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ORIGINAL PAPER

Molecular identification of yeast, lactic and acetic acid bacteria species during spoilage of tchapalo, a traditional sorghum beer from Côte d'Ivoire

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Abstract
Yeasts, lactic and acetic acid bacteria are responsible of microbial spoilage of alcoholic beverages. However species involved in deterioration of sorghum beer produced in Côte d'Ivoire has not been investigated. This study was carried out to identify species of yeast, LAB and AAB during spoilage of tchapalo in order to define the best strategy for beer preservative. Thus, a total of 210 yeasts, LAB and AAB were isolated from samples of *tchapalo* stored at ambient temperature and at 4 °C for 3 days. Based on PCR–RFLP of the ITS region and the sequencing of D1/D2 domain, yeast isolates were assigned to seven species (*Saccharomyces cerevisiae, Candida tropicalis, Rhodotorula mucilaginosa, Trichosporon asahii, Kluyveromyces marxianus, Meyerozyma guilliermondii* and *Trichosporon coremiiforme*). During the storage at ambient temperature and at 4 °C, *S. cerevisiae* was the predominant species (> 76%). Excepted *R. mucilaginosa*, occurrence of non-*Saccharomyces* species was sporadic. LAB species detected in fresh samples using molecular methods were *Pediococcus acidilactici, Lactobacillus paracasei, Lb. curvatus, Lb. fermentum* and *Weisssella paramesenteroides. P. acidilactici* was the dominant species (47.8%) followed by *Lb. paracasei* (17.5%). *W. paramesenteroides* and *Lb. fermentum* were not detected during the spoilage at ambient temperature while at 4 °C *W. paramesenteroides* and *Lb. paracasei* have not been detected. For AAB, the species found were *Acetobacter pasteurianus sub paradoxus* and *Acetobacter cerevisiae*. These species were common to all samples during spoilage and *A. pasteurianus sub paradoxus* was the most frequently detected.

Keywords Acetic acid bacteria · Lactic acid bacteria · PCR–RFLP · Spoilage · Tchapalo · Yeast

Introduction

In Africa, most of the cereals such as maize, millet and sorghum are often transformed into beverage whose manufacture includes an essential step of alcoholic fermentation. Traditional alcoholic beverage manufactured with malted sorghum, take different names according to regions (Djè et al. 2008; Kayodé et al. 2007; Sawadogo-Lingani et al. 2007).

In Côte d'Ivoire, tchapalo play an important role as an alcoholic beverage in both urban and rural areas. It is consumed in various festivals and Ivorian ceremonies (marriage, birth, baptism, dowery, etc.) and constitutes a source of economic return for the women manufacturers. The production of tchapalo includes two main fermentation steps composed of spontaneous lactic fermentation and uncontrolled alcoholic fermentation.

Lactic acid bacteria are responsible for the lactic fermentation which is followed by the alcoholic fermentation conducted by yeasts (Aka et al. 2008). Studies have been devoted to the isolation and identification of microorganisms responsible for these fermentations. Lactic acid bacteria implicated in the spontaneous fermentation are *Lactobacillus fermentum, Lactobacillus cellobiosus, Lactobacillus brevis, Lactobacillus coprophilus, Lactobacillus plantarum* and *Lactobacillus hilgardii* (Djè et al. 2009). Yeast species associated to the alcoholic fermentation are *Saccharomyces cerevisiae*-like, *Candida tropicalis, Pichia kluyveri, Pichia kudriavzevii, Kodamaea ohmeri* and *Meyerozyma carribbica* (N'guessan et al. 2011). In order to alleviate the problems of variations in organoleptic quality, the use of starter cultures

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was suggested by N'guessan et al. (2010). Recently, Coulibaly et al. (2016) evaluated the capacity of *S. cerevisiae* and *C. tropicalis* to produce sorghum beer as freeze–dried starter in mixed or pure culture.

However, like most of African traditional beverage, tchapalo has short shelf life. Attchelouwa et al. (2017) showed that sensory rejection time of tchapalo during storage was 48 h at ambient temperature. According to Kutyauripo et al. (2009), the deleterious changes are primarily due to the objectionable off-flavour or over souring induced by continued microbial activities after production. Attchelouwa et al. (2017) reported the presence of yeast, lactic and acetic acid bacteria during storage of tchapalo. These microorganisms were reported as the most problematic organisms in alcoholic beverage spoilage (Amoa-awua et al. 2007; Bartowsky et al. 2003; Hutzler et al. 2013; Sanni et al. 1999). In tchapalo, no investigation has dealt in detail with the involvement microbial species during the spoilage, while effectiveness of preservatives agents depends on the microbial species (Delfini et al. 2002; Renouf et al. 2008).

Thus, in order to define adapted strategies for shelf life extension programs for this traditional beer, this work was carried to identify yeast, lactic and acetic acid bacteria associated with the spoilage of the Ivorian traditional sorghum beer.

Materials and methods

Sample collection

Samples of tchapalo were collected from three local producers located in three production areas in Abidjan. Seven samples of 250 ml from each producer were simultaneously collected at the end of the fermentation period (fresh samples). Samples were collected into pre-sterilized Plexiglas containers which were immediately immersed in an isothermal box, and brought to the laboratory. Once at the laboratory, one sample was used for the analysis (fresh tchapalo). Then three samples were stored at ambient temperature (27–30 °C) and three other at 4 °C in a refrigerator. Each day, one sample at each storage temperature was used for analysis. All experiments were carried out independently in triplicate. A total of 63 samples were collected.

Isolation procedure

Isolation of microorganisms from the fresh and stored tchapalo samples were carried out at different storage time: at the time 0 h, meaning immediately after sampling (fresh tchapalo), and again after 24, 48 and 72 h at each storage temperature. For yeast isolation, samples were plated on Sabouraud Chloramphenicol agar medium (BIO-RAD, France) and plates were incubated at 30 °C for 48 h. Lactic acid bacteria (LAB) were isolated on MRS agar (BIO-RAD, France), supplemented with cycloheximide (100 mg/l; Sigma–Aldrich, Steinheim, Germany) after incubation at 30 °C for 48 h under anaerobic conditions. For acetic acid bacteria (AAB), GYC agar (yeast extract, 10 g; $CaCO_3$, 20 g; glucose, 100 g; agar, 15 g in 1 l of distilled water) supplemented with cycloheximide and penicillin was used as isolation medium and plates were incubated at 30 °C for 2–4 days under aerobic conditions. At each storage time and temperature, 10 colonies were randomly picked from the countable plate from each sample. About 210 colonies were picked from each culture media (30 from fresh tchapalo, 90 from tchapalo stored at ambient temperature and 90 from tchapalo stored at 4 °C). Isolates were maintained in 20% of glycerol at − 20 °C.

DNA extraction

Fresh colonies from 24 h old growing on Sabouraud Chloramphenicol agar for yeast, MRS agar for LAB and GYC agar for AAB were collected in sterile conditions. Colonies were suspended in 1.5 ml of Sabouraud broth, MRS broth and GY broth and incubated at 30 °C for 24 h (yeast and LAB) or for 48 h (AAB). A volume of 1.5 ml of growth medium was centrifuged (14,000 rpm, 7 min), the supernatant discarded and the pellet resuspended in lysis buffer described by Hassaïne et al. (2009). Then 0.3 g of microbead 0.5 mm (Sigma G8772) and 200 µl of chloroform/isoamyl alcohol (24:1) were added for Lab and AAB. For yeast, the pellet was suspended in 700 µl of CTAB (cetyl trimethyl ammonium bromide) solution (Devi et al 2013) and incubated at 65 °C for 30 min after vortexing. It was centrifuged and supernatant suspended in chloroform/isoamyl alcohol (24:1). Mixture was vortex and centrifuged (13,000 rpm, 10 min). The upper phase was collected and 20 µl of sodium acetate and 600 µl of ethanol were added. After centrifugation, solution was discarded and pellet was washed in 70% ethanol. Mixture was centrifuged again (13,000, 2 min), the supernatant discarded, precipitated DNA was dried at room temperature and the DNA resuspended in 100 µl of TE buffer (Tris, 10 mM; EDTA, 1 mM, pH 8). DNA solutions obtained were stored at − 20 °C for PCR.

PCR conditions

All the amplifications were performed in a thermal cycler (TECHNE, 3PRIMEBASE/02, UK). The amplification of the ITS1-5.8S-ITS2 region of yeasts was carried out in 50 µl of reaction mixture containing 25 µl of PCR Master Mix 2× (Promega, Madison, WI, USA), 1 µM each of forward and reverse primers (ITS1 5′-TCCGTAGGTGAACCTGCGG-3′, ITS4 5′-TCCTCCGCTTATTGATATGC-3′) (Eurofins

genomic, Munich, Germany) (White et al. 1990), and 15 µl nuclease free water (Promega). The cycling program was started with an initial denaturation step at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 2 min, annealing at 60 °C for 1 min and elongation at 72 °C for 2 min. The PCR was ended with a final extension at 72 °C for 7 min.

PCR of LAB isolates included 16S rDNA and rpoB gene which codes for the RNA polymerase beta submit. The gene encoding 16S rRNA of LAB was amplified using primers EGE1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and EGE2 (5′-CTACGGCTACCTTGTTACGA-3′) (Eurofins genomic) (Yavuz et al. 2004). PCR mixtures contained 10 µl of DNA solution, 25 µl of PCR-master mix 2× (Promega), 0.2 µM of each primer. The PCR cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, and 40 cycles with denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min and primer extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. The primers rpoB1 (5′-ATTGACCACTTGGGTAACCGTCG-3′), rpoB1o (5′-ATCGATCACTTAGGCAATCGTCG-3′), rpoB2 (5′-ACG ATCACGGGTCAAACCACC-3′), described by Renouf et al. (2006) were used to amplify a 300 pb fragment of a partial sequence of rpoB gene. PCR amplifications were performed in a PCR mixture with a total volume of 50 µL consisting of 25 µl of PCR-master mix 2× and 0.2 µM of both primer. Amplification reaction included an initial denaturation at 95 °C for 10 min. Then a touchdown cycle followed during that the annealing temperature was lowered from 59 to 45 °C, 1 °C increments every cycle and 15 additional cycles were carried out with annealing of 45 °C. A final 10 min extension step at 72 °C achieved the amplification procedure.

Primers used to amplify the 16S rDNA of AAB were 16Sd (5′-GCTGGCGGCATGCTTAACACAT-3′) and 16Sr (5′-GGAGGTGATCCAGCCGCAGGT-3′) (Eurofins genomic) based on the conserved regions and described by Ruiz et al. (2000). PCR amplification was carried out in final volume of 50 µl comprising 10 µl of DNA solution, 25 µl of PCR master mix 2× (Promega), 15 pmol of each primer. The reactions were set as follows: denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min; and a final extension at 72 °C for10 min.

Amplified samples were kept at − 20 °C until further use.

Nuclease restriction

Amplicons, without purification, were used for restriction analysis by *Hae* III, *Hinf* I and *Cfo* I (Promega) for yeast (N'guessan et al. 2011); *Hinf* I and *Cfo* I for AAB (Ruiz et al. 2000) and *Hae* III and *Hinf* I for LAB (Claisse et al. 2007; Yavuz et al. 2004). A volume of 10 µl of PCR products were used for each of the restriction enzyme digestion with 0.5 µl of enzyme, 2 µl of BSA (Promega), and 1.5 µl of the corresponding buffer supplied by manufacturer. The digestion was conducted at 37 °C for 5 h and digested DNA fragments were detected by electrophoresis on 2% agarose gel. The gel was run for 1.5 h at 90 V in 0.5× TBE buffer and stained with ethidium bromide. Lengths of restriction fragment were detected by comparing against 100 bp DNA ladder (Promega) for RpoB and 5.8S rDNA restriction fragments and 200 bp DNA ladder (Promega) for 16S restriction fragments. Lengths of restriction fragments were determinated using UVI band max software v15.06a (Uvitec, England).

Sequencing

Representative strains of each PCR–RFLP profile were identified at the species level by sequencing. The D1/D2 region of the 26S rRNA gene of yeasts was amplified with the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett 1998). PCR mix was prepared as for the amplification of the ITS region and amplification was conducted as follows: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 54 °C for 40 s and DNA extension at 72 °C for 1 min 30 s. A final extension was completed at 72 °C for 5 min.

For LAB and AAB, the 16S rRNA gene was amplified with primers PA (5′-AGAGTTTGATCCTGGCTCAG-3′) and PE (CCGTCAATTCCTTTGAGTTT), primers 16Sd (5′-GCTGGCGGCATGCTTAACACAT-3′) and 16Sr (5′-GGA GGTGATCCAGCCGCAGGT-3′) respectively following the same conditions as previously described.

The amplified fragments were then sequenced by Eurofins MWG Operon (Ebersberg, Germany). Sequences were processed using the package phred/phrap/consed. The obtained sequences were compared to sequences at NCBI (http://www.ncbi.nlm.nih.gov) using blastn.

Results

Yeast identification

A total of 210 yeasts isolated from samples of tchapalo during storage at ambient temperature and 4 °C were taken for this study. The amplification of the ITS region showed bands ranging from 520 to 890 pb (Table 1). When the PCR product were digested with *Hinf* I, *Cfo* I and *Hae* III, the results allowed us to distinguish seven different ITS-PCR profiles which were indicated by roman numerals. Two of the seven patterns obtained were identified

Table 1 Length in pb of the PCR amplified products of 5.8S rDNA-ITS region, ITS-RFLP and identified species of yeast isolated during spoilage of tchapalo at ambient temperature and 4 °C

ITS-RFLP group	ITS size (pb)	Restriction fragments size (pb)						
		Cfo I	*Hinf* I	*Hae* III	Literature corresponding speciesa	Genbank corresponding species	Percent of identity	Number of strain isolated
I	890	390 + 360	380 + 140	340 + 250 + 190 + 110	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632	99	179
II	730	290 + 200 + 180	260 + 200 + 130 + 60	680	nd	*Kluyveromyces marxianus* CBS 712	100	21
III	650	300 + 260	350 + 220	420 + 220	nd	*Rhodotorula mucilaginosa* CBS 316	99	3
IV	650	300 + 260	230 + 230	420 + 130 + 100	nd	*Meyerozyma guilliermondii* NRRL Y-2075	99	2
V	540	270 + 270	240 + 240	500	nd	*Trichosporon asahii* CBS 2479	100	1
VI	540	270 + 270	260 + 260	500	nd	*Trichosporon coremiiforme* CBS 2482	99	2
VII	520	280 + 240	260 + 260	430 + 90	*C. tropicalis*	*C. tropicalis* ATCC 750	100	2

nd not determined

[a]Yeast were identified to species level by comparison of pattern obtained for each group with the pattern of the strain described by Jeyaram et al. (2008); Llanos-Frutos et al. (2004); N'guessan et al. (2011)

to the species level after comparing the molecular size of the restriction product with those previously described (Llanos-Frutos et al. 2004; Jeyaram et al. 2008; N'guessan et al. 2011). These two groups corresponded to *S. cerevisiae* and *C. tropicalis*.

In order to find the identity of the species of others groups and confirm the identity of group I and group VII, strains of each profile were randomly chosen for the sequencing of the D1/D2 domain of the 26S rDNA. When the sequences obtained were compared to those present in GenBank database using BLAST N 2.7 software, strains of PCR–RFLP groups I, III, IV and VI showed 99% identity to *S. cerevisiae* NRRL Y-12632, *Rhodotorula mucilaginosa* CBS 316, *Meyerozyma guilliermondii* NRRL Y-2075 and *Trichosporon coremiiforme* CBS 2482 respectively. Those of PCR–RFLP groups II, V and VII showed 100% identity to *Kluyveromyces marxianus* CBS 712, *Trichosporon asahii* CBS 2479 and *C. tropicalis* ATCC 750 respectively.

Yeast diversity during storage

Figure 1 shows the percentages of each species isolated during storage of tchapalo at ambient temperature and 4 °C. *S. cerevisiae* was the predominant species in fresh tchapalo and during its storage at ambient temperature and 4 °C. This species represented over 75% of isolates at all stages. Among non-*Saccharomyces* species, *R. mucilaginosa* was the most frequent species. The species *C. tropicalis, T. asahii, K. marxianus, M. guilliermondii* and *T. coremiiforme* were isolated at specific times. At 4 °C, the species *C. tropicalis, T. coremiiforme* and *K. marxianus* were not founded.

LAB identification

As shown in Table 2, rpoB primers set produced amplicons of 300 pb for all species. PCR amplification products were further used to obtain enzymatic restriction patterns with *Hinf* I and *Hae* III. *Hae* III produced a non-digested fragment

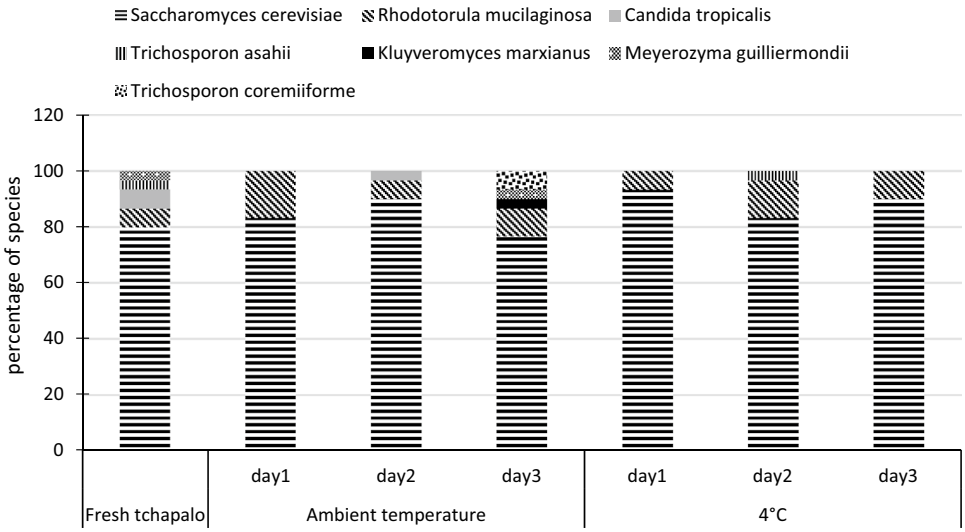

Fig. 1 Dynamic of yeast species (%) during spoilage of tchapalo at different temperatures

Table 2 Length in pb of the PCR amplified products of 16S rDNA and rpoB region, RFLP and identified species of LAB isolated during spoilage of tchapalo at ambient temperature and 4 °C

PCR–RFLP Group	RpoB fragment			16S rDNA fragment					
	rpoB PCR size (pb)	Restriction fragments size (pb)		16S rDNA PCR size (pb)	Restriction fragments size (pb)		GenBank corresponding species	Percent of identity	Number of strain isolated
		Hae III	*Hinf* I		*Hinf* I	*Hae* III			
I	300	300	270 + 30	1500	1000 + 400	650 + 500 + 350 + 130 + 80	*Lb. fermentum* NBRC 15885	99	11
II	300	300	280	1500	1000 + 370	650 + 500 + 350 + 130 + 80	*Lb. paracasei* JCM 1171	100	32
III	300	300	280	1500	1000 + 400	650 + 500 + 350 + 130 + 80	*Lb. curvatus* JCM 1096	100	50
IV	300	300	270	1500	1000 + 400	1250 + 370 + 80	*Weissella paramesenteroides* JCM 9890	99	3
V	300	300	270 + 30	1500	500 + 450	650 + 400 + 280 + 200 + 120 + 80	*P. acidilactici* DSM 20284	99	114

while *Hinf* I cleaved the amplicon and generated three patterns. The length of the amplification products with EGE primers was 1500 pb. The digestion of the PCR products yielded three patterns with *Hinf* I and three patterns with *Hae* III (Table 2). When the two PCR–RFLP results were combined, five groups were found among the 210 isolates. A representative strains of each group was sequenced for 16S rRNA gene and the sequences obtained were compared with the known data stored in the NCBI gene databank by standard methods of comparison. The DNA sequences that were used for the identification were a minimum of 750 pb in length. The similarity degree of RFLP groups II and III reached 100%, compared with *L. fermentum* NBRC 15885, *Lactobacillus paracasei* JCM1171 respectively. DNA sequences of strains belonging to PCR–RFLP groups I, IV and V showed 99% similarities with *Lactobacillus curvatus* JCM 1096, *Weissella paramesenteroides* JCM 9890 and *Pediococcus acidilactici* DSM 20284.

LAB diversity during storage

The species identified and their frequencies are shown in Fig. 2. *P. acidilactici* was the dominant LAB in the fresh tchapalo (47.8% of total isolates). The other species present in the fresh tchapalo were *Lb. paracasei* (17.5%), *Lb. curvatus* (13%), *W. paramesenteroides* (8.7%) and *Lb. fermentum* (13%). During the storage at ambient temperature, a rapid disappearance of *W. paramesenteroides* and *Lb. fermentum* were observed. *P. acidilactici* remained the dominant species. When *tchapalo* was stored at 4 °C, *W. paramesenteroides* and *Lb. paracasei* were not detected. On contrary,

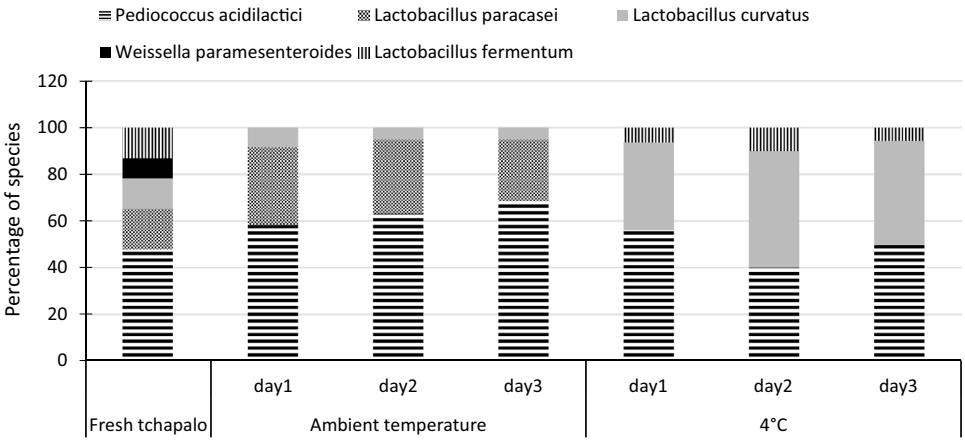

Fig. 2 Dynamic of LAB species (%) during spoilage of tchapalo at different temperatures

the population of *Lb. curvatus* increased and shared higher occurrence with *P. acidilactici*.

AAB identification

The PCR fragment was about 1400 pb for all the isolates. Base on f the restriction profiles obtained with *Hinf* I and *Cfo* I, the 210 isolates were divided into two groups, groups I and II. The isolates which produced *Cfo* I bands of 500, 410, 200, 150 pb and *Hinf* I bands of 350, 275, 200 pb were placed in group I. Group II included isolates that *Cfo* I digestion generated bands of 500, 350, 200 and 150 pb and *Hinf* I fragments of 950, 200, 80 pb (Table 3). The sequences of 16S rDNA obtained from randomly selected strains of each PCR–RFLP group were compared with 16S rDNA sequences of NCBI database (Table 3). The strains of group I showed 99% similarity with *Acetobacter pasteurianus sub paradoxus* LMG 1591 and group II showed 100% similarity with *Acetobacter cerevisiae* JCM 17273.

AAB diversity during storage

Among the two AAB species found in fresh tchapalo, *A. pasteurianus sub paradoxus* with a percentage of 83.3% was the predominant species (Fig. 3). During the storage at ambient temperature, this species remained predominant. Its prevalence increased during the first 2 days of storage, passing from 83.3 to 96.3% and then decreased to 72.7%. In the samples stored at 4 °C, a contrary evolution was observed for the same species. Thus, its prevalence had lowered to 33.3% after 1 day of storage and increased up to 79.2% during the remaining 2 days.

Discussion

The data presented in this study extend our knowledge on the sequential development of yeast, LAB and AAB species during spoilage of tchapalo at ambient temperature and 4 °C. Yeasts associated to tchapalo during the spoilage have been identified by ITS region of the rDNA and the partial sequencing of the D1/D2 domain of 26S rDNA. PCR–RFLP was used as a very efficient tools in many cases of yeast identification (Jeyaram et al. 2008; N'guessan et al. 2011). In this study, *S. cerevisiae, R. mucilaginosa, M. guilliermondii, T. asahii*, and *C. tropicalis* were yeast species identified in fresh tchapalo and *S. cerevisiae* was the dominant species (80%). This preponderance of *S. cerevisiae* and its coexistence with non-*Saccharomyces* species in traditional sorghum beer were earlier reported by several authors (Greppi et al. 2013; Lyumugabe et al. 2010; Maoura et al. 2005; N'guessan et al. 2011; Sefa-Dedeh et al. 1999). However presence of various species could contribute to the variations in organoleptic quality of beer. The use of starter culture

Table 3 Length in pb of the PCR amplified products of 16S rDNA, RFLP and identified species of AAB isolated during spoilage of tchapalo at ambient temperature and 4 °C

16S rDNA-RFLP group	16S rDNA size (pb)	Restriction fragments size (pb)				
		Cfo I	*Hinf* I	Genbank corresponding species	Percent of identity	Number of strain isolated
I	1400	500 + 410 + 200 + 150	350 + 275 + 200	*A. pasteurianus subsp. paradoxus* LMG 1591	99	156
II	1400	500 + 350 + 200 + 150	950 + 200 + 80	*Acetobacter cerevisiae* JCM 17273	100	54

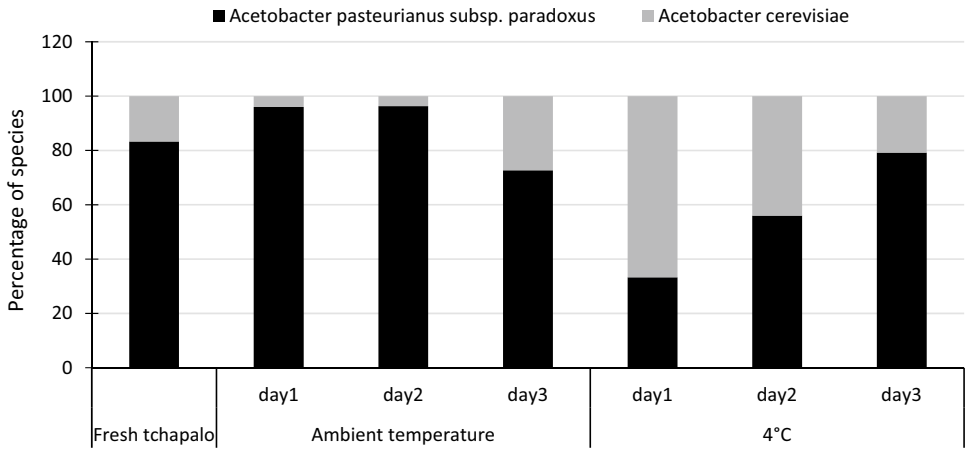

Fig. 3 Dynamic of AAB species (%) during spoilage of tchapalo at different temperatures

composed to *S. cerevisiae* and *C. tropicalis* was so suggested by N'guessan et al. (2010) as the appropriate approach to alleviate the problems. Furthermore, several non-*Saccharomyces* species identified in this study have emerged as opportunistic pathogens. Thus, *M. guilliermondii* can cause severe fungal infections like candidemia. It was the third yeast species most commonly isolated from blood cultures (Guler et al. 2017). *M. guilliermondii* fungemia may occur in children with underlying conditions other than cancer (Shah and Bhatia 2012). In the same way, *T. asahii* (formerly *T. beigelii*) is an emerging fungal pathogen seen particularly in immunologically compromised patients. There are reported cases of hematogenously disseminated infections with this life-threatening yeast, and no effective antifungal therapy is available (Ebright et al. 2001).

During spoilage of tchapalo at ambient temperature and 4 °C, *S. cerevisiae* remained the predominant species with a detection frequency above 76%. At room temperature all yeasts grow well because alcohol percentage in tchapalo (about 5–6%) (Attchelouwa et al. 2017) is not enough to prevent growth of non-*Saccharomyces* species. On the other hand, at 4 °C most yeast species grow so slowly that it looks like they are not able to grow. Moreover, low temperature and 5–6% ethanol together are sure affecting very negatively yeast growth (Charoenchai et al. 1998; Stanley et al. 2010).

Presence of yeast species during storage of tchapalo could be detrimental to organoleptic quality. Both *Saccharomyces* strains and non-*Saccharomyces* yeasts can produce off-flavors (du Toit and Pretorius 2000). Thus, *M. guilliermondii, C. tropicalis* and *K. marxianus* have been reported as non-*Saccharomyces* spoiler agents. They spoil beer through the production of off-flavors, haze, sediment or surface films. Furthermore, they were found common throughout breweries, especially in unwashed sampling ports and on other surfaces contacting beer (Bokulich and Bamforth 2013). They are opportunistic contaminants, causing spoilage when conditions are favorable, but are generally not an issue in modern brewing practices, due to improved oxygen control. These yeasts are more of an issue in barrel-fermented beers, where oxygen ingress stimulates their growth, hence the need to limit the headspace during barrel maturation (Priest 2003). In traditional alcoholic beverage, *Candida* sp., *Meyerozyma* sp. and *Rhodotorula* sp. produce pellicle which render the texture of the beer unacceptable to consumers (Lyumugabe et al. 2012). *S. cerevisiae* is regarded as a spoilage organism by causing re-fermentation of residual sugars turbidity and sediment. According to Clemente-Jimenez et al. (2004), *S. cerevisiae* have a significant influence on the taste and character of wine by the production of 2-butanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol.

LAB isolated during the storage of tchapalo have been identified by PCR–RFLP of the 16S rDNA region and rpoB gene followed by the partial sequencing of the 16S rDNA. PCR linked with restriction fragment length polymorphism analysis are easy, rapid and inexpensive ways to identify LAB species. The 16S rRNA gene sequence has been widely used as a molecular method for LAB identification. However the sensitivity of this approach has been questioned (Fox et al. 1992). Renouf et al. (2006) demonstrated that the housekeeping rpoB gene, coding the RNA polymerase beta submit, exhibits greater differences between species than the 16S RNA gene. In this study, combination of the patterns of amplified product of both regions using restriction enzymes *Hae* III and *Hinf* I showed five groups which corresponded to *L. fermentum, Lb. paracasei, Lb. curvatus, Weissella paramesenteroides* and *P. acidilactici*. These finding disagree with the report of Aka et al. (2008) who found only species belonging to genera *Leuconostoc* and *Lactobacillus* based on morphological and biochemical criteria. *Weisella* are often misidentified as *Lactobacillus* by traditional and commercial phenotypic identification methods. This genus was previously grouped along with *Lactobacillus, Leuconostoc* and *Pediococcus*. Utilization of more sensitive methods like DNA sequencing has facilitated identification of *Weissella* as a unique genus (Kamboj et al. 2015). Presence of these

LAB species in other side may be detrimental for tchapalo shelf life. In fact, most of scientific reports considered species of *Lactobacillus* and *Pediococcus* as predominant alcoholic beverages spoilers by the fact that they can produce off-flavors (mainly due to diacetyl and lactic acid), change in color and increase in turbidity (Menz et al. 2010; Rouse and Van Sinderen 2008). In modern beer, approximately 60–90% of the microbiological spoilage incidents were caused by *Lactobacillus* (Sakamoto and Konings 2003). The spoilage incidents by *Lb. paracasei* appear to have increased since 2010 and this species is known to cause diacetyl off-flavor in beer. *Lb. curvatus* have also been found commonly in beer and recognized as beer LAB spoilers species (Garofalo et al. 2015; Sakamoto and Konings 2003). In wine, *Pediococcus* and *Lactobacillus* strains can survive and they were found as potent spoilage agents after winemaking (Lonvaud-Funel 1999).

AAB were identified by PCR–RFLP and sequencing of the 16S rDNA region. This technique was used by several authors (Gonzalez et al. 2004; Poblet et al. 2000; Ruiz et al. 2000) to identify AAB and it is appropriate for differentiating and characterizing all the AAB species on the basis of their phylogenetic relationships. In this work, it allowed us to assign the isolates to *A. pasteurianus sub paradoxus* and *A. cerevisiae*. These two species were detected during the 3 days of storage at ambient temperature and 4 °C. In alcoholic beverages, species of the *Acetobacter* genus were regularly reported as spoilage AAB (van Vuuren and Priest 2003). Thus, in European beer, *A. aceti, A. liquefaciens, A. pasteurianus* and *A. hansenii* are most frequently occurring species (Priest 2006, 2003). In wine, during fermentation and storage, *A. pasteurianus, A. aceti* and *A. liquefaciens* were also identified (Bartowsky et al. 2003; Gonzalez et al. 2004). Sanni et al. (1999) isolated from Nigerian traditional alcoholic beverages at post-production time of 72 h, *A. aceti, A. hansenii* and *A. pasteurianus*. They were the cause for the development of a vinegary taste and high volatile acidity (Amoa-awua et al. 2007; Bartowsky et al. 2003). Presumably, occurrence of *Acetobacter* species in tchapalo and other alcoholic beverages is facilitated by the production of ethanol by yeasts. Ukwo et al. (2010) reported that *Acetobacter* species are better adapted to the higher ethanol concentrations. These species prefer ethanol as carbon source. Ethanol is first oxidized to acetaldehyde followed by further oxidation to acetic acid, and the reactions are catalyzed by cytoplasmic membrane bound enzymes alcohol dehydrogenase and aldehyde dehydrogenase, respectively. Though these enzymes are important in industrial production of acetic acid, they are nevertheless spoilage molecules for many types of food and juices including tchapalo (Ameh et al. 2011). The low pH values of tchapalo (3.2–3.4) may also explain the presence of *Acetobacter* strain only. According to Matsushia et al. (2003), under acidic conditions, the alcohol dehydrogenase activity of *Acetobacter* is more stable than the activity in other genera.

Conclusion

S. cerevisiae, R. mucilaginosa, M. guilliermondii, T. asahii, K. marxianus, T. coremiiforme and *C. tropicalis* were yeast species identified during spoilage of tchapalo. Among these, *S. cerevisiae, M. guilliermondii, K. marxianus* and *R. mucilaginosa* have been reported in various alcoholic beverages as spoilage yeasts.

For LAB, species detected were *Lb. fermentum, Lb. paracasei, Lb. curvatus, W. paramesenteroides* and *P. acidilactici* with *P. acidilactici, Lb. paracasei* and *Lb. curvatus* as potential spoilage agents. *A. pasteurianus* and *A. cerevisiae* were AAB detected during spoilage of tchapalo. These species are responsible for souring in alcoholic beverage. Because of the spoilage potential of most of yeasts, LAB and AAB species detected during tchapalo storage, their growth must be controlled. Thus, in the way to define the best preservation strategy of tchapalo, susceptibility of each identified species to preservative agents should be evaluated.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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