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ORIGINAL PAPER

Profile of microbial communities present in tibico (sugary kefir) grains from different Brazilian States

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Abstract The microorganisms associated with Brazilian tibico (sugary kefir) grains from eight different Brazilian States were investigated using a combination of culture-dependent and culture-independent methods. The bacterial genera included *Lactobacilllus*, *Acetobacter*, *Gluconobacter*, *Bacillus* and yeast genera included *Pichia*, *Saccharomyces*, *Kazachstania*, *Candida*, *Zygosaccharomyces* and *Yarrowia*. Some bacteria and yeast detected by sequence analysis of DGGE (denaturing gradient gel electrophoresis) bands were not recovered at some Brazilian tibico grains by plating. Conversely, DGGE fingerprints did not reveal bands corresponding to some of the species isolated by culturing methods. The bacteria's *Gluconobacter liquefaciens* and *Bacillus cereus* and the yeast *Pichia cecembensis*, *Pichia caribbica* and *Zygosaccharomyces fermentati* are described for the first time in tibico grains. Our findings are relevant to the knowledge of tibico grains used as starter culture for fermented beverages consumed by the Brazilian population.

Keywords Kefir · Tibico · Bacteria · Yeast · PCR-DGGE

Introduction

Kefir is a consortium of microorganism that is mainly used in the production of the low alcoholic, traditional Russian beverage ''kefir'', where milk constitutes the initial fermenting substrate. This mixed culture consists of various yeasts (*Kluyveromyces*, *Candida* and *Saccharomyces*) (Garbers et al. 2004; Latorre-García et al. 2007; Magalhães et al. 2010a, b), various lactic acid bacteria of the genus *Lactobacillus, Lactococcus* and acetic acid bacteria (Miguel et al. 2010; Magalhães et al. 2010a). In the preparation of traditional kefir, a starter culture must be produced from the kefir grains. Kefir grains are added to milk and incubated with stirring at 24–26°C until the pH is decreased to 4.6. After incubation, the kefir grains are removed via sieving and are reused in subsequent starter culture preparation. With each subsequent incubation, the size of the kefir grains increases slightly. The fresh kefir starter culture is added to pasteurized milk at the rate of 2–3% (v/v) and incubated at 24–26°C for approximately 18–48 h. After incubation, the product is refrigerated promptly (Güzel-Seydim et al. 2000).

Kefir grains can be also cultivated in a sugary solution. The kefir grains are usually inoculated in sugary solution in the proportion of 3–10%. The kefir grains cultived in sugary solution are known as tibico or tibi (Rubio et al. 1993). These tibico grains are very similar to the milk kefir grains, in relation to their structure and microbiological constitution (Magalhães et al. 2010a). The tibico beverage is mainly consumed in Mexico and Brazil. The microbial populations of tibico grains include LAB, such as *Lactobacillus*, *Leuconostoc*, *Streptococcus*, acetic acid bacteria such as *Acetobacter* and a small proportion of yeasts, such as *Saccharomyces*, *Zygosaccharomyces*, *Candida*, and *Kloekera* (Rubio et al. 1993; Magalhães et al. 2010a).

For many years, research on the microbiota of kefir grains has relied on conventional culturing methods followed by phenotypic and/or genotypic identification of a (randomly) selected subset of purified isolates. Recently, molecular culture-independent approaches have proven to be powerful tools in providing a more complete inventory

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of the microbial diversity in food samples (Giraffa 2004) and PCR-DGGE has been applied successfully to assess the microbiota in milk kefir grains (Chen et al. 2008; Jianzhong et al. 2009). Despite the limitations of conventional culturing methods, this remains the only way to obtain microbial strains of importance for biotechnology purposes and may still provide a useful means for investigating microbial succession by enumerating viable cells at every stage of fermentation. Thus, a combination of conventional culturing methods and PCR-DGGE is needed to describe in maximal detail the microbiota present within food (Chen et al. 2008).

In Brazil, tibico grains are used in private households spontaneously fermented by inoculating in sugary solution. The different Brazilian States are characterized by different climatic zones that include cold temperatures (South Region States), average temperatures (Southeast Region States) and hot temperatures (North and Northeast States). Tibico grains have been adapted to these areas during a process of continuous propagation and the exact impact of climate, environment and cultivation methods of microbial populations remains unclear.

The main aim of this study was to assess the profile microbial communities present in tibico grains from different Brazilian States. For this purpose, a combined approach of conventional culturing identification with culture-independent analysis using PCR-DGGE was performed.

Materials and methods

Samples of Brazilian tibico grains

The samples of tibico grains were obtained from families (private household) that traditionally consumed the tibico beverage from eight different Brazilian States (One sample of each Brazilian State): São Paulo (Santos), Goiás (Caiaponia), Minas Gerais (Governador Valadares), Alagoas (Maceió), Rio de Janeiro (Rio de Janeiro), Distrito Federal (Brasília), Espírito Santo (Aracruz) and Bahia (Vitória da Conquista). The samples of the tibico grains were sent in plastic bags, involved in powdered brown sugar. Each sample (50 g) was aseptically removed for phenotypic and genotypic analysis.

Enumeration of different groups of bacteria and yeast

Bacteria and yeasts were enumerated by the surface spread technique, plating in triplicate 100 µl of each diluted sample according to Magalhães et al. (2010a). Enumeration of microorganisms was carried out using two different culture media. Bacteria were enumerated in De Man, Rogosa and Sharpe Agar (MRS, Oxoid/Brazil) supplemented with 0.4 mg/ml nystatin (Sigma, St. Louis, USA). Yeasts were enumerated on malt yeast glucose peptone extract (MYGP, Oxoid/Brazil) containing 100 mg chloramphenicol (Sigma, St. Louis, USA) and 50 mg chlortetracycline (Sigma, St. Louis, USA) to inhibit bacterial growth. After spreading, plates were incubated at 28°C for yeast and 28 and 35°C for bacteria (the incubation at 28 and 35°C for bacteria was done in order to reproduce the average room temperature of North and Northeast States of Brazil favoring only the microbiota already present in the grains, since they were plated the way they were received). The plates were incubed for 48 h for bacteria, and 5 days for yeasts; and colony forming units ($\log_{10}$ cfu/ml) were quantified. For each type of medium containing isolated colonies, the square root of the number of colonies was taken at random for identification.

Identification of bacteria and yeast by conventional methods

Gram-negative bacteria were identified using Bac-Tray Kits I, II and III (Difco) according to the manufacturer's instructions. Gram-positive bacteria were identified using the API 50 CHB and API 50 CHL galleries (Bio-Merieux).

Yeasts were identified according to Magalhães et al. (2010a).

DNA extraction and PCR-DGGE analysis

Each sample (5 g) (tibico grains of Brazilian States different) was transferred into a plastic tube and was subjected to DNA extraction using a NucleoSpin Tissue kit (Macherey–Nagel, Düren, Germany), according to the manufacturer's instructions. Genomic DNA was used as template for PCR amplification of bacterial or yeast ribosomal target regions, for denaturing gradient gel electrophoresis (DGGE) analyses. Two primers sets were used for the analysis of each microbial community. Table 1 presents information about the primers and conditions of PCR and DGGE.

DGGE bands sequencing and multivariate analysis

Selected bands from the PCR-DGGE gels were excised with a sterile blade, and the DNA was separated using the kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. DNA recovered from each DGGE band was reamplified using the primers same for bacteria's and yeasts (Table 1) without GC clamp. The PCR amplicons were sequenced by Macrogen Inc. (Seoul, South Korea). GenBank searches (http://www.ncbi.nlm.nih.gov/BLAST/) were performed to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

Table 1 DGGE-PCR primers used to detect yeasts and bacteria's in Brazilian tibico grains

Primer	Sequence (5′–3′)	Community	Target	PCR conditions	DGGE conditions	References
968fGC	AAC GCG AAG AAC CTT AC	Bacteria	V6-V8 region of the 16S rRNA gene	Condition 1	16 h at 70 V at 60°C	b
	GC clamp connected to the 5′ end of 968f					
1401r	CGG TGT GTA CAA GAC CC					
ITS1fGC	TCC GTA GGT GAA CCT GCG G	Yeast	ITS region of the rDNA	Condition 1	16 h at 70 V at 60°C	a
	GC clamp connected to the 5′ end of ITS1gc					
ITS4r	TCCTCCGCTTATTGATATGC					
338fGC	GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG	Bacteria	V3 region of the 16S rRNA gene	Condition 1	6 h at 70 V at 60°C	b
	GC clamp connected to the 5′ end of 338fgc					
518r	ATT ACC GCG GCT GCT GG					
NS3fGC	GCA AGT CTG GTG CCA GCA GCC	Yeast	18S region of the rDNA	Condition 2	16 h at 70 V at 60°C	b
	GC clamp connected to the 5′ end of NS3gc					
YM951r	TTG GCA AAT GCT TTC GC					

GC clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GG, *f* forward primer, *r* reverse primer

[a] White et al. (1990)

[b] Magalhães et al. (2010b)

Condition 1 denatured for 5 min at 95°C. 30 cycles: denaturing at 92°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 60 s. Final extension for 10 min at 72°C. *Condition 2* 35 cycles instead of 30

The similarity of the composition of microbial communities at different Brazilian tibico grains was determined based on the presence or absence of amplicons detected by DGGE. The gels were analysed using the Diversity Database program for determining the diversity of amplicons. The hierarchical clustering was performed using the program Systat 8.0, based on similarity matrices generated by the method of agreement (simple matching), using the algorithm of Ward and Euclidean distance. Data from microorganisms isolated and identified from Brazilian sugary kefir grains was subjected to statistical analysis (principal component analysis, or PCA) using The Unscrambler® 9.7 software (CAMO, Oslo, Norway).

Results and discussion

Evaluation of different primers to assess bacterial and yeast communities in Brazilian tibico grains

Although many studies have clearly demonstrated the broad applicability of PCR-DGGE to discriminate among target bacteria/yeast, the displayed community profiles can be highly dependent on the PCR primers used (Jianzhong et al. 2009). It has been shown that targeting different rDNA regions may, sometimes, lead to different results in terms of microbial composition. PCR bias (Kanagawa 2003), co-migration of DNA from different species in the same band (Sekiguchi et al. 2001) and formation of multiple bands in amplification of genes from single genomes (Nübel et al. 1996), may provide incorrect information about dominance and diversity of certain ribotypes in the community.

In this study, four of the mostly used primers for PCR-DGGE, were selected to profile microbial communities in Brazilian tibico grains: two primer sets targeting different regions of bacterial 16S rDNA, namely 968fGC/1401r (V6–V8 region) and 338fGC/518r (V3 region), and the primer pairs ITS1fGC/ITS4r and NS3fGC/YM951r targeting fungal ITS (internal transcribed spacer) and 18S rDNA regions, respectively. All the analyzed primer pairs gave satisfactory amplification of the samples (Figs. 1, 2). For yeast community, both ITS and 18S rDNA PCR-DGGE analyses yielded the same microbial DGGE profile in both gels (Figs. 1b, 2a). For bacteria, however, a different profile was generated by the two primer pairs tested. The primer pair 968fGC/1401r, targeting the 16S rDNA V6–V8 regions, yield patterns with two main bands (high intensity) in the microbial profile from Alagoas sample grains (Fig. 1b), whereas the pair 338fGC/518r generated profiles with absence of bands in Alagoas sample grains (Fig. 1a). The other bacteria profiles were similar in both gels (Fig. 1a, b). In this study the primers were capable to differentiate species of *Lactobacillus* and uncultured bacterium besides yeast species.

Other authors tested the feasibility of different primers pairs for molecular detection of microbial communities. Randazzo et al. (2002) used the 16S rDNA V6–V8 regions to examine the evolution of bacterial community during manufacturing of Ragusano cheese. This PCR-DGGE

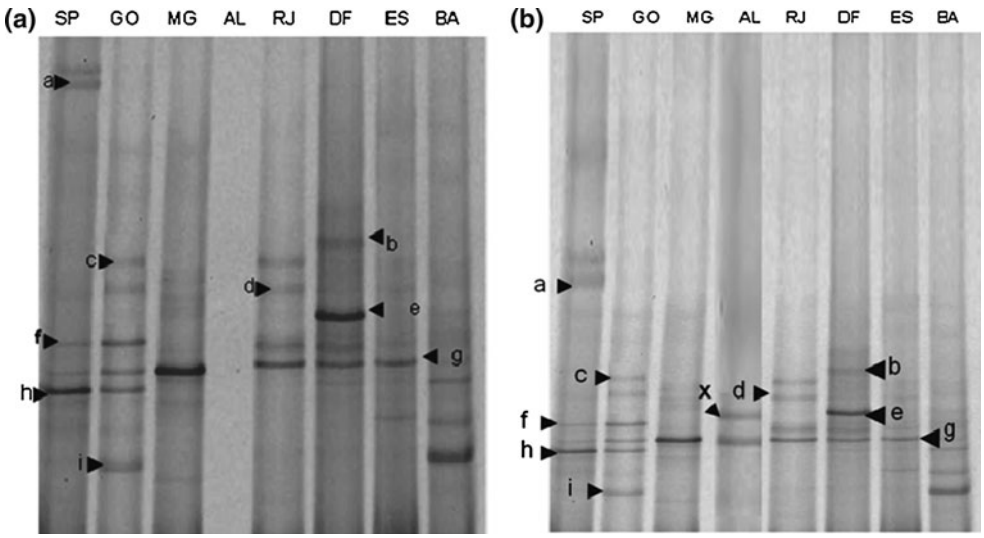

Fig. 1 DGGE profiles of bacterial 16S rDNA V3 region (**a**) and V6–V8 regions (**b**) amplified from tibico grains samples. *Abbreviations*: *SP* São Paulo, *GO* Goiás, *MG* Minas Gerais, *AL* Alagoas, *RJ* Rio de Janeiro, *DF* Distrito Federal, *ES* Espírito Santo, *BA* Bahia. *Bands a, c—Lactobacillus helveticus* AB026985.1/AB061775.1. *Band b—Lactobacillus sunkii* AB366385.1. *Bands d, f—Lactobacillus kefiranofaciens* AJ575259.1. *Band g—Lactobacillus parakefiri* AB429373.1. *Bands h, i*—uncultured bacterium EF014703.1. *Band e, x*—not identified

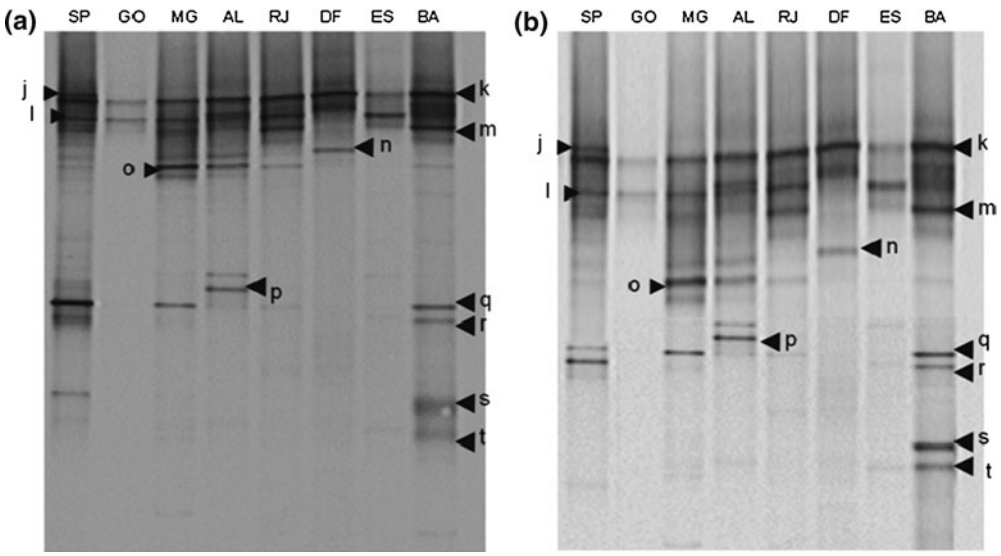

Fig. 2 DGGE profiles of fungal 18S (**a**) and ITS (**b**) rDNA fragments amplified from tibico grains samples. *Abbreviations*: *SP* São Paulo, *GO* Goiás, *MG* Minas Gerais, *AL* Alagoas, *RJ* Rio de Janeiro, *DF* Distrito Federal, *ES* Espírito Santo, *BA* Bahia. *Band k—Saccharomyces cerevisiae* EU649673.1. *Band l—Zygosaccharomyces* sp. AF017728.1. *Band o—Candida* sp. GI190714325. *Band n—Pichia fermentans* FJ770542.1. *Band s—Kluyveromyces lactis* AJ229069.1. *Band p—Kluyveromyces marxianus* GM620595. *Band t—Lachancea meyersii* AY645661.1. *Bands j, q and r*—not identified

analysis was able to successfully identify and differentiate between species of *Lactococcus, Leuconostoc, Streptococcus, Lactobacillus* and *Macrococcus.* Van Beek and Priest (2002) monitored lactic acid bacteria communities during fermentation of Malt whisky by PCR-DGGE of V3 and RT-PCR-DGGE of V6–V8 regions of 16S rDNA. These authors optimized the separation of *Lactobacilli* in DGGE by adopting the V6–V8 region as a target, giving better resolution of several species due to higher heterogeneity in sequences of species from *Lactobacillus.* In a recent study Magalhães et al. (2010a) could not differentiate some species of *Lactobacillus* by PCR-DGGE migration of fragments of the 16S rDNA V3 region. Additionally, some individual *Lactobacillus* spp. were found to correspond to more than one band in the DGGE profile, probably due to target sequence heterogeneity among multiple copies of the 16S rDNAs. The same authors in also recent studies evaluated the milk kefir grains (Magalhães et al. 2010b). For yeast community, both ITS and 18S rDNA PCR-DGGE analyses yielded the same microbial DGGE profile. For bacteria, however, a different profile was generated by the two primer pairs tested. The primer pair 968fGC/1401r, targeting the 16S rDNA V6–V8 regions, yield patterns with two main bands (high intensity) in the microbial profile, whereas the pair 338fGC/518r generated profiles with five bands (high intensity).

Altogether, the results obtained in this work using different pairs of primers, show a similar DGGE profile either in tibico grains, under different Brazilian States suggesting the presence of a robust dominant microbial consortium.

Enumeration and identification of microbial communities present in Brazilian tibico grains

Using conventional culture techniques, we have monitored the microbial diversity present in the Brazilian tibico grains. Previous results have shown that two groups of microorganisms co-exist in tibico grains: lactic acid bacteria and yeast (Rubio et al. 1993; Magalhães et al. 2010a). The same authors observed that the tibico grains demonstrate larger bacterial population in comparison with yeast population.

The tibico grains of the eight different Brazilian States showed similar macroscopic characteristics. Tibico grains from São Paulo, Goiás, Rio de Janeiro, Distrito Federal and Espírito Santo presented a distribution of size between 6 and 9 mm, while Minas Gerais, Alagoas and Bahia grains presented size $\leq$5 mm. Magalhães et al. (2010a) observed tibico grains measuring 5 mm.

The enumeration values (cfu/g) of the isolated viable bacteria and yeast are given in Table 2. The yeast counts varied between the minimum value of 5.92 $\log_{10}$ cfu/g in the sample from Minas Gerais and the maximum 8.30 $\log_{10}$ cfu/g in the sample from Rio de Janeiro. In MRS the average number of cfu/g varied between 6.04 $\log_{10}$ cfu/g at 28°C and 6.36 $\log_{10}$ cfu/g at 35°C in the samples from Alagoas and São Paulo respectively and a maximum 9.18 $\log_{10}$ cfu/g at 28°C and 9.08 $\log_{10}$ cfu/g at 35°C in the sample from Bahia.

In order to establish the different species of bacteria and yeast present in Brazilian tibico grains, all the microbial isolates were identified. A total of 286 isolates were obtained from all samples. Among the isolates examined, 201 (70%) were found to be bacteria and 85 (30%) were yeasts. The Table 3 displays the distribution of the isolated microorganisms of the tibico grains of Brazilian States different. The 9 yeast species identified in the tibico grains in this work by conventional technique were, *Saccharomyces cerevisiae, Pichia cecembensis, Yarrowia lipolytica, Pichia membranifaciens, Pichia caribbica, Pichia fermentans, Candida valdiviana, Zygosaccharomyces fermentati* and *Kazachstania aerobia* (Table 3). The species *Candida valida, Pichia membranafaciens, Kazachstania aerobia* and *Saccharomyces cerevisiae* were also identified in tibico grains from different locals of Mexico (Ulloa et al. 1994). *Saccharomyces cerevisiae, Pichia fermentans* and *Yarrowia lypolytica* are species that were described as microorganisms present in the milk kefir and tibico grains (Rubio et al. 1993; Jianzhong et al. 2009; Magalhães et al. 2010a), whereas *Pichia cecembensis, Pichia caribbica* and *Zygosaccharomyces fermentati* have not been reported as microorganisms present in kefir grains.

Table 2 Microbial population of Brazilian tibico grains from different origins ($\log_{10}$ cfu/g)

Grains origin (Brazilian States)	28°C MRS	35°C MRS	28°C MYGP
SP	7.57 ± 0.01	6.36 ± 0.01	8.04 ± 0.07
GO	8.43 ± 0.02	8.28 ± 0.02	7.82 ± 0.01
MG	7.08 ± 0.01	6.98 ± 0.01	5.92 ± 0.08
AL	6.04 ± 0.03	7.87 ± 0.04	7.81 ± 0.05
RJ	6.08 ± 0.03	7.89 ± 0.01	8.30 ± 0.01
DF	7.65 ± 0.01	7.67 ± 0.03	7.61 ± 0.04
ES	7.23 ± 0.02	7.37 ± 0.01	5.99 ± 0.01
BA	9.18 ± 0.01	9.08 ± 0.01	6.49 ± 0.02
Min	6.04 ± 0.03	6.36 ± 0.01	5.92 ± 0.08
Max	9.18 ± 0.01	9.08 ± 0.01	8.30 ± 0.01

ND not detected, *MRS* medium for bacterial growth, *MYGP* medium for yeast growth, ± standard deviation

SP São Paulo, *GO* Goiás, *MG* Minas Gerais, *AL* Alagoas, *RJ* Rio de Janeiro, *DF* Distrito Federal, *ES* Espírito Santo, *BA* Bahia

A new species of the genus *Pichia,* proposed name *Pichia cecembensis* sp. nov., was isolated in papaya fruits (Bhadra et al. 2007). This species was identified from tibico grain sample from São Paulo, Bahia and Alagoas. *Pichia caribbica* was found in tibico grain sample from Distrito Federal, Rio de Janeiro and Goiás. *Pichia caribbica* has been described as the teleomorphic state of *Candida fermentati* and *Candida guilliermondii* based on molecular data (Vaughan-Martini et al. 2005). The *Zygosaccharomyces fermentati*, identified in tibico grain from all the Brazilian States except Espírito Santo, were also found in grape musts and orange juice samples obtained in Surat Thani Province (Sukkasem et al. 2007). In Espírito Santo only the specie *Kazachstania aerobia* was identified.

The 11 bacterial species identified in the tibico grains samples from different Brazilian states were *Lactobacillus casei, Lactobacillus sunkii, Lactobacillus helveticus, Lactobacillus buchneri, Lactobacillus paracasei, Lactobacillus satsumensis, Lactobacillus kefiri, Lactobacillus kefir, Bacillus cereus, Acetobacter lovaniensis, Gluconobacter liquefaciens* (Table 3). Species of *Lactobacillus* and *Acetobacter lovaniensis,* have been previously reported as members of the tibico grains population (Magalhães et al. 2010a).

The species belonging *Bacillus* genus such as *B. brevis, B. polymyxa, B. circulans, B. coagulans, B. firmus, B. macerans* and *B. pumilus* were identified in tibico grains derived from Mexico (Rubio et al. 1993). The specie *Bacillus cereus* is a saprophytic bacteria commonly found in soil and its presence in fermented products suggest the

Table 3 Microbial isolates present in tibico grains

Samples		Isolates identified	Identified microorganism number/ total isolates
SP	Bacteria	*Lactobacillus casei*	4/201
		Lactobacillus sunkii	8/201
		Lactobacillus kefiri	12/201
	Yeast	*Saccharomyces cerevisiae*	7/85
		Pichia cecembensis	3/85
		Yarrowia lipolytica	2/85
		Zygosaccharomyces fermentati	1/85
GO	Bacteria	*Lactobacillus satsumensis*	4/201
		Lactobacillus paracasei	6/201
		Gluconobacter liquefaciens	12/201
	Yeast	*Pichia membranifaciens*	6/85
		Pichia caribbica	4/85
		Saccharomyces cerevisiae	1/85
		Zygosaccharomyces fermentati	2/85
MG	Bacteria	*Lactobacillus kefiri*	8/201
		Lactobacillus paracasei	3/201
	Yeast	*Candida valdiviana*	7/85
		Zygosaccharomyces fermentati	1/85
RJ	Bacteria	*Lactobacillus kefiri*	7/201
		Lactobacillus casei	2/201
	Yeast	*Pichia membranifaciens*	6/85
		Pichia caribbica	3/85
		Candida valdiviana	2/85
		Zygosaccharomyces fermentati	1/85
DF	Bacteria	*Lactobacillus satsumensis*	2/201
		Lactobacillus casei	1/201
		Lactobacillus kefir	12/201
		Acetobacter lovaniensis	18/201
		Bacillus cereus	11/201
	Yeast	*Saccharomyces cerevisiae*	1/85
		Pichia caribbica	3/85
		Pichia fermentans	6/85
		Zygosaccharomyces fermentati	1/85
BA	Bacteria	*Lactobacillus satsumensis*	3/201
		Lactobacillus casei	1/201
		Gluconobacter liquefaciens	11/201
		Acetobacter lovaniensis	14/201
		Bacillus cereus	9/201
	Yeast	*Saccharomyces cerevisiae*	6/85
		Zygosaccharomyces	1/85
		fermentati Pichia cecembensis	3/85
AL	Bacteria	*Lactobacillus casei*	18/201
		Lactobacillus kefir	1/201
	Yeast	*Saccharomyces cerevisiae*	4/85
		Zygosaccharomyces	1/85
		fermentati Pichia cecembensis	2/85
ES	Bacteria	*Lactobacillus helveticus*	6/201
		Lactobacillus buchneri	2/201
		Lactobacillus kefir	4/201
	Yeast	*Saccharomyces cerevisiae*	6/85
		Zygosaccharomyces	1/85
		fermentati Pichia cecembensis	3/85

SP São Paulo, *GO* Goiás, *MG* Minas Gerais, *RJ* Rio de Janeiro, *DF* Distrito Federal, *ES* Espírito Santo, *BA* Bahia, *AL* Alagoas

need of hygienic-sanitary improvements in the whole fluxogram of milk processing (Vidal-Martins et al. 2005). The presence of *Bacillus cereus* in the tibico grains sample from Bahia and Distrito Federal may be due to contamination at some stage in the preparation or submission of grain. The production of fermented foods under controlled conditions and its safety assurance depend on the knowledge and control of their microbiota. Traditional fermented foods are obtained by natural fermentations in which no inoculum is added and contain microbial complexes. These results show the importance of the identification of the microorganisms present in the tibico grains in the preparation of tibico beverages for human consumption.

Lactobacillus were present in all tibico grains samples in this study, indicating the importance of this group for the production of the beverage (Table 3). Previous studies showed that *Lactobacillus* species also have been isolated and identified in the tibico grains samples (Rubio et al. 1993; Magalhães et al. 2010a). *Lactobacillus satsumensis,* which was isolated from tibico kefir grains obtained from Goiás, Bahia and Distrito Federal, was also isolated from shochu mashes a traditional Japanese distilled spirit made from fermented rice, sweet potato, barley and other starchy materials (Endo and Okada 2005). *Lactobacillus satsumensis* clustered in the *Lactobacillus casei—Pediococcus* group and was closely related to *Lactobacillus nagelii* and *Lactobacillus mali* on the basis of 16S rDNA gene sequence similarity. The isolate was considered to represent a novel species, for which the name *Lactobacillus satsumensis* was proposed (Endo and Okada 2005).

The acetic acid species *Acetobacter lovaniensis* isolated from the tibico grains from Bahia and Distrito Federal. Magalhães et al. (2010a) identified *Acetobacter* in tibico grains. *Gluconacetobacter liquefaciens* isolated from the tibico grains from Bahia and Goiás although present in other fermented food (Seerunruangchai et al. 2004); their presence in tibico was first reported in this study.

To determinate the total composition of microbiota in the Brazilian tibico grains, PCR-DGGE analysis was used. Recently Miguel et al. (2010) evaluated the constitution bacterial population of Brazilian milk kefir grains for technique of PCR-DGGE. The authors showed that the dominant bacterial genus was *Lactobacillus*.

The bands of the gels from Figs. 1a, b were excised from the acrylamide gel, re-amplified and the eluted DNA fragments were used for sequencing with the primers 338f/518r and 968f/1401r respectively. This sequencing exhibited higher than 97% identity with sequences in the GenBank. The bands a and c were identified as *Lactobacillus helveticus,* the band b as *Lactobacillus sunkii*. The bands d and f as *Lactobacillus kefiranofaciens*. The band g as *Lactobacillus parakefiri*, h and i uncultured bacterium. Band e and x could not be assigned to any bacterial species. The *Lactobacillus helveticus* (bands a and c) and *Lactobacillus kefiranofaciens* (bands d and f) were found in bands at different positions. These multiple banding patterns may be due to sequence microheterogeneity between multiple copies of the 16S rDNA gene of this strain (Nübel et al. 1999). A single species with multiple rDNA copies can overestimate a community diversity detected by DGGE because the technique could have favored the extraction of this specie.

The *Lactobacillus helveticus* was identified in the sample from Espirito Santo by the culture dependent method, but the analysis of DGGE bands was not observed in the same positions (bands a and c) in São Paulo and Goiás samples. This apparent contradiction between culture dependent and independent methods could be explained by the difficulty to obtain good DNA extraction and PCR reaction of the grain samples from Espirito Santo. Initial template DNA ratio and template competition may affect the detection of microorganisms present in low abundance in microbial complexes (Zhang et al. 2005). Also, it is interesting to note that *L. helveticus* was not identified by the culture dependent technique in the samples from São Paulo, Goiás and Rio de Janeiro, but was identified in samples analyzed by DGGE. The possible explanation might be that this microorganism was inviable to grow in the plates of these samples. DGGE analysis does not provide information on the viability of the microorganisms. The bands d and f correspond to *Lactobacillus kefiranofaciens* that appeared in samples from São Paulo, Rio de Janeiro and Goiás in DGGE gel. These multiple banding patterns may be also due to sequence heterogeneity between multiple copies of the 16S rDNA of these strains (Miguel et al. 2010). This bacterium was not isolated in plates of the grains sampled, probably for being inviable. In the Rio de Janeiro samples it was not possible to isolated bacteria in plates without diluting the sample. In DGGE analysis of Rio de Janeiro samples bands were observed being identified as *Lactobacillus kefiranofaciens* and *Lactobacillus parakefiri.* In the São Paulo tibico sample *Lactobacillus kefiranofaciens* and *Lactobacillus helveticus* were identified. These species also were isolated from Taiwanese milk kefir grains (Chen et al. 2008).

Bands g which corresponds to *Lactobacillus parakefiri* appeared in all of the samples with the exception of Bahia grains in DGGE gel. This specie was not isolated by culture dependent methods, which may be the result of the fact that this bacterium was damaged in transit or dead. Previous studies showed that *Lactobacillus parakefiri* specie has been reported a member of several milk kefir grains (Garrote et al. 2001; Miguel et al. 2010). The *Lactobacillus sunkii* bacterium identified from the São Paulo sample was unique, found by both independent and culture dependent methods. The specie *Lactobacillus casei, Lactobacillus kefir, Lactobacillus satsumensis, Gluconobacter liquefaciens, Acetobacetr lovaniensis* and *Bacillus cereus* were identified only by culture dependent methods, not being identified by culture independent methods. This may be due to biases inherent in the PCR technique, which reinforces the idea that PCR based methods cannot account for all organisms from a given sample and a polyphasic approach is needed when a more comprehensive assessment of microbial diversity is sought.

In yeast analysis, PCR-DGGE showed a good correlation with the culture-independent methods. The bands of the gels from Figs. 1b and 2a were excised from the acrylamide gel, re-amplified and the eluted DNA fragments were used for sequencing with the primers NS3f/YM951r and ITS1f/ITS4r respectively. This sequencing exhibited higher than 97% identity with sequences in the GenBank. Band k represented the *Saccharomyces* sensu stricto group (Figs. 2a, b). This group of yeast consists of very closely related species which, in some cases, never seem to show a clear-cut separation (Giraffa 2004). Among the yeasts present in the *Saccharomyces* sensu stricto group, *Saccharomyces cerevisiae* was the most probable strain identified because according to culture-based isolations, this species was the most commonly recovered yeast in the Brazilian tibico grains (Table 3). *S. cerevisiae* has been reported previously to play a significant role in the fermentation of tibico grains (Magalhães et al. 2010a).

Fragments most closely related to band l as *Zygosaccharomyces* sp., band o as *Candida* sp. were also detected. Despite the fact that these amplicons were derived only at the genus level, *Zygosaccharomyces fermentati* and *Candida valdiviana,* were the most probable species to be identified because they were sporadically isolated during the fermentation processes (Table 3). Band n represented the *Pichia fermentans.* Band s—*Kluyveromyces lactis*, band t—*Lachancea meyersii* and band p—*Kluyveromyces marxianus.* This species were not isolated by culture

dependent methods. This species were not isolated by culture dependent methods. *Lachancea meyersii* and *Kluyveromyces lactis* were previously indentificadas in tibico for Magalhães et al. (2010a). It was not possible to identify the band j; the band was excised from the gel, but could not be recovered for sequencing. Band r and q could not be assigned to any bacterial species.

Recently, Magalhães et al. (2010a) observed similar microbiota present in grains and beverage tibico using conventional method and DNA analysis (by denaturing gradient gel electrophoresis (DGGE). The most common microbial species isolated were related to *Lactobacillus paracasei, Acetobacter lovaniensis, Lactobacillus parabuchneri, Lactobacillus kefir, Lactococcus lactis, Saccharomyces cerevisiae*, and *Kluyveromyces lactis*.

Multivariate analysis

Microbial community comparisons were performed by cluster analysis of DGGE gel (V6–V8 regions for bacteria and ITS region for yeast) according to Pearson correlation, and dendograms were constructed using UPGMA methods (Figs. 3a, b). DGGE profile analysis of samples of tibico grains from Brazilian States showed two distinct groups for both prokaryotic (Fig. 3a) and eukaryotic (Fig. 3b) communities. For the prokaryotic community, the first group included the samples from Goiás, Rio de Janeiro, São Paulo, and the latter group contained the samples from Minas Gerais, Alagoas, Bahia, Distrito Federal, and Espírito Santo. The eukaryotic communities present in all the samples were grouped together, except for Bahia. Similar results were observed on multivariate analysis using the frequency value for all microorganisms found in Brazilian tibico grains, as identified by culture-dependent methods.

Multivariate analysis was performed using frequency values for all microorganisms isolated and identified in Brazilian tibico grains. In the graphical representation (Figs. 4, 5), each axis displays the percentage of total variation between organisms over different tibico grains of

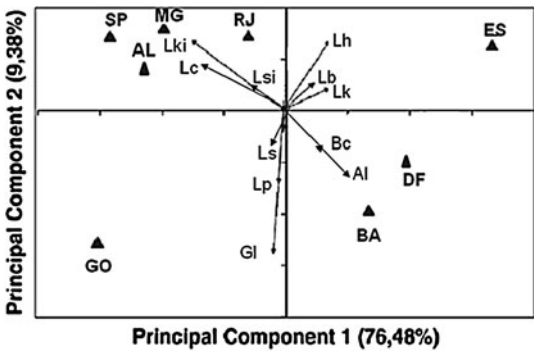

Fig. 4 Principal component analysis (*PCA*) of bacteria isolated and identified by culture-dependent method. *Abbreviations*: *SP* São Paulo, *GO* Goiás, *MG* Minas Gerais, *AL* Alagoas, *RJ* Rio de Janeiro, *DF* Distrito Federal, *ES* Espírito Santo, *BA* Bahia. (Gl) *Gluconobacter liquefaciens*, (Lp) *Lactobacilllus paracasei*, (Ls) *Lactobacillus satsumensis*, (Lki) *Lactobacillus kefiri*, (Lc) *Lactobacillus casei*, (Lsi) *Lactobacillus sunkii*, (Lh) *Lactobacillus helveticus*, (Lk) *Lactobacillus kefir*, (Lb) *Lactobacillus buchneri*, (Al) *Acetobacter lovaniensis*, (Bc) *Bacillus cereus*

the Brazilian States. Principal components 1 and 2 together explained 76.48% of the variation that occurred between bacteria's from different tibico grains of the Brazilian States (Fig. 4). Principal component analysis (PCA) shows that the most distant vectors from zero correspond to a variation with the greatest influence on the value of the main component. The sample Goiás and Espírito Santo were different in comparison to the Brazilian States, showing high frequency of *Gluconobacter liquefaciens* in Goiás and *Lactobacillus helveticus* in Espírito Santo. For the hight frequency of *Lactobacillus kefiri*, *Lactobacillus casei* and *Lactobacillus sunkii,* the first group included the samples from São Paulo, Minas Gerais, Rio de Janeiro, Alagoas, and the latter group contained the hight frequency of *Acetobacter lovaniensis*, *Bacillus cereus* in samples from Bahia and Distrito Federal. In the graphical representation, obtained by multivariate analysis of the frequency of all yeasts found in each sample of Brazilian tibico, it was found that principal components 1 and 2

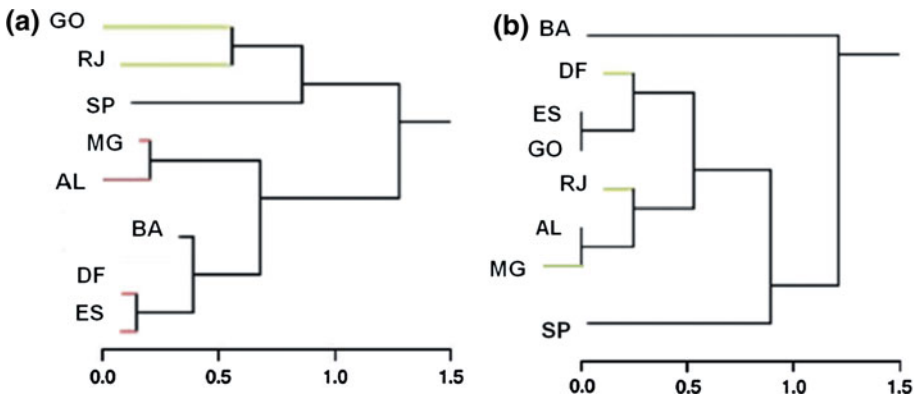

Fig. 3 Microbial Clusters. **a** Cluster analysis of V6–V8 regions of 16S rDNA amplicons present in tibico grains. **b** Cluster analysis of ITS rDNA amplicons present in tibico grains. *Abbreviations*: *SP* São Paulo, *GO* Goiás, *MG* Minas Gerais, *AL* Alagoas, *RJ* Rio de Janeiro, *DF* Distrito Federal, *ES* Espírito Santo, *BA* Bahia

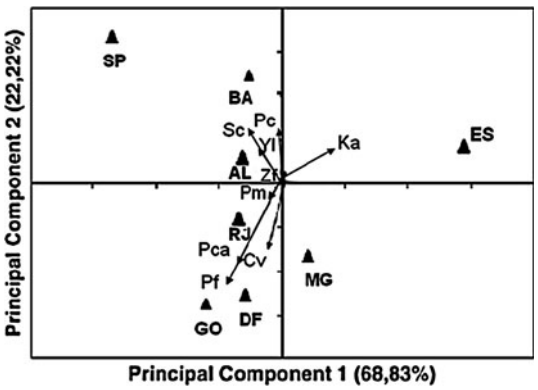

Fig. 5 Principal component analysis (*PCA*) of yeast isolated and identified by culture-dependent method. *Abbreviations*: *SP* São Paulo, *GO* Goiás, *MG* Minas Gerais, *AL* Alagoas, *RJ* Rio de Janeiro, *DF* Distrito Federal, *ES* Espírito Santo, *BA* Bahia. (Sc) *Saccharomyces cerevisiae*, (Zf) *Zygosaccharomyces fermentati*, (Yl) *Yarrowia lipolytica*, (Pc) *Pichia cecembensis*, (Cv) *Candida valdiviana*, (Pf) *Pichia fermentans*, (Pca) *Pichia caribbica*, (Pm) *Pichia membranifaciens*, (Ka) *Kazachstania aerobia*

together explained 68.83% of the yeast species variation that occurred between the kefir grains (Fig. 5). The sample from Espírito Santo, as observed in the multivariate analysis carried out for bacterial isolates, was different with respect to other times of fermentation, showing a high frequency of *Kazachstania aerobia* (Table 3; Fig. 5). Except for the Espírito Santo samples, the remaining sugary kefir grains were similar with respect to the composition of the microbiota (Fig. 5). Knowledge about microorganisms present in tibico grains used is crucial for the development of a higher quality tibico beverage.

Conclusion

Since the origins of these tibico (sugary kefir) grains are different and your manipulation process is not known and it can not involve health care precautions, the study of microbiota becomes very important, particularly due to possible pathogenic microorganisms associated with chronic diseases. Our study provided a more comprehensive estimate of the diversity of microbial communities found in Brazilian tibico grains used as starter culture for fermented beverages. This was the first study to characterise the microbial community involved in the tibico grains from different Brazilian States. Our findings are relevant to the knowledge of tibico grains used as starter culture for fermented beverages consumed by the Brazilian population.

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