and adhesiveness of *L. acidophilus* M92, this relationship also seems to exist for the studied isolates. *L. brevis* has the GRAS status (EFSA, 2009) however, it is not typically used in probiotic products although Collins et al. (1998) have mentioned *L. brevis* in a list of strains used in probiotic products. *L. brevis* FFC199 may be considered a potential probiotic. Although *L. fermentum* is well known probiotic species (Mikelsaar and Zilmer, 2009), only one of our isolates (CH58) was selected based on its high auto-aggregation, co-aggregation with *E. coli* JM109, antagonistic activity towards *L. monocytogenes* 4446 and *S. aureus*, and adhesion capacity to Caco-2 cells. However, *L. fermentum* CH58 did not present significant ($p < 0.05$) increase in the TEER ratio. Although the

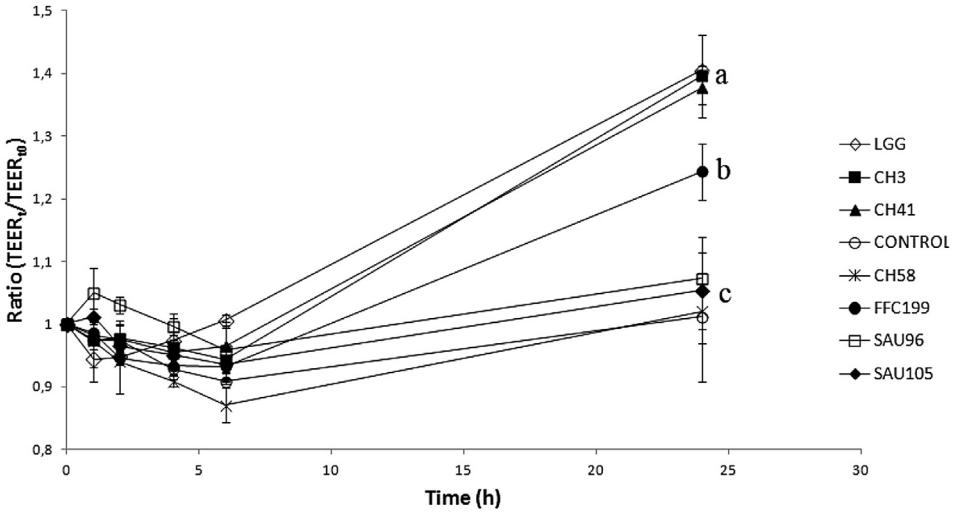

Fig. 4. TEER of polarized Caco-2 monolayers exposed to 6 LAB strains at a concentration of 10^6 cfu mL^{-1}, or without bacteria (control). TEER is expressed as the ratio of TEER at time *t* in relation to the initial value (*t*0) for each strain. Bars indicate standard deviation from the mean. Lines followed by different letters differ significantly ($p < 0.05$) by Scott–Knott test.

fermented dairy products are the most typical food matrices for probiotic bacteria, it is possible to obtain probiotic strains from several matrices (Rivera-Espinoza and Gallardo-Navarro, 2010).

In the selection process of probiotic strains, safety aspects including specifications such as origin, identity and lack of harmful activities, should be considerate (FAO/WHO, 2002; Giraffa, 2012). Haemolytic activity was evaluated and no activity (γ activity) was observed for the 51 LAB isolates (data not shown).

In conclusion, potential probiotic isolates were identified and characterized from different Brazilian fermented foods. Probiotic characteristics of the LAB isolates from cauim, cocoa fermentation and fresh sausage have not been studied before. It was clearly observed that microorganisms belonging to the same species may develop different mechanisms and present different characteristics. The results of this work provided a preliminary selection of some potential isolates, *L. brevis* FFC199 (from cauim) and *L. plantarum* CH3 and *L. plantarum* CH41 (from cocoa) could be used as probiotic, due to tolerance to low pH and bile salts, which are conditions imposed by the GIT environment. In addition, they were able to adhere to human intestinal epithelial cell line (Caco-2), and then increased the TEER of Caco-2 cell line.

Acknowledgements

This study was supported by University of Copenhagen and CNPq and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) which provided the scholarship.

References

Almeida, E.G., Rachid, C.C.T.C., Schwan, R.F., 2007. Microbial population present in fermented beverage 'cauim' produced by Brazilian Amerindians. International Journal of Food Microbiology 120, 146–151.

Ammor, M.S., Mayo, B., 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: an update. Meat Science 76, 138–146.

Anderson, R.C., Cookson, A.L., McNabb, W.C., Park, Z., McCann, M.J., Kelly, W.J., Roy, N.C., 2010. *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiology 10, 316–326.

Argyri, A.A., Zoumpopoulou, G., Karatzas, K.-A.G., Tsakalidou, E., Nychas, G.-J.E., Panagou, E.Z., Tassou, C.C., 2013. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiology 33, 282–291.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B.K., Lim, Y.W., 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. International Journal of Systematic and Evolutionary Microbiology 57, 2259–2261.

Collins, J.K., Thornton, K., Sullivan, G.O., 1998. Selection of probiotic strains for human applications. International Dairy Journal 8, 487–490.

Del Re, B., Sgorbati, B., Miglioli, M., Palenzona, D., 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology 31, 438–442.

EFSA Panel on Biological Hazards (BIOHAZ), 2009. Scientific Opinion on the maintenance of the list of QPS microorganisms intentionally added to food of feed (2009 update). EFSA Journal 7 (12), 1431. Available online: www.efsa.europa.eu.

FAO/WHO, 2002. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food London. Ontario, Canada, April 30 and May1, 2002.

Ferreira, D.F., 2008. SISVAR: um programa para análise e ensino de estatística. Revista Symposium 6, 36–41.

Fuller, R., 1992. Probiotics: the Scientific Basis, vol. 1. Chapman and Hall, London.

Georgieva, R., Iliev, I., Chipeva, V.A., Dimitonova, S.P., Samelis, J., Danova, S., 2008. Identification and vitro characterization of *Lactobacillus plantarum* strains from artisanal Bulgarian white brined cheeses. Journal of Basic Microbiology 48, 234–244.

Gilliland, S.E., Staley, T.E., Bush, L.J., 1984. Importance in bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. Journal of Dairy Science 67, 3045–3051.

Giraffa, G., 2012. Selection and design of lactic acid bacteria probiotic cultures. Engineering in Life Sciences 12, 391–398.

Giraffa, G., Chanishvili, N., Widyastuti, Y., 2010. Importance of lactobacilli in food and feed biotechnology. Research in Microbiology 161, 480–487.

Guo, Z., Wang, J., Yan, L., Chen, W., Liu, X., Zhang, H., 2009. In vitro comparison of probiotic properties of *Lactobacillus casei* Zhang, a potential new probiotic, with selected probiotic strains. LWT – Food Science and Technology 42, 1640–1646.

Hammes, W.P., Hertel, C., 2006. The genus *Lactobacillus* and *Carnobacterium*. In: Dworkin, M., Flakow, S., Rosenberg, E., Schleifer, K.H., Stackbrandt, E. (Eds.), The Prokaryotes. LLC: Springer Science and Business Media, New York, pp. 320–403.

Jensen, H., Grimmer, S., Naterstad, K., Axelsson, L., 2012. In vitro testing of commercial and potential probiotic lactic acid bacteria. International Journal of Food Microbiology 153, 216–222.

Klingberg, T.D., Axelsson, L., Naterstad, K., Elsser, D., Budde, B.B., 2005a. Identification of potential probiotic starter cultures for Scandinavian-type fermented sausages. International Journal of Food Microbiology 105, 419–431.

Klingberg, T.D., Pedersen, M.H., Cencic, A., Budde, B.B., 2005b. Application of measurements of transepithelial electrical resistance of intestinal epithelial cell monolayers to evaluate probiotic activity. Applied and Environmental Microbiology 71, 7528–7530.

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J., Matosic, S., 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of Applied Microbiology 94, 981–987.

Kotzamanidis, C., Kourelis, A., Litopoulou-Tzanetaki, E., Tzanetakis, N., Yiangou, M., 2010. Evaluation of adhesion capacity, cell surface traits and immunomodulatory activity of presumptive probiotic *Lactobacillus* strains. International Journal of Food Microbiology 15, 154–163.

Maldonado, N.C., Ruiz, C.S., Otero, M.C., Sesma, F., Nader-Macías, 2012. Lactic acid bacteria isolated from young calves – characterization and potential as probiotic. Research in Veterinary Science 92, 342–349.

Mathara, J.M., Schillinger, U., Kutima, P.M., Mbugua, S.K., Guigas, C., Franz, C., Holzapfel, W.H., 2008. Functional properties of *Lactobacillus plantarum* strains

isolated from Maasai traditional fermented milk products in Kenya. Current Microbiology 56, 315–321.
Mikelsaar, M., Zilmer, M., 2009. *Lactobacillus fermentum* ME-3 – an antimicrobial and antioxidative probiotic. Microbial Ecology in Health and Disease 21, 1–27.
Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H., 2007. The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture independent methods. International Journal of Food Microbiology 114, 168–186.
Padonou, S.W., Nielsen, D.S., Hounhouigan, J.D., Thorsen, L., Nago, M.C., Jakobsen, M., 2009. The microbiota of Lafun, an African traditional cassava food product. International Journal of Food Microbiology 133, 22–30.
Parkouda, C., Thorsen, L., Compaore, C.S., Nielsen, D.S., Tano-Debrah, K., Jensen, J.S., Diawara, B., Jakobsen, M., 2010. Microorganisms associated with Maari, a Baobab seed fermented product. International Journal of Food Microbiology 142, 292–301.
Pereira, G.V.M., Miguel, M.G.C.P., Ramos, C.L., Schwan, R.F., 2012. Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacteria strains for the development of a defined starter culture. Applied and Environmental Microbiology 78, 5395–5405.
Peres, C.M., Peres, C., Hernández-Mendoza, A., Malcata, F.X., 2012. Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria – with an emphasis on table olives. Trends in Food Science & Technology 26, 31–42.
Ramos, C.L., Almeida, E.G., Pereira, G.V.M., Cardoso, P.G., Dias, E.S., Schwan, R.F., 2010. Determination of dynamic characteristics of microbiota in a fermented beverage produced by Brazilian Amerindians using culture-dependent and culture-independent methods. International Journal of Food Microbiology 140, 225–231.
Ramos, C.L., Almeida, E.G., Freire, A.L., Schwan, R.F., 2011. Diversity of bacteria and yeast in the naturally fermented cotton seed and rice beverage produced by Brazilian Amerindians. Food Microbiology 28, 1380–1386.
Riedel, C.U., Foata, F., Goldstein, D.R., Blum, S., Eikmanns, B.J., 2006. Interaction of bifidobacteria with Caco-2 cells – adhesion and impact on expression profiles. International Journal of Food Microbiology 110, 62–68.
Rivera-Espinoza, Y., Gallardo-Navarro, Y., 2010. Non-dairy probiotic products. Food Microbiology 27, 1–11.
Shah, N.P., 2007. Functional cultures and health benefits. International Dairy Journal 17, 1262–1277.
Taked, S., Yamasaki, K., Takeshita, M., Kikuchi, Y., Tsend-Ayush, C., Dashnyam, B., Ahhmed, A.M., Kawahara, S., Muguruma, M., 2011. The investigation of probiotic potential of lactic acid bacteria isolated from traditional Mongolian dairy products. Animal Science Journal 82, 571–579.
Zago, M., Fornasari, M.E., Carminati, D., Burns, P., Suàrez, V., Vinderola, G., Reinheimer, J., Giraffa, G., 2011. Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. Food Microbiology 28, 1033–1040.