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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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ABSTRACT

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.

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

triplicate.

2.6. Inhibiting activity against food pathogens

2.6.1. Deferred inhibition assay

The antimicrobial activity was tested according to Nardi et al. (1999). A sample (5 μl) of an overnight culture of the strain was spotted on MRS agar (Fluka, Sigma-Aldrich). After incubation (37 °C, 48 h, anaerobiosis), colony cells were killed by exposure to chloroform (100 μl) for 30 min. Residual chloroform was evaporated off and the Petri dish overlayed with 3.5 ml of soft agar (0.75% w/v) previously inoculated with 10^6 CFU of the indicator strain (*Salmonella enterica* var. Typhimurium ATTCC 14028, *Shigella flexi* ATTCC11060, *Escherichia coli* ATCC 25922, and *Listeria monocytogenes* ATCC 15313). After 48 h of incubation at 37 °C under aerobiosis, the plate was evaluated for the presence of inhibition zone.

2.6.2. Co-cultivation assay

In order to test the direct antagonism of the strains under study against food pathogens (*S. enterica* var. Typhimurium ATTCC 14028, *S. flexi* ATTCC 11060 and *E. coli* ATCC 25922), a co-cultivation test was done. The method was adapted from Hütt, Shchepetova, Lõivukene, Kullisaar, and Mikelsaar (2006). An overnight culture of each pathogen and the *Lactobacillus* strain were inoculated together (1% v/v) in 1 ml of BHI broth (Acumedia, Neogen, Lansing, MI, USA) and incubated for 16 h (37 °C, aerobiosis). Control cultures were also prepared. Cell counts of the pathogen after incubation were done using MacConkey agar (Acumedia), incubated for 24 h (37 °C, aerobiosis). The growth of the pathogen in BHI broth, inoculated alone or co-inoculated with a strain of lactobacilli was compared.

2.6.3. Antagonistic activity of lactobacilli supernatants

The antagonism test using culture supernatants was adapted from Hütt et al. (2006). Briefly, overnight cultures of the strains under study in MRS broth were centrifuged (5000 × *g*, 5 min). The supernatant was collected and filter-sterilized (0.22 μm Millex, Merck Millipore, Darmstadt, Germany). Overnight cultures of the food pathogens (*S. enterica* var. Typhimurium ATTCC 14028, *S. flexi* ATTCC11060 and *E. coli* ATCC 25922) were inoculated (1% v/v) in BHI and 1% (v/v) of the lactobacilli supernatant was added to the broth. The broth was incubated for 16 h (37 °C, aerobiosis). Cell counts of pathogens were conducted on MacConkey agar (Acumedia) for 24 h (37 °C, aerobiosis).

2.7. Co-aggregation assay

The co-aggregation assay was conducted according to Kos et al. (2003). Overnight cultures of the strains under study and *S. enterica* var. Typhimurium ATTCC 14028, *S. flexi* ATTCC11060 or *E. coli* ATCC 25922 were centrifuged (5000×*g*, 5 min, 5 °C) and supernatants were discarded. Cell pellets were resuspended in PBS (pH 7.5). Aliquots of 500 μl of each cell suspension (lactobacilli-pathogen) were mixed, vortexed and left to stand at 37 °C for 4 h. The absorbance (600 nm) of the upper portion of the samples was measured in a microplate reader device (Sunnyvale, CA USA). Control samples (containing only the lactobacilli or the pathogen) were also measured at the beginning of the experiment. The test was performed in duplicates. The co-aggregation percentage was determined according to the formula: $[((Ax + Ay)/2) - A(x + y)]/[(Ax + Ay)/2]$, in which x represents the lactobacilli absorbance at time zero, y represents the pathogen concentration at time zero and (xy) the absorbance of the mix at 4 h of co-incubation.

2.8. Antibiotic susceptibility

Antibiotic susceptibility test was done according to Charteris, Kelly, Morelli, and Collins (1997). The strains were grown on MRS agar (Acumedia) in aerobiosis (37 °C, 24 h). Colonies were transferred to tubes containing 3.5 ml of saline solution (0.85% w/v NaCl) and adjusted to 0.5 McFarland turbidity. Microorganisms were inoculated on MRS agar (Acumedia-Neogen, Lansing, MI, USA) and disks (Oxoid, Basingstoke, England) containing the different antibiotics were distributed on the agar surface. Tested antibiotics and their concentrations were: chloramphenicol (30 μg), vancomycin (30 μg), amoxicillin (10 μg), penicillin (10 μg), tetracycline (30 μg), ceftriaxone (30 μg), sulfatrim (25 μg), nalidixicacid (30 μg) of laborclin (Pinhais, PA, Brasil). Plates were incubated at 37 °C for 24 h. Inhibition zones were measured after incubation. The results were subjected to a qualitative classification of microorganisms as sensitive, moderately susceptible or resistant to the antimicrobial drug tested.

2.9. Statistical analysis

A Completely Randomized Design (CRD) was used. Results were expressed as the mean ± standard deviation of at least two independent assays. Data were analyzed using the one-way ANOVA procedure of GraphPad Prism version 6.0 (2007) software. The difference among means was detected by Duncan's multiple range test (GraphPad, 2007). Data were considered significantly different when $^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$, $^{****} = p < 0.0001$.

3. Results

The selection of probiotic candidates out of a group of isolates from any natural source for application in food or medicine involves a number of steps that include proper species identification, study of strain diversity and *in vitro* technological and functional studies. In this paper, a group of *L. plantarum* and *L. fermentum* isolates obtained from natural cocoa fermentation were subjected to different *in vitro* assays with the purpose of determining their potential as probiotics. RAPD analysis with 6 different primers was done to evaluate the genetic diversity among isolates (Fig. 1). The RAPD profiles showed that *L. fermentum* 260 and *L. fermentum* 244 were indeed the same strain, while *L. fermentum* 266 was the same as *L. fermentum* 263. For the rest of this work, *L. fermentum* 260 and 266 were no longer considered, reducing the study to 8 strains.

Results of cell hydrophobicity and resistance to heat shock and to simulated gastrointestinal conditions are shown in Table 1. ANOVA analysis of hydrophobicity results showed significant differences among values ($p = 0.013$). However, Tuckey test showed that only *L. plantarum* 281 displayed higher hydrophobicity (16.9%) than *L. plantarum* 289 and *L. fermentum* 263. Concerning resistance to heat shock, reduction in counts of viable cells ranged from 1.9 to 3.4 log orders, but no significant differences were observed, maybe because of experimental errors among independent repetitions for each strain, as can be deduced from the magnitude of the standard deviations. In view of the resistance to simulated gastrointestinal digestion conditions, the test allowed to establish three groups of strains that had significant differences in this parameter ($p = 0.000$). Strains 277, 286 and 291 displayed a cell death less than 1 log order in simulated gastrointestinal digestion assay. Strains 244, 263, 265 and 289 demonstrated a reduction in cell counts that ranged from 1 to 2 log orders, while the most sensitive strain was *L. plantarum* 281 (more than 2 log orders of cell death).

The inhibiting activity of the lactobacilli strains against *S. enterica* var. Typhimurium, *S. flexi*, *E. coli* and *L. monocytogenes* was assessed through tree different *in vitro* assays. Table 2 shows the

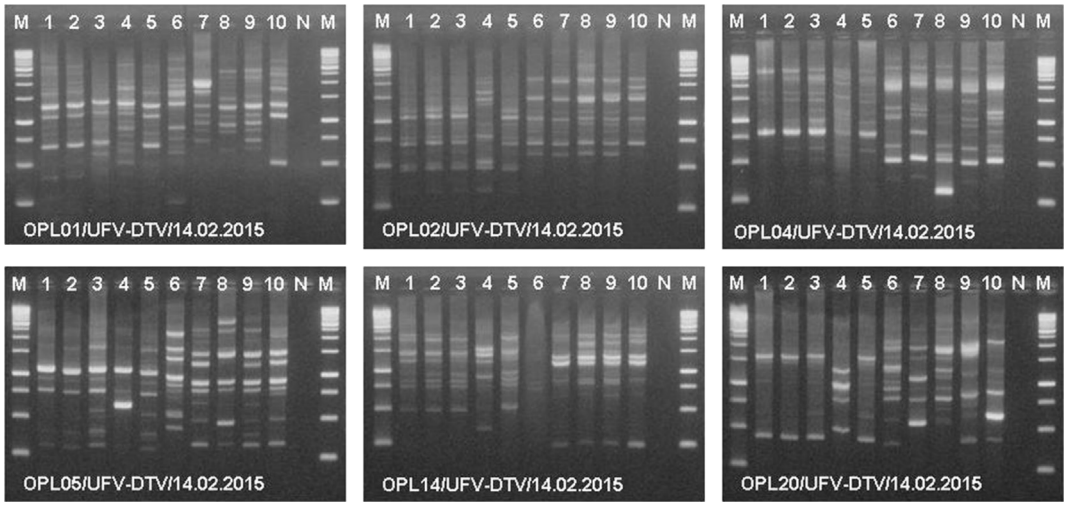

Fig. 1. RAPD profiles of *Lactobacillus* isolates obtained with primersOPL01, OPL02, OPL04, OPL05, OPL14 and OPL20. M: Tracklt 1 Kb plus DNA Ladder (Invitrogen), lane 1: *L. fermentum* 244, lane 2: *L. fermentum* 260, lane 3: *L. fermentum* 263, lane 4: *L. fermentum* 265, lane 5: *L. fermentum* 266, lane 6: *L. plantarum* 277, lane 7: *L. plantarum* 281, lane 8:*L. plantarum* 286, lane 9: *L. plantarum* 289, lane 10:*L. plantarum* 291, lane N: negative control.

Table 1
Heat resistance, hydrophobicity (% H) and resistance to simulated gastrointestinal digestion of *L. fermentum* (strains 244, 263, 265) and *L. plantarum* (strains 277, 281, 286, 289, 291).Values are means (±SD) of 3 repetitions.

Strain	Heat death (Δlog)[a]	% H	Log CFU/mL during simulated gastrointestinal digestion at t (min)								Cell death (Δlog)[b]
			Simulated gastric digestión			Bile shock			Intestinal digestión		
			0	30 60	90	0	10		0	180	
244	2.40 ± 0.83	10.78 ± 0.01	8.56 ± 0.31	8.59 ± 0.24	8.37 ± 0.33	8.14 ± 0.64	7.93 ± 0.58	7.72 ± 0.69	7.62 ± 0.40	7.26 ± 0.57	1.30 ± 0.34
263	1.94 ± 0.32	14.22 ± 0.04	9.04 ± 0.09	8.92 ± 0.23	8.89 ± 0.20	8.88 ± 0.17	8.68 ± 0.18	8.80 ± 0.11	8.67 ± 0.19	7.93 ± 0.17	1.11 ± 0.26
265	2.80 ± 0.93	8.00 ± 0.05	9.60 ± 0.13	9.47 ± 0.15	9.54 ± 0.13	9.46 ± 0.09	9.04 ± 0.33	8.97 ± 0.40	8.80 ± 0.43	8.34 ± 0.32	1.26 ± 0.26
277	3.27 ± 0.84	3.50 ± 0.03	9.47 ± 0.06	9.29 ± 0.07	9.27 ± 0.07	9.29 ± 0.06	9.19 ± 0.02	9.20 ± 0.05	9.20 ± 0.04	9.20 ± 0.09	0.25 ± 0.03
281	3.28 ± 0.78	16.87 ± 0.01	9.34 ± 0.05	6.69 ± 0.40	5.35 ± 1.20	4.90 ± 1.78	5.29 ± 1.71	5.34 ± 1.76	5.33 ± 1.72	6.84 ± 0.18	2.49 ± 0.23
286	2.90 ± 0.29	11.73 ± 0.00	9.62 ± 0.04	9.41 ± 0.09	9.35 ± 0.11	9.38 ± 0.13	9.31 ± 0.02	9.28 ± 0.07	9.24 ± 0.12	9.31 ± 0.11	0.32 ± 0.14
289	3.39 ± 0.49	14.42 ± 0.01	9.61 ± 0.06	7.88 ± 0.63	7.72 ± 0.54	7.71 ± 0.55	7.63 ± 0.49	7.64 ± 0.57	7.57 ± 0.59	7.79 ± 0.49	1.82 ± 0.44
291	2.21 ± 1.46	8.98 ± 0.02	9.67 ± 0.06	9.36 ± 0.04	9.10 ± 0.45	8.96 ± 0.35	8.85 ± 0.34	8.98 ± 0.44	8.92 ± 0.35	8.89 ± 0.32	0.77 ± 0.28

[a] Difference in cell counts (log CFU/ml) after and before heat treatment (60 °C, 5 min).
[b] Difference in cell counts (log CFU/ml) after and before gastrointestinal digestion (gastric digestion + bile shock + intestinal digestion).

Table 2
Antagonistic activities of lactobacilli strains according to the Deferred Inhibition Assay.

Lactobacilli strain	Pathogen			
	ST	LM	EC	SF
L. fermentum 244	+	+	+	+
*L. fermentum*263	+	+	+	+
*L. fermentum*265	+	−	−	−
L.plantarum 277	+	+	−	+
L.plantarum 281	+	+	−	+
L.plantarum 286	+	+	−	+
L.plantarum 289	+	−	+	+
L.plantarum 291	+	−	+	+

ST: *Salmonella enterica* var. Typhimurium ATTCC 14028, LM: *Listeria monocytogenes* ATCC 15313, EC: *Escherichia coli* ATCC 25922 and SF: *Shigella flexi* ATTCC 11060. Presence of inhibition halo (+), absence of inhibition halo (−).

results from the deferred inhibition assay. *L. fermentum* 244 and 263 demonstrated the widest inhibition spectrum, whereas *L. fermentum*265 the narrowest one. The rest of the strains showed inhibitory activity against 3 of the 4 pathogenic strains.

Co-cultivation test showed that all tested strains were able to inhibit partially and significantly the growth of *E. coli* ATCC 25922. Only L. *plantarum* 286 inhibited the growth of *S. enterica* var. Typhimurium ATTCC 14028 and *S. flexi* ATTCC11060 (Fig. 2).

The supernatants of all strains under study were able to partially but significantly inhibit the growth of *E. coli* ATCC 25922, whereas only supernatants from *L. plantarum* 286, 289 and 291 were able to significantly inhibit the growth of *S. enterica* var. Typhimurium ATTCC 14028 and *S. flexi* ATTCC11060 (Fig. 3).

The results of the co-aggregation assay (Fig. 4) showed that *L. plantarum* 289 displayed significantly higher co-aggregation ($p < 0.05$) with the three food pathogens assayed compared to the other 7 strains of lactobacilli (except when *L. plantarum* 289 was compared to *L. fermentum* 244 in co-aggregation with *S. enterica* var. Typhimurium ATTCC 14028). *L. plantarum* 286 also displayed co-aggregation with *E. coli* ATCC 25922 that was also significantly higher than the values observed for the strains 263, 265, 281 and 291. The co-aggregation capacity of *L. plantarum* 286 with *S. flexi* ATTCC 11060 was higher than the strains 263, 277 and 291 and finally its co-aggregation ability with *S. enterica* var. Typhimurium ATTCC 14028 was higher than the values observed for the strains 265 and 291.

Finally, strains were tested for their sensitivity to different antibiotics (Table 3). All strains showed sensitivity to both amoxicillin and chloramphenicol but resistance to both vancomycin and nalidix acid. In particular, *L. fermentum* 265 and *L. plantarum* 281, 286, 289 and 291 were sensitive to moderately sensitive to the rest of

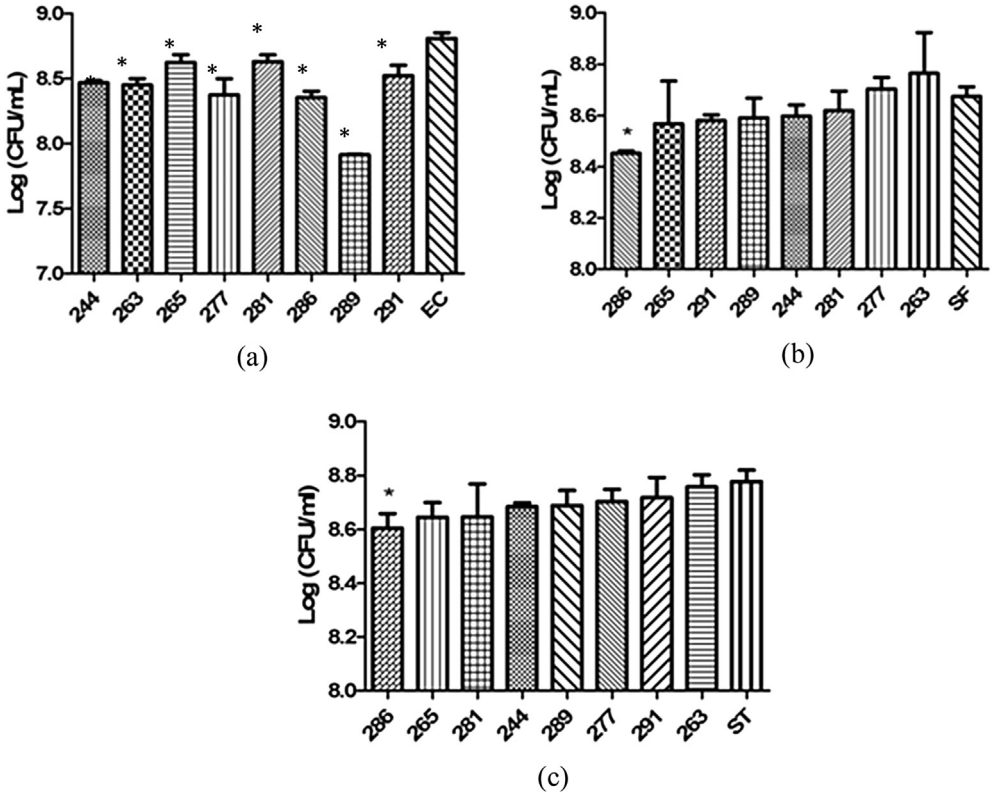

Fig. 2. Cell counts (log CFU/ml) of *Escherichia coli* ATCC 25922 (a), *Shigella flexi* ATTCC 11060 (b) or *Salmonella enterica* var. Typhimurium ATTCC 14028 (c), alone (EC, SF, ST, respectively) or after 24 h of co-cultivation with a single lactobacilli (the number of the strain is shown on the X axis).

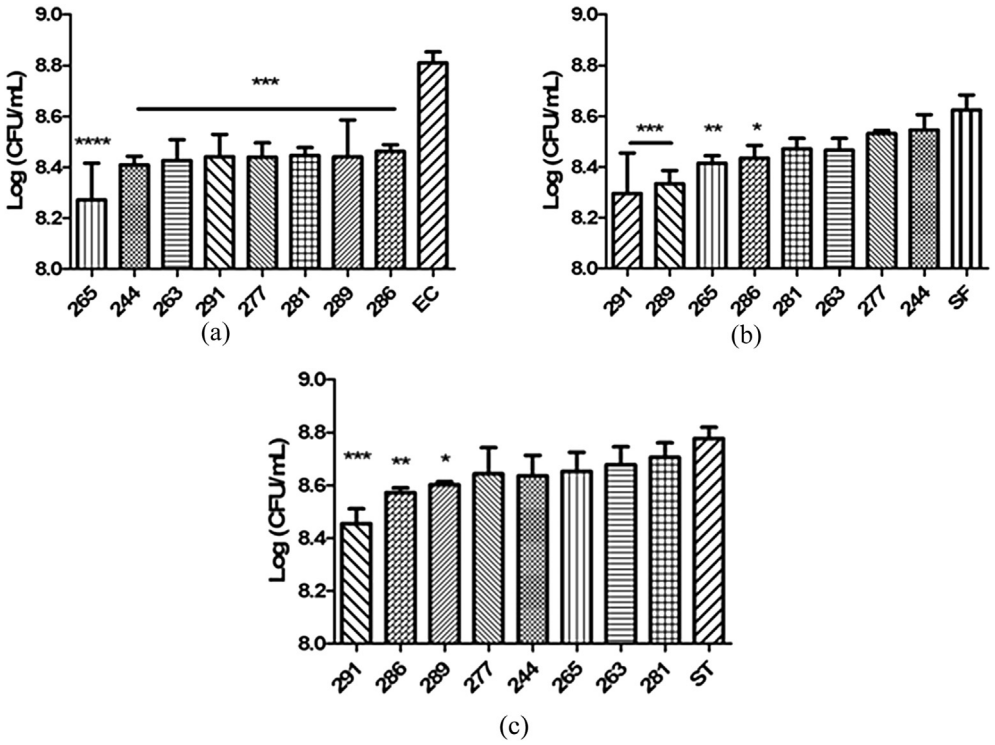

Fig. 3. Cell counts (log CFU/ml) of *Escherichia coli*ATCC 25922 (a), *Shigella flexi* ATTCC 11060 (b) or *Salmonella enterica* var. Typhimurium ATTCC 14028 (c), after growing alone (EC, SF, ST, respectively) or after 24 h of cultivation in supernatant of lactobacilli (the number of the strain is shown on the X axis).

the antibiotics assessed. The rest of the strains showed additional resistance to other antibiotics.

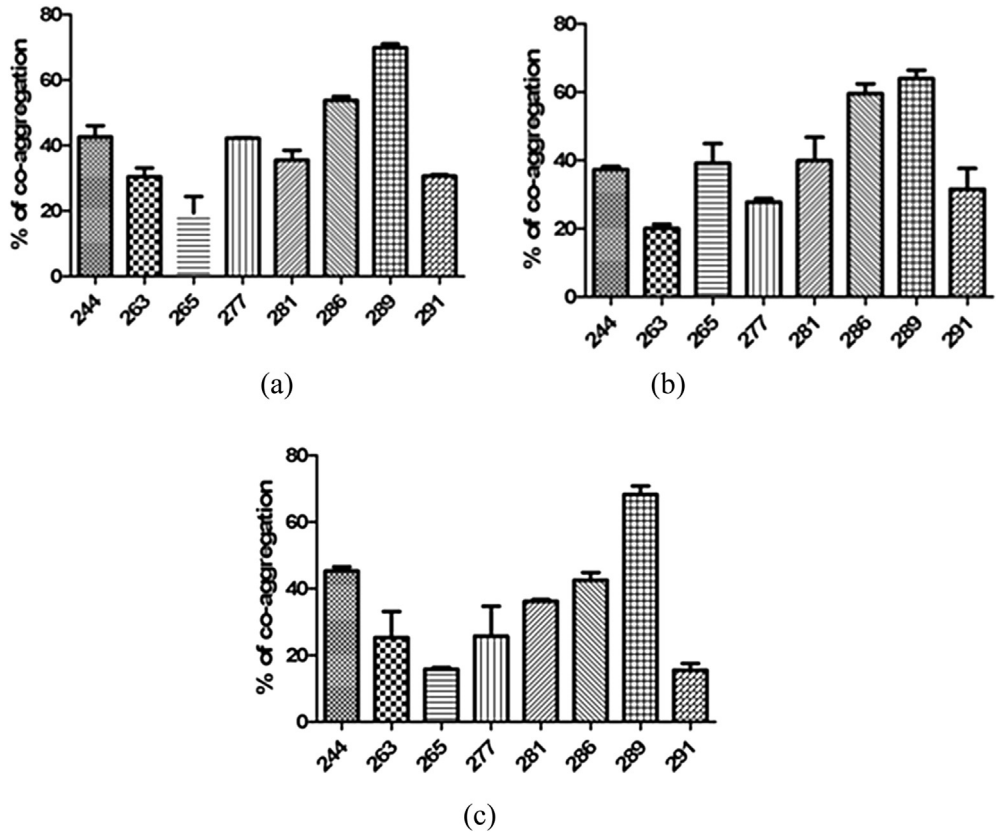

Fig. 4. Co-aggregation percentage of *Escherichia coli* ATCC 25922 (a), *Shigella flexi* ATTCC 11060 (b) or *Salmonella enterica* var. Typhimurium ATTCC 14028 (c) after 4 h of incubation in the presence of lactobacilli (the number of the strain is shown on the X axis).

Table 3
Antibiotic resistance of *L. fermentum* and *L. plantarum* strains isolated from cocoa fermentation.

Strain	Antibiotic[a]							
	A	Clo	V	P	T	Cef	S	NA
L. fermentum 244	S	S	R	S	S	S	R	R
L. fermentum 263	S	S	R	S	S	S	R	R
L. fermentum 265	S	S	R	S	S	S	S	R
L. plantarum 277	S	S	R	MS	R	S	S	R
L. plantarum 281	S	S	R	MS	S	S	S	R
L. plantarum 286	S	S	R	MS	S	S	S	R
L. plantarum 289	S	S	R	MS	MS	S	S	R
L. plantarum 291	S	S	R	MS	S	S	S	R

[a] Amoxicillin (A), chloramphenicol (Clo) (30 μg), vancomycin (V) (30 μg), penicillin (P) (10 μg), tetracycline (T) (30 μg), ceftriaxone (Cef) (30 μg), sulfatrim (S) (25 μg), nalidix acid (NA) (30 μg). R: resistant, MS: moderately susceptible, S: sensitive.

4. Discussion

Locally sourced isolates of *L. fermentum* and *L. plantarum* obtained from Brazilian cocoa fermentation were characterized by RAPD and tested *in vitro* for their probiotic potential. Physiological traits as heat tolerance, hydrophobicity, resistance to simulated gastrointestinal digestion and antibiotic susceptibility were studied. Activity against food pathogens was also tested through different assays: deferred inhibition, co-aggregation, co-cultivation and antagonism of lactobacilli supernatants.

The analysis of RAPD profiles allowed us to recognize 8 strains among 10 isolates: 5 different profiles for *L. plantarum* whereas 3 profiles for *L. fermentum*. All strains were isolated from cocoa fermentation and no significant differences were observed among them for heat tolerance, which might suggest that tested strains were similarly adapted to the relative high temperature stress found during spontaneous cocoa fermentation. In this process, a natural microbial succession of yeasts, lactic-acid, and acetic-acid bacteria takes place, in which temperature reaches up to 50 °C (Schwan & Wheals, 2004).

An important factor for bacterial maintenance in the human gastrointestinal tract (GIT) is to attach to the surface of epithelial cells (Naidu, Bidlack, & Clemens, 1999), especially for probiotics. The attachment of bacteria to epithelial cells depends on factors such as Brownian movement, van der Waals attraction, gravitational forces and surface electrostatic charges (Van Loosdrecht, Norde, & Zehnder, 1990). The hydrophobic nature of the outermost surface of microorganisms has been also shown to be involved in the attachment of bacteria to host tissues (Rosenberg, Gutnick, & Rosenberg, 1980). Although lactobacilli are generally regarded as possessing cell surfaces of an overall hydrophobic nature (García-Cayuela et al., 2014), in our study, *L. plantarum* 281 displayed the highest hydrophobicity value (16.87%). In spite of that, this value can be considered low when compared to other strains of lactobacilli (Burns et al., 2008). Vinderola and Reinheimer found hydrophobicity percentages ranging from 10.9% to 67.8% for strains of probiotic lactobacilli demonstrating that the hydrophobicity can be highly variable within this group (Vinderola & Reinheimer, 2003). The facts that the strains under study were isolated from cocoa fermentation and that cocoa pulp presents a matrix consisting of 82–87% water, 10–15% sugar, 2–3% pentosans, 1–3% citric acid and 1–1.5% pectin (Roelofsen, 1958), might explain their low hydrophobicity. The link between a high hydrophobicity and positive

outcomes of human clinical studies is still controversial. For instance, a strain with a high number of positive functional traits as *L. rhamnosus* GG presents low values of hydrophobicity *in vitro* compared to other isolates of lactobacilli of human origin (Strus, Kukla, Rurańska-Smutnicka, Przondo-Mordarska, & Heczko, 2001).

A desirable trait in a probiotic culture is to show preferably high resistance to gastric acidity, bile salts, pepsin, pancreatin and other enzymes and antimicrobial compounds found in the GIT. Lactobacilli have variable resistance to gastrointestinal conditions being a strain-dependent characteristic (Begley, Gahan, & Hill, 2005; Burns et al., 2008). A range of studies have demonstrated that being from intestinal origin do not necessarily implies resistance to gastrointestinal conditions (Jacobsen et al., 1999; Sanni, Sefa-Dedeh, Sakyi-Dawson, & Asiedu, 2002). In this work, *L. plantarum* strains exhibited higher resistance to simulated gastrointestinal *in vitro* digestion than *L. fermentum* strains. Saito et al. (2014) utilizing the methodology used in this study for the determination of gastrointestinal resistance, but using soy yogurt as food matrix, studied *L. plantarum* TcUESC02 and *L. fermentum* TcUESC01 both isolated from cocoa fermentation. They observed that cell counts decreased less than 1 log cycle for both strains at pH 2.5 and 3.0 (Saito et al., 2014). Higher values of gastrointestinal digestion resistance found by Saito et al. could be due to the use of a food matrix, which can confer protection (Saito et al., 2014). As observed by Saito et al. (2014) there was a decrease in cell viability specially in step I (acid digestion) whereas in steps II (bile shock) and III (intestinal digestion) cell viability remained within the same order of magnitude throughout the process, except for *L. plantarum* 281 (Saito et al., 2014). Considering that acid-sensitive strains can be buffered through the stomach (Huang & Adams, 2004) or microencapsulated with low pH-resistant materials (De Vos, Faas, Spasojevic, & Sikkema, 2010), resistance to bile salts and to conditions found in the small intestine is perhaps more important than gastric survival. The strains here studied maintained cell viability during bile shock and intestinal digestion.

Three methodologies were used to test the antimicrobial potential of *L. fermentum* and *L. plantarum* against food pathogens. Activity against *S. enterica* var. Typhimurium ATTCC 14028, *S. flexi* ATTCC11060, *E. coli* ATCC 25922 were observed in all methodologies used for all the lactobacilli strains studied. The deferred inhibition assay allowed to detect antimicrobial activity for the highest number of lactobacilli strains. We were able to notice that *L. monocytogenes* ATCC 15313 was inhibited by *L. fermentum* strains 244 and 263 when using the deferred inhibition assay. While in the deferred inhibition assay a solid medium was used to detect antagonisms, the co-cultivation assay and the antagonistic activity of lactobacilli supernatants assay were performed in broth. According to Rios et al. a diffusion test (solid medium) is more suitable for the mobility of apolar inhibitory compounds (Rios, Recio, & Villar, 1988).

As an initial screening for potential probiotic, antibiotic susceptibility was performed for all tested strains against antibiotics inhibitors of cell wall (penicillin, amoxicillin, vancomycin and ceftriaxone), nucleic acid (nalidixic acid), aminoglycosides (chloramphenicol and tetracycline), and folic acid (sulfatrim) synthesis. The strains showed sensitivity in different target sites. Most of them were sensitive to most tested antibiotics, except for vancomycin and nalidix acid, probably because of their wild origin (cocoa fermentation). In general, lactobacilli present general resistance to vancomycin due to the absence of target site of action of this antibiotic, having no risk of horizontal transfers (DeLisle & Perl, 2003). Resistance to antibiotics site of action in nucleic acids such as nalidixic acid is commonly found in *Lactobacillus* (Ammor et al., 2008; Charteris et al., 1997; D'Aimmo, Modesto, & Biavati, 2007).

4.1. Conclusions

Considering the *in vitro* resistance to simulated gastrointestinal digestion and antimicrobial and co-aggregation tests together, *L. plantarum* 286 showed the most promising results, followed by *L. plantarum* 289, for further *in vitro* and *in vivo* studies, in order to determine their probiotic value for use in new functional foods developed with locally sourced strains.

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References

Ammor, M. S., Flórez, A. B., van Hoek, A. H. A. M., de los Reyes-Gavilán, C. G., Aarts, H. J. M., Margolles, A., et al. (2008). Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *Journal of Molecular Microbiology and Biotechnology, 14*(1–3), 6–15. http://dx.doi.org/10.1159/000106077. Retrieved from.

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *{FEMS} Microbiology Reviews, 29*(4), 625–651. http://dx.doi.org/10.1016/j.femsre.2004.09.003.

Burns, P., Vinderola, G., Binetti, A., Quiberoni, A., de los Reyes-Gavilán, C. G., & Reinheimer, J. (2008). Bile-resistant derivatives obtained from non-intestinal dairy lactobacilli. *International Dairy Journal, 18*(4), 377–385. http://dx.doi.org/10.1016/j.idairyj.2007.10.012.

Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J. S., … De Vuyst, L. (2007). Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of Cocoa beans in Ghana. *Applied and Environmental Microbiology, 73*(6), 1809–1824. http://dx.doi.org/10.1128/AEM.02189-06.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1997). Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and Bifidobacterium species in mixed bacterial populations. *International Journal of Food Microbiology, 35*(1), 1–27. http://dx.doi.org/10.1016/S0168-1605(96)01222-6.

De Vos, P., Faas, M. M., Spasojevic, M., & Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal, 20*(4), 292–302. http://dx.doi.org/10.1016/j.idairyj.2009.11.008.

DeLisle, S., & Perl, T. M. (2003). Vancomycin-resistant enterococci: A road map on how to prevent the emergence and transmission of antimicrobial resistance. *Chest, 123*(5 suppl), 504S–518S. http://dx.doi.org/10.1378/chest.123.5_suppl.504S.

D'Aimmo, M. R., Modesto, M., & Biavati, B. (2007). Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology, 115*(1), 35–42. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.10.003.

García-Cayuela, T., Korany, A. M., Bustos, I. P., Gómez de Cadiñanos, L., Requena, T., Peláez, C., et al. (2014). Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. *Food Research International, 57*, 44–50. http://dx.doi.org/10.1016/j.foodres.2014.01.010.

Huang, Y., & Adams, M. C. (2004). *In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiology, 91*(3), 253–260. http://dx.doi.org/10.1016/j.ijfoodmicro.2003.07.001.

Hütt, P., Shchepetova, J., Lõivukene, K., Kullisaar, T., & Mikelsaar, M. (2006). Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *Journal of Applied Microbiology, 100*(6), 1324–1332. http://dx.doi.org/10.1111/j.1365-2672.2006.02857.x.

Jacobsen, C. N., Rosenfeldt Nielsen, V., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Pærregaard, A., … Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology, 65*(11), 4949–4956. Retrieved from http://aem.asm.org/content/65/11/4949.abstract.

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology, 94*(6), 981–987. http://dx.doi.org/10.1046/j.1365-2672.2003.01915.x.

Lagunes Gálvez, S., Loiseau, G., Paredes, J. L., Barel, M., & Guiraud, J.-P. (2007). Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. *International Journal of Food Microbiology, 114*(1), 124–130. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.10.041.

Mahasneh, A. M., & Abbs, M. M. (2010). Probiotics and traditional fermented foods: The eternal connection (Mini-Review). *Jordan Journal of Biological Sciences, 3*(4), 133–140.

Naidu, A. S., Bidlack, W. R., & Clemens, R. A. (1999). Probiotic spectra of lactic acid bacteria (LAB). *Critical Reviews in Food Science and Nutrition, 39*(1), 13–126. http://dx.doi.org/10.1080/10408699991279187.

Nardi, R. D., Santos, A. R. M., Carvalho, M. A. R., Farias, L. M., Benchetrit, L. C., & Nicoli, J. R. (1999). Antagonism against anaerobic and facultative bacteria through a diffusible inhibitory compound produced by a *Lactobacillus* sp. isolated from the rat fecal microbiota. *Anaerobe, 5*(3–4), 409–411. http://dx.doi.org/10.1006/anae.1999.0217.

Nielsen, D. S., Hønholt, S., Tano-Debrah, K., & Jespersen, L. (2005). Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). *Yeast, 22*(4), 271–284. http://dx.doi.org/10.1002/yea.1207.

Ortu, S., Felis, G. E., Marzotto, M., Deriu, A., Molicotti, P., Sechi, L. A., … Zanetti, S. (2007). Identification and functional characterization of *Lactobacillus* strains isolated from milk and Gioddu, a traditional Sardinian fermented milk. *International Dairy Journal, 17*(11), 1312–1320. http://dx.doi.org/10.1016/j.idairyj.2007.02.008.

Paéz, R., Lavari, L., Vinderola, G., Audero, G., Cuatrin, A., Zaritzky, N., et al. (2012). Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion. *Food Research International, 48*(2), 748–754. http://dx.doi.org/10.1016/j.foodres.2012.06.018.

Pimm, S. L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., … Sexton, J. O. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science, 344*(6187), 897–1000. http://dx.doi.org/10.1126/science.1246752.

Puerari, C., Magalhães-Guedes, K. T., & Schwan, R. F. (2015). Physicochemical and microbiological characterization of chicha, a rice-based fermented beverage produced by Umutina Brazilian Amerindians. *Food Microbiology, 46*(0), 210–217. http://dx.doi.org/10.1016/j.fm.2014.08.009.

Ramos, C. L., Sousa, E. S. O., de, Ribeiro, J., Almeida, T. M. M., Santos, C. C. A. do A., Abegg, M. A., et al. (2015). Microbiological and chemical characteristics of tarubá, an indigenous beverage produced from solid cassava fermentation. *Food Microbiology, 49*(0), 182–188. http://dx.doi.org/10.1016/j.fm.2015.02.005.

Ramos, C. L., Thorsen, L., Schwan, R. F., & Jespersen, L. (2013). Strain-specific probiotics properties of *Lactobacillus fermentum, Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. *Food Microbiology, 36*(1), 22–29. http://dx.doi.org/10.1016/j.fm.2013.03.010.

Rios, J. L., Recio, M. C., & Villar, A. (1988). Screening methods for natural products with antimicrobial activity: A review of the literature. *Journal of Ethnopharmacology, 23*(2–3), 127–149. http://dx.doi.org/10.1016/0378-8741(88)90001-3.

Roelofsen, P. A. (1958). Fermentation, drying, and storage of cacao beans. In F. Research (Ed.), *E. M. M. and G. F. S. B. T.-A* (Vol. 8, pp. 225–296). Academic Press. http://dx.doi.org/10.1016/S0065-2628(08)60021-X.

Rosenberg, M., Gutnick, D., & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters, 9*(1), 29–33. http://dx.doi.org/10.1111/j.1574-6968.1980.tb05599.x.

Saito, V. S. T., dos Santos, T. F., Vinderola, C. G., Romano, C., Nicoli, J. R., Araújo, L. S., … Uetanabaro, A. P. T. (2014). Viability and resistance of lactobacilli isolated from Cocoa fermentation to simulated gastrointestinal digestive Steps in Soy Yogurt. *Journal of Food Science, 79*(2), M208–M213. http://dx.doi.org/10.1111/1750-3841.12326.

Sanni, A., Sefa-Dedeh, S., Sakyi-Dawson, E., & Asiedu, M. (2002). Microbiological evaluation of ghanaian maize dough co-fermented with cowpea. *International Journal Food Science Nutrition, 53*(5), 367–373. http://dx.doi.org/10.1080/0963748021000044705.

Santos, A. O., Ávila, C. L. S., & Schwan, R. F. (2015). Selection of tropical lactic acid bacteria for enhancing the quality of maize silage. *Journal of Dairy Science, 96*(12), 7777–7789. http://dx.doi.org/10.3168/jds.2013-6782.

Schroth, G., Farias, D., Araujo, M., Bede, L., Bael, V. A. S., Cassano, R. C., … Delabie, C. H. J. (2011). Conservation in tropical landscape mosaics: The case of the cacao landscape of southern Bahia, Brazil. *Biodivers Conserv.* http://dx.doi.org/10.1007/s10531-011-0052-x.

Schwan, R. F., & Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role in chocolate quality. *Critical Reviews in Food Science and Nutrition, 44*(4), 205–221. http://dx.doi.org/10.1080/10408690490464104.

Strus, M., Kukla, G., Rurańska-Smutnicka, D., Przondo-Mordarska, A., & Heczko, P. (2001). Surface properties of *Lactobacillus* strains. II. Adherence to tissue culture surfaces. *Medycyna Doswiadczalna Mikrobiologia, 53*(3), 253–258.

Sybesma, W., Kort, R., & Lee, Y.-K. (2015). Locally sourced probiotics, the next opportunity for developing countries? *Trends in Biotechnology, 33*(4), 197–200. http://dx.doi.org/10.1016/j.tibtech.2015.01.002.

Todorov, S. D., Ho, P., Vaz-Velho, M., & Dicks, L. M. T. (2010). Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Science, 84*(3), 334–343. http://dx.doi.org/10.1016/j.meatsci.2009.08.053.

Van Loosdrecht, M. C. M., Norde, W., & Zehnder, A. J. B. (1990). Physical chemical description of bacterial adhesion. *Journal of Biomaterials Applications, 5*(2), 91–106. Retrieved from http://jba.sagepub.com/content/5/2/91.abstract.

Vinderola, C. G., & Reinheimer, J. A. (2003). Lactic acid starter and probiotic bacteria: A comparative “in vitro” study of probiotic characteristics and biological barrier resistance. *Food Research International, 36*(9–10), 895–904. http://dx.doi.org/10.1016/S0963-9969(03)00098-X.