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ORIGINAL PAPER

Phenotypical and molecular characterization of yeast content in the starter of "*Tchoukoutou*," a Beninese African sorghum beer

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Abstract *Tchoukoutou* is a Beninese African sorghum beer obtained by mixed fermentation including different yeast and lactic acid bacteria (LAB). Until now, the make-up of the starter's whole microbial communities and the main reason for the diversity of this special beer's organoleptic quality (taste and flavor) have remained unknown. A total of 240 yeasts isolated from a *Tchoukoutou* starter have been characterized following the polyphasic approach and using yeast phenotype (morphology and physiology), proteins (MALDI-TOF MS), ITS1-5.8S-ITS4 and D1/D2 of the large subunit (LSU) rRNA gene as biomarkers. The microbial ecology of the starter used to produce *Tchoukoutou* is diverse and belongs to different strains of four species including *Saccharomyces cerevisiae* (75.17 %) as the dominant yeast, followed by *Pichia kudriavzevii* (17.24 %), *Candida ethanolica* (4.14 %) and *Debaryomyces hansenii* complex (3.45 %). *D. hansenii* complex and *C. ethanolica* are two yeast species which have never yet been isolated from *Tchoukoutou*. Some *S. cerevisiae* with an interesting fermentative profile are able to metabolize lactic acid (lactic acid bacteria metabolite) and therefore may increase the beer pH, thereby allowing the growth of LAB for further beer maturation and flavor enhancement during Yeast-LAB mixed fermentation. The co-presence of the non-*Saccharomyces* with *S. cerevisiae* in sorghum beer starter depends for the agroecology zones.

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Introduction

Tchoukoutou is an African opaque sorghum beer obtained by mixed fermentation including different yeasts and lactic acid bacteria. This traditional beer is present in all African countries and is also called *pito* or *burukutu* in Ghana and Nigeria, *kefir* beer in South Africa, *pombe* in Tanzania and "Seven days" beer in Zambia [1, 2]. African sorghum beer is very rich in calories, B-group vitamins (thiamine, folic acid, riboflavin, nicotinic acid) and amino acids such as lysine [3]. Present in popular bars and a dedicated local market, sorghum beer is a refreshing drink much appreciated by a large number of consumers. In different local *Tchoukoutou* markets and *Cabaret* (beer gardens), consumers discriminate producers by the organoleptic quality (flavor and taste) and alcohol content of their beer. As observed by Kayode et al. [4] in Benin and Schoustra et al. [5] in Zambia, producers and consumers claim that the traditional sorghum beer offers health benefits such as preventing and curing diarrhea. The health benefits as well as good aroma and flavor of *Tchoukoutou* are due to the combination of yeast and the LAB content in this traditional starter. The LAB content may also cooperate with yeasts for the bio-protection of the beer by producing organic acid and bacteriocins [6]. During the mashing processes of producing *Tchoukoutou* and other African beers, the sorghum mash pH is decreased by spontaneous LAB acidification. This bio-acidification, if efficient, should improve amylase, protease, endo-β-glucanase and phosphatase activities [7, 8], lautering

performance [9], organoleptic qualities, mineral bioavailability [10] and vitamin production [11].

African sorghum beers are not standardized, and in addition to the variation of organoleptic qualities, several safety problems limit the marketability of these drinks. Indeed, *Tchoukoutou,* because of its instability as well as short shelf life resulting from uncontrolled and unlimited fermentation, is only consumed in the areas where it is produced. Furthermore, African sorghum beer is consumed while it is still fermenting, and the drink contains many fragments of insoluble materials, like undigested starch and protein, which still remain in the beer. This is likely due to the inappropriate gelatinization time, temperature and the rudimentary materials (basket and cloth) used for filtration. The growth of mold during germination is also a permanent exogenous risk that compromises the safety of African sorghum beer. Indeed, aflatoxin produced by *Aspergillus flavus* is the most frequently occurring mycotoxin identified in South African traditional beer [12], in Nigeria's opaque beer [13] and in Malawi's traditional maize beer [14]. In addition to this exogenous contaminant, the raw material used during beer processing, i.e., sprouted grains of *sorghum bicolor*, contains a cyanogenic glycoside [15–18]. If this secondary metabolite of sorghum grain remains in the beer, it generates poisonous hydrogen cyanide by the action of enzymes of the intestinal microbiota after consumption by humans [19]. Because of these safety problems, which hinder the marketability of African sorghum beer, a controlled mixed fermentation may be a potential key to upgrading this traditional beer. This challenge may help producers to move from an ancestral, neolithic household process to industrial production using the endogenous *Tchoukoutou* microorganisms to guarantee its particular organoleptic quality. This will solve the safety problem, increase the shelf life and make this beer as competitive as Berliner Weißbier, Belgian lambic beer and American coolship ales, which are also obtained by mixed fermentation including yeast and LAB. For this purpose, selecting performant yeast and LAB that produce aryl-ß-D-glucosidase is a good approach. Through this approach, dhurrin (sorghum cyanogenic glucoside) and other sorghum glucose conjugate compounds are hydrolyzed thereby contributing to sorghum beer detoxification and flavor enhancement, respectively. To achieve this goal, the first challenge is the identification of different yeasts and LAB which occur during this spontaneous mixed fermentation. In this study, following a polyphasic approach, the different yeasts in the traditional starter used for *Tchoukoutou* production are isolated, discriminated and classified based on their phenotype, proteins and DNA sequence analysis. Furthermore, based on their metabolic profile, yeasts with interesting biotechnological properties are selected.

Materials and methods

Sample collection, yeast isolation and purification

Thirty-six samples of *Tchoukoutou* starter were collected in 12 localities of two agroecological zones with high production of African sorghum beer in Benin. Yeast strains were isolated from the starter using the standard serial method. A hundred microliters of the dilution (10^{-4}–10^{-8}) was plated using the spread plate method on oxytetracycline glucose yeast agar (yeast extract 5 g/l, glucose 20 g/l, agar 15 g/l). The selectiveness of the medium was improved by adding 1 % of oxytetracycline per liter of OGYA medium for LAB inhibition. The inoculated plates were incubated at 27 °C for 72 h. The pure yeast colonies were obtained by picking single colonies and streaking them on the same medium following the quadrant streaking technique. The plates were incubated at 27 °C for 48 h.

Phenotypical characterization

Morphological and physiological characterization

Two hundred and forty pure yeast isolates were characterized on the basis of their morphological and physiological characteristics following the methods described by Yarrow [20]. The morphological and physiological data of the online CBS database (www.cbs.knaw.nl) and the key procedure described by Back [21] were used for yeast identification. The results of this phenotypical characterization were labeled as negative (−), positive (+) or weak (w), and R software was used for heat map construction.

MALDI-TOF analysis based on yeast proteome mass spectrum

A hundred and forty-five yeasts, selected from 240 yeasts which were isolated and phenotypically characterized, were submitted to MALDI-TOF test using the ethanol extraction method. After the growth of the different yeast strains on YPG agar for 48 h at 27 °C, a single yeast colony was suspended in 300 µL of ultrapure water, and then, 900 µL of absolute ethanol was added. The proteome of the precipitated cell material was extracted with formic acid and acetonitrile. One microliter of this yeast extract supernatant was dropped on the target, allowed to dry, overlaid with 1 µL of a matrix solution (α-cyano-4-hydroxy-cinnamic acid). The measurement was performed on a Microflex LT spectrometer (Bruker Daltonik). The Bruker database (Biotyper 3.1) and an in-house database (Technische Mikrobiologie, Weihenstephan) were used for the automatic online yeast identification. According to the similarity between the applied yeast proteome mass spectrum and the mass spectrum of the target yeast strain in the database, the

score value is generated. The identification is highly probable at species level when the score value is between 2.300 and 3.000. No reliable identification is observed when the score value is below 1.699. The applied yeast strain being scored from 1.700 to 2.000 limits the identification to genus level. On the basis of the yeast mass spectra, groupings were visualized by similarity calculations and multidimensional scaling (MDS) proposed by Usbeck et al. [22].

Molecular characterization

The overnight culture was obtained by growing the yeast strain in YPG medium at 27 °C. The DNA of this overnight culture was isolated according to the method described by Hanna and Xiao [23].

Amplification of ITS region and RFLP analysis

The PCR was performed with ITS_1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS_4 (5′-TCCTCCGCTTATTGATATGC-3′) primers to amplify the ITS region [24]. This amplified DNA of 50 yeasts which were characterized on the basis of MALDI-TOF analysis was randomly selected from different sub-clusters obtained after multidimensional scaling (MDS). It was digested with three endonucleases *HindIII*, *HinfI* and *HaeIII,* as per the instruction of the manufacturer (New England Biolabs, Frankfurt, Germany). The digested PCR–DNA was run on 2 % agarose gel at constant voltage (130 V) for 80 min and visualized by UV. The yeasts were identified on the basis of their restriction patterns using the Spanish Type Culture Collection CECT database (www.yeast-id.org).

Amplification of D1/D2 of 26S rDNA and sequencing

The D1/D2 domain of the large subunit ribosomal RNA gene was amplified with NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′GGTCCGTGTTTCAAGACGG-3′) primers [25]. The amplified PCR–DNA of D1/D2 of 22 yeasts which were randomly selected from different RFLP patterns was purified following the manufacturer's (PeqLab, Erlangen) instructions, and it was sequenced with NL_1 primer (5′-GCATATCAATAAGCGGAGGAAAAG-3′). The different yeast strains were identified using the NCBI database (Blastn).

Results

Metabolic spectrum as biomarker for yeast classification and identification

The yeast content of the traditional starter, which is used for *Tchoukoutou* production, varies from 10^8 to 10^9 CFU/g of liquid starter. The colonies of isolated yeasts are creamy-white and yellowish, butyrous in texture, farinose and of smooth appearance. The different yeasts exhibited round, oval and cylindrical shapes at microscopic observation. The fermentation test discriminated the isolated yeasts into different fermentative groups with distinct metabolic profiles. After the classification of the isolated yeasts based on their phenotypic characteristics, two principal clusters were obtained (Fig. 1). The online yeast identification based only on their morphological and physiological characteristics generates ambiguous and inconclusive results. The key procedure described by Back [21] has been used to discriminate *S. cerevisiae* (lysine agar negative) from the non-*Saccharomyces* yeast (lysine agar positive). Cluster 2 probably consists of only the non-*Saccharomyces* yeasts (lysine agar positive) while cluster 1 consists of *S. cerevisiae* (lysine agar negative) with the exception of the yeast strain BE-227. Most of the lysine and crystal violet negative yeast strains do not ferment melibiose. They therefore have the same fermentative aptitude as *S. cerevisiae* top-brewing yeast [21]. Twenty-five of the isolated yeasts including *S. cerevisiae* and non-*Saccharomyces* are able to

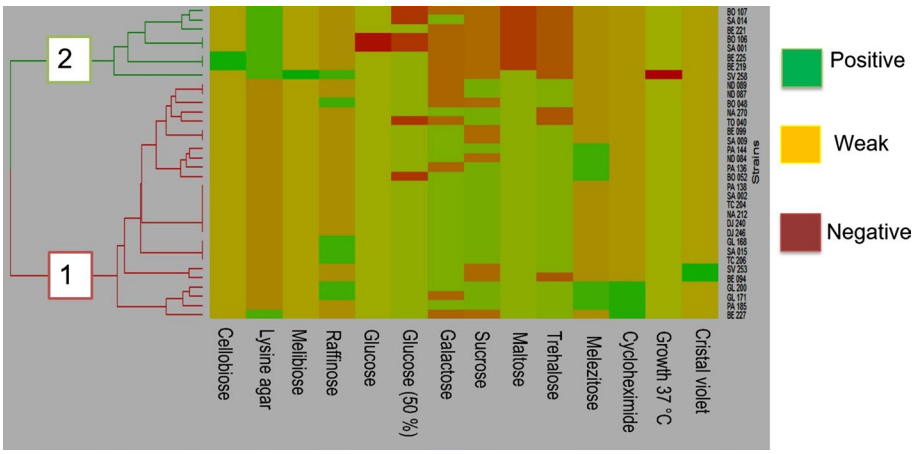

Fig. 1 Yeast phenome heat map showing the repartition of the isolated yeasts based on their fermentative aptitude and capacity to grow under stress conditions using the Ward method and Euclidean distance. The color play ranges from *green* to *red* via *yellow* when the reaction is positive, weak or negative

use lactic acid as a substrate. Two of the isolated yeasts fermented cellobiose and therefore express β-D-glucosidase (cellobiase). Most (99.58 %) of the isolated yeasts were able to grow at 37 °C (Table 1).

Proteome as biomarker for yeast discrimination and identification

A hundred and forty-five yeasts with the largest differences in their metabolic profiles were selected and submitted to MALDI-TOF analysis. After this analysis, 92 yeasts were identified, and among them, 69.51 % were identified at species level (score value $\geq$ 2.3) as *P. kudriavzevii* and *S. cerevisiae,* and 30.49 % were identified at genus level as *Saccharomyces* sp. and *Debaryomyces* sp. After similarity calculations and multidimensional scaling (MDS) based on the yeast proteome mass spectra, the 145 yeasts were distributed to four clusters (Fig. 2). All the yeasts identified as *S. cerevisiae* and *Saccharomyces* sp. as well as 45 non-identified yeasts were grouped in cluster A and those yeasts that were identified as *P. kudriavzevii* belonged to cluster B. Cluster C consisted of only the yeasts belonging to the *Debaryomyces* genus and two unidentified yeasts. The out-cluster yeast which does not belong to cluster C was also identified as *Debaryomyces*. Six of the yeasts that were unidentified because they scored below 1.77 were grouped in cluster D. These yeasts have finally been identified as *C. ethanolica* on the basis of the combination of the partial domains 1 and 2 of the large subunit ribosomal RNA gene and the restriction patterns of the 5.8S-ITS region. The different mass spectra generated from the different groups obtained after MALDI-TOF MS analysis were different and discriminated the isolated yeasts to genus and species levels (Fig. 3).

Yeast molecular characterization on the basis of ITS1-5.8S-ITS4 region and D1/D2 of large subunit rRNA gene

Fifty isolated yeasts were randomly selected from the different protein patterns (MALDI-TOF MS) and submitted to the restriction fragment length polymorphism (RFLP) analysis. After this RFLP analysis with three different endonucleases (*HindIII*, *HinfI* and *HaeIII*), only *HinfI* (Hf) and *HaeIII* (He) digested the ITS1-5.8S-ITS4 region into 2, 3, 4 or 5 fragments according to the strain as shown by the restriction patterns obtained after electrophoresis on agarose gel (Fig. 4). Six different restriction patterns were obtained after RFLP analysis. The restriction profiles 14-Hf (250 and 200 bp) and 14-He (300 and 150 bp) were obtained after the digestion with *HinfI* and *HaeIII,* respectively, and correspond to *C. ethanolica* yeast species. All the yeasts grouped in cluster D, which were obtained after their classification on the basis of protein patterns (MALDI-TOF MS analysis), have the same restriction patterns. The same observation was made with the different yeasts grouped in clusters B and C. In fact, the isolated yeast strains 337 and 375 have the same restriction profile that corresponds to *P. kudriavzevii* while those of 233, 219 and 221 (Fig. 4b) belong to *D. hansenii* complex yeast species. The different yeast strains in clusters B, C and D, which were obtained after yeast classification based on their protein patterns, are therefore homogeneous at species level. The RFLP analysis subdivided the *S. cerevisiae* yeast into different groups corresponding to different genotypes (Fig. 4a and c). The whole strain selected from the three different restriction patterns and belonged to cluster A which includes identified and non-identified yeasts was successfully identified as *S. cerevisiae* on the basis of D1/D2 domain. The ambiguous results corresponding to *C. ethanolica* and *P. deserticola* with a similarity of 100 and 99, respectively, were obtained when the d1 and d2 domain sequence of yeast belonging to cluster D was used. The yeasts grouped in clusters C and D, on the basis of MALDI-TOF analysis, were, respectively, identified as *D. hansenii* and *C. ethanolica* by using 5.8S-ITS region (Table 2).

Discussion

The yeast characterization on the basis of their morphological and physiological (fermentation and assimilation) traits does not enable to discriminate the non-*Saccharomyces*. The variability of the fermentative and assimilation profiles of the isolated strains, all belonging to *S. cerevisiae* yeast species, limits their identification. However, all yeasts which were identified as *S. cerevisiae* are lysine negative and, contrary to the non-*Saccharomyces* yeasts (lysine positive), show a distinct fermentative profile (Fig. 1). In contrast to the isolated yeasts' morphology and physiology, the yeast proteome obtained after MALDI-TOF MS analysis seems to be a good biomarker enabling yeast discrimination and grouping. The restriction patterns of the 5.8S-ITS sequence and comparing the D1/D2 domain of 26 ribosomal LSU sequence show the homogeny inside the different clusters generated on the basis of protein patterns. Despite the accuracy of MALDI-TOF analysis for yeast grouping, the absence of *C. ethanolica* in the used database hinders its identification. Kurtzman [26], therefore, proposed the expansion of the databases with additional yeast species to achieve complete yeast identification. The MALDI-TOF MS analysis based on microorganism proteome prior to molecular characterization may yet help to accurately group yeast. This rapid yeast grouping may be a good approach for an accurate, high throughput and inexpensive alternative to a large number of yeast characterizations and

Table 1 Physiological characteristics of yeasts isolated from Beninese African sorghum beer starter

Strain codes	Fermentation							
	Glucose	Galactose	Sucrose	Maltose	Cellobiose	Trehalose	Lactose	Melibiose
SA-001	+	−	−	−	−	−	−	−
SA-002	+	+	+	+	−	+	−	−
SA-009	+	+	−	+	−	+	−	−
SA-014	+	+	−	−	−	−	−	−
SA-015	+	+	+	+	−	+	−	−
TO-040	+	−	+	+	−	−	−	−
BO-048	+	−	−	+	−	+	−	−
BO-052	+	+	+	+	−	+	−	−
ND-084	+	+	−	+	−	+	−	−
ND-087	+	−	+	+	−	+	−	−
ND-089	+	−	+	+	−	+	−	−
BE-094	+	+	−	+	−	−	−	−
BE-099	+	+	−	+	−	+	−	−
BO-106	+	−	−	−	−	−	−	−
BO-107	+	−	−	−	−	−	−	−
PA-136	+	−	+	+	−	+	−	−
PA-138	+	+	+	+	−	+	−	−
PA-144	+	+	+	+	−	+	−	−
GL-168	+	+	+	+	−	+	−	−
GL-171	+	−	+	+	−	+	−	−
PA-185	+	+	+	+	−	+	−	−
GL-200	+	+	+	+	−	+	−	−
TC-204	+	+	+	+	−	+	−	−
TC-206	+	+	+	+	−	+	−	−
NA-212	+	+	+	+	−	+	−	−
DJ-240	+	+	+	+	−	+	−	−
BE-219	+	−	−	−	+	−	−	−
BE-227	+	−	−	+	−	+	−	−
BE-221	+	−	−	−	−	−	−	−
NA-270	+	+	+	+	−	−	−	−
NA-278	+	+	−	+	−	+	−	−
DJ-246	+	+	+	+	−	+	−	−
SV-253	+	+	−	+	−	+	−	−

Table 1 continued

Strain codes	Assimilation of carbon and Nitrogen													
	Glucose	Galactose	Sorbose	Sucrose	Maltose	Celllobiose	Trehalose	Lactose	Melibiose	Raffinose	Melibiose	Inulin	Starch	Xylose
SA-001	+	+	−	+	+	−	−	−	−	+	+	+	−	−
SA-002	+	−	−	+	+	+	−	−	−	−	−	+	−	−
SA-009	+	+	−	+	+	−	−	−	−	−	−	−	−	−
SA-014	+	+	−	+	+	−	+	−	−	−	+	+	+	−
SA-015	+	−	−	+	+	−	−	−	−	−	−	−	−	−
TO-040	+	−	+	+	+	−	+	−	+	+	+	+	−	+
BO-048	−	+	−	+	+	+	−	+	−	−	−	−	−	−
BO-052	+	−	−	+	+	−	−	−	−	−	+	+	−	−
ND-084	+	+	−	+	+	−	+	−	+	+	+	+	+	−
ND-087	+	−	w	+	+	−	−	−	−	−	+	−	−	−
ND-089	+	−	+	+	+	−	+	−	−	+	+	+	+	−
BE-094	+	+	−	+	+	−	+	−	−	+	+	+	+	−
BE-099	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BO-106	+	+	−	+	+	−	+	−	−	w	+	+	+	−
BO-107	+	+	w	+	+	−	−	−	−	−	+	+	−	−
PA-136	+	−	−	−	+	−	−	−	−	−	−	−	+	−
PA-138	+	−	−	w	−	+	−	−	−	−	−	−	−	−
PA-144	+	+	−	+	+	−	−	−	−	+	+	+	+	−
GL-168	+	+	+	+	+	−	+	−	+	+	+	+	+	−
GL-171	+	−	−	−	+	−	+	+	−	−	w	−	−	−
PA-185	+	+	−	−	+	−	−	−	−	−	−	−	−	−
GL-200	+	+	−	+	+	−	−	−	−	−	+	w	+	−
TC-204	+	+	−	−	+	−	−	−	−	−	−	−	−	−
TC-206	+	+	−	+	+	−	−	−	−	+	−	+	−	−
NA-212	+	−	−	+	+	+	−	−	−	−	−	+	−	−
DJ-240	+	+	−	−	+	−	−	+	−	−	w	−	−	−
BE-219	+	+	+	+	+	+	+	−	+	+	+	+	+	+
BE-227	+	+	+	+	+	+	+	w	+	+	+	+	+	w
BE-221	+	+	−	+	+	−	−	−	−	+	+	+	−	−
NA-270	+	−	−	+	+	−	+	−	−	−	+	−	−	−
NA-278	+	−	−	−	+	−	+	+	−	−	−	−	+	
DJ-246	+	+	+	+	+	w	+	−	+	−	+	−	+	w
SV-253	+	+	−	+	+	−	−	−	−	−	−	−	−	−

Table 1 continued

Strains codes	Assimilation of carbon and Nitrogen															
	L-arab-inose	D-arab-inose	Ribose	Rham-nose	Erythri-tol	Mannitol	Sorbitol	Methyl glucoside	Salicin	Lactic acid	Succinic acid	Inositol	Esculin	Mannose	Lysine agar	Nitrate
SA-001	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+
SA-002	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
SA-009	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
SA-014	−	−	−	−	−	−	−	+	−	+	+	+	−	+	+	+
SA-015	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
TO-040	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+
BO-048	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
BO-052	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
ND-084	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	+
ND-087	−	−	w	−	−	−	−	−	−	−	−	−	−	+	−	+
ND-089	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	+
BE-094	−	−	−	−	−	+	−	−	−	+	−	−	+	+	−	+
BE-099	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
BO-106	−	−	−	−	−	−	−	w	−	+	+	−	−	+	+	+
BO-107	−	−	−	−	−	−	−	+	+	−	−	−	+	−	+	+
PA-136	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
PA-138	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
PA-144	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+
GL-168	+	−	+	−	−	−	−	−	+	−	−	−	+	+	−	+
GL-171	−	−	−	−	−	−	−	w	−	−	−	+	w	+	−	+
PA-185	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
GL-200	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	+
TC-204	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
TC-206	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
NA-212	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
DJ-240	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
BE-219	−	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+
BE-227	−	w	+	−	+	−	+	−	−	−	+	−	+	+	+	+
BE-221	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
NA-270	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
NA-278	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+
DJ-246	−	w	+	−	−	−	−	+	−	−	−	W	+	+	−	+
SV-253	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+

Table 1 continued

Strain codes	Growth			
	Growth at 37 °C	Cycloheximide	50 % Glucose	Cristal violet
SA-001	+	−	+	−
SA-002	+	−	+	−
SA-009	+	−	+	−
SA-014	+	−	−	−
SA-015	+	−	+	−
TO-040	+	−	−	−
BO-048	+	−	+	−
BO-052	+	−	−	−
ND-084	+	−	+	−
ND-087	+	−	+	−
ND-089	+	−	+	−
BE-094	+	−	+	+
BE-099	+	−	+	−
BO-106	+	−	−	−
BO-107	+	−	−	−
PA-136	+	−	+	−
PA-138	+	−	+	−
PA-144	+	−	+	−
GL-168	+	−	+	−
GL-171	+	−	+	−
PA-185	+	+	+	−
GL-200	+	+	+	−
TC-204	+	−	+	−
TC-206	+	−	+	−
NA-212	+	−	+	−
DJ-240	+	−	+	−
BE-219	+	−	+	−
BE-227	+	+	+	−
BE-221	+	−	+	−
NA-270	+	−	+	−
NA-278	+	−	+	−
DJ-246	+	−	+	−
SV-253	+	−	+	+

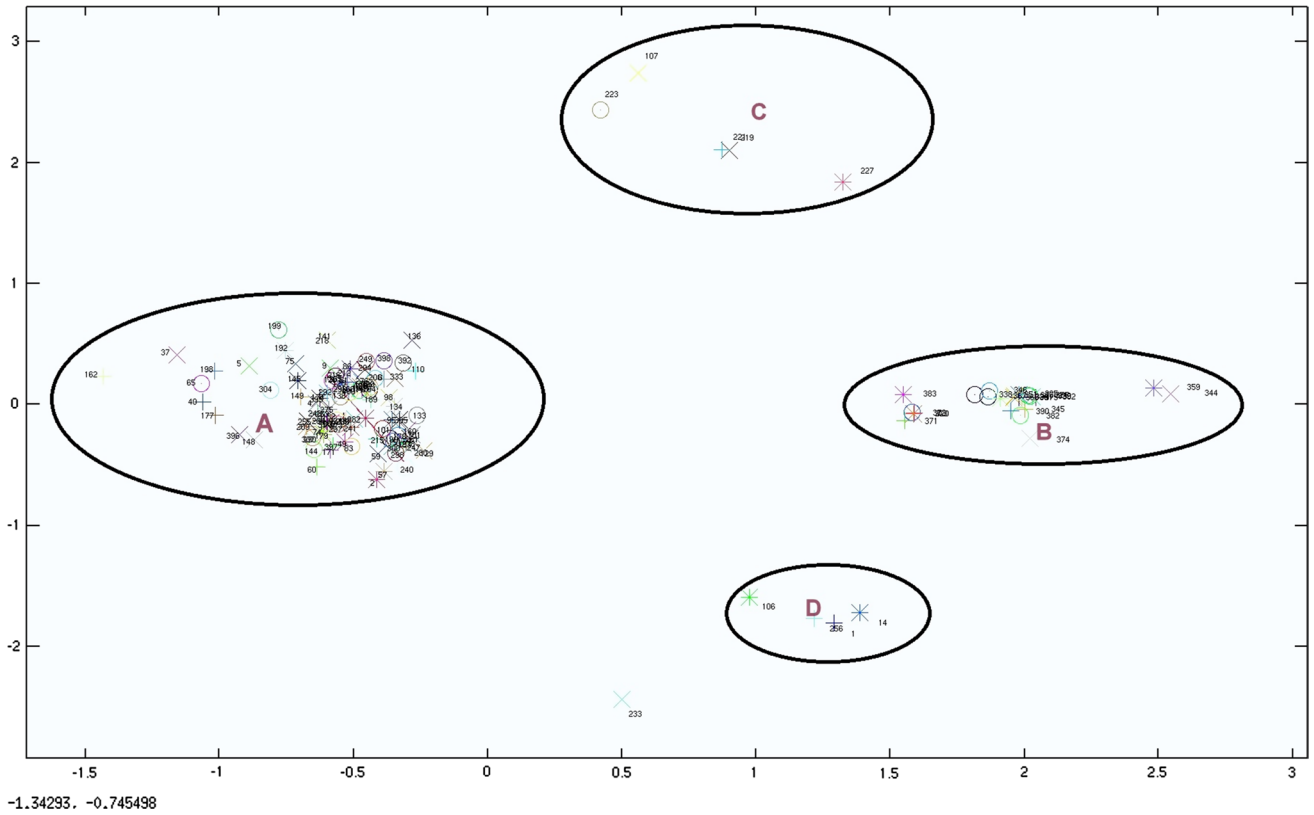

Fig. 2 Multidimensional scaling (MDS) based on protein patterns showing the repartition of different yeasts isolated from the starter of African sorghum beer. *S. cerevisiae* yeasts were separated from non-*Saccharomyces* yeasts. The non-*Saccharomyces* yeasts were distributed into three different groups belonging to *Pichia*, *Candida* and *Debaryomyces* genus. *Saccharomyces cerevisiae* is the dominant yeast (*A*) followed by *P. kudriavzevii* (*B*), *D. hansenii* (*C*) and *C. ethanolica* (*D*)

classifications. However, grouping the *D. hansenii* complex yeast is difficult and its identification is limited to genus level. The discrimination of yeast belonging to *D. hansenii* complex such as *D. fabryi*, *D. macquariensis*, *D. nepalensis*, *D. propopidis*, *D. subglobosus*, *D. tyrocola*, *D. vietnamensis*, *D. vindobonensis* and solving the limit of the 26S rRNA gene to differentiate these ascomycetous yeasts still remains a challenge. According to the work carried out by Prista et al. [27] and Martinez et al. [28], the *Debaryomyces* is very sensitive to the presence of some ionic substances such as sodium and potassium, which induce a variation of their proteome. This high susceptibility of *D. hansenii* complex, causing a small variation of the growth conditions to strongly affect their proteome, will probably preclude obtaining a protein pattern similar to that content in the used library for a reliable *Debaryomyces* yeast identification. Comparing the D1/D2 of the 26S rDNA sequence does not enhance the result obtained with yeast identification on the basis of the protein patterns. The limits of differentiating yeast belonging to *D. hansenii* complex due to the high similarity among their D1/D2 domain of the 26S rRNA gene was also observed by Kurtzman et al. [29], Groenewald et al. [30] and Martorell et al. [31]. Sequence analysis of the D1/D2 domain also indicated that *C. ethanolica* and *P. deserticola* are closely related, and they were both identified with a similarity of 100 and 99 %, respectively. The 5.8S-ITS region seems to be more discriminative and, contrary to the D1/D2 domain of 26 LSU, allows the differentiation of *C. ethanolica* from *P. deserticola*. Indeed, the polymorphic restriction endonuclease *HaeIII* generated different restriction patterns corresponding to two DNA fragments (110, 310 bp) for *C. ethanolica* (Table 2) and three DNA sequences (80, 100, 280 bp) for *P. deserticola* according to the restriction patterns obtained from the used CECT database. The *Debaryomyces* yeasts were successfully identified to species level as *D. hansenii* on the basis of the restriction patterns generated from the digestion of the 5.8S-ITS region with *HinfI* and *HaeIII*. This result is likely due to the absence of the others *Debaryomyces* yeasts in the used CECT database. The RFLP analysis of the 5.8S-ITS sequence is limited and does not allow the differentiation of some closely related *Debaryomyces* yeasts. Martorell et al. [31] shows the limit of the RFLP analysis of the 5.8S-ITS gene to differentiate to

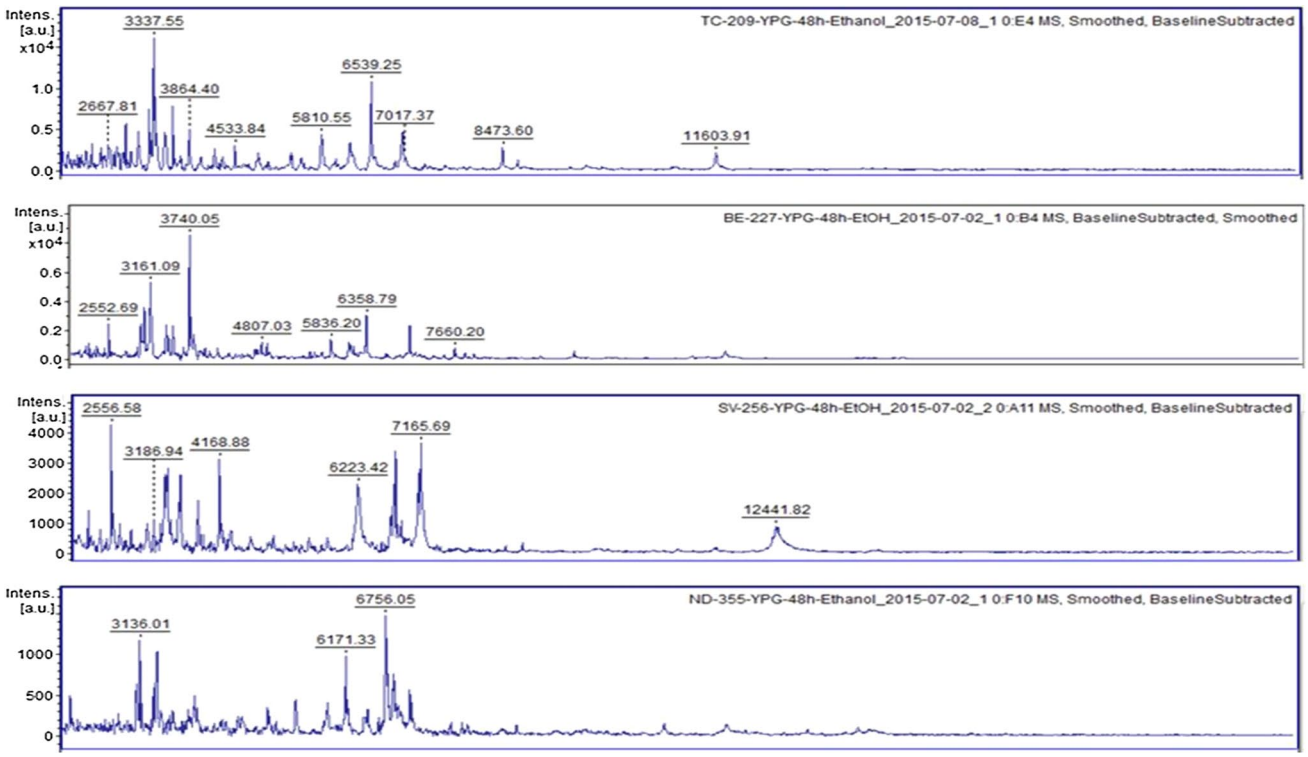

Fig. 3 Proteome mass spectrum belonging to each of the four groups of identified yeast: *S. cerevisiae* (TC-209), *D. hansenii* (BE-227), *P. kudriavzevii* (ND-355) and *C. ethanolica* (SV-256) obtained after matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) using Bruker software, α-cyano-4-hydroxy-cinnamic acid as matrix after the ethanol extraction method

species level some *Debaryomyces* yeast such as *D. castellii*, *D. courdertii*, *D. nepalensis*, *D. polymorphus*, *D. pseudopolymorphus*, *D. robertsiae*, *D. udenii* and *D. vanrifiae*. The recent work of Wrent et al. [32] also shows the limit of the RFLP of the 5.8S-ITS region to discriminate the *D. hansenii* complex such as *D. hansenii*, *D. fabryi* and *D. subglobosus*. After RFLP of the 5.8S-ITS region, the polymorphic endonucleases *HaeIII* and *HinfI,* therefore, lead to restriction patterns which discriminated the isolated *Candida, Pichia* and probably *Debaryomyces* yeasts to species level and *S. cerevisiae* to strain level (Fig. 4). Indeed, the yeast strains belonging to *S. cerevisiae* have been distributed in three different patterns corresponding to three different genotypes of these species. This biodiversity of the *S. cerevisiae* component in *Tchoukoutou* starter has been confirmed by their phenotype, characterized by different metabolic profiles (Fig. 1, sub-clusters I) and protein patterns (Fig. 2) even if the D1/D2 sequence analysis does not evidence any high diversity among these isolated *Saccharomyces* yeasts. Yet the polyphasic approach that integrates yeast phenotype, protein patterns generated by MALDI-TOF MS, and DNA sequence comparison therefore provides reliable information about relationships among yeasts and helps to avoid a mis-identification. Based on this polyphasic approach, it became evident that *S. cerevisiae* is present in the whole starter and is the dominant or only yeast species (55–100 %) of 11 starters out of the 12 localities where the starters were collected (Fig. 5). This predominance of *S. cerevisiae* yeast was also observed by Kayode et al. [33], by Djegui et al. [34] and Greppi et al. [35]. The outgrowth of non-*Saccharomyces* yeasts by *S. cerevisiae* was also observed during wine production [36]. As observed by Csoma et al. [37], it seems that *S. cerevisiae*, according to its genome, is more flexible than some other yeasts, which allows this dominant yeast species to more efficiently adapt to the continuously changing environment during fermentation. *S. cerevisiae* yeast contained in the Beninese traditional sorghum beer starter have different and interesting fermentative aptitudes. Indeed, some specific strains of *S. cerevisiae* ferment sucrose and therefore express invertase. Djegui et al. [34] indexed *S. cerevisiae* with invertase gene as a performant strains for the brewing process. In addition to invertase properties, many isolated yeast strains belonging to *S. cerevisiae* species show a good fermentative profile. They ferment the mains sorghum's wort sugar and are able to use lactic acid as one of their substrates. These *S. cerevisiae* yeast species may increase the beer pH during yeast and LAB symbiotic interaction, thereby allowing the growth of LAB that may contribute to further beer maturation and enhance the beer flavor and

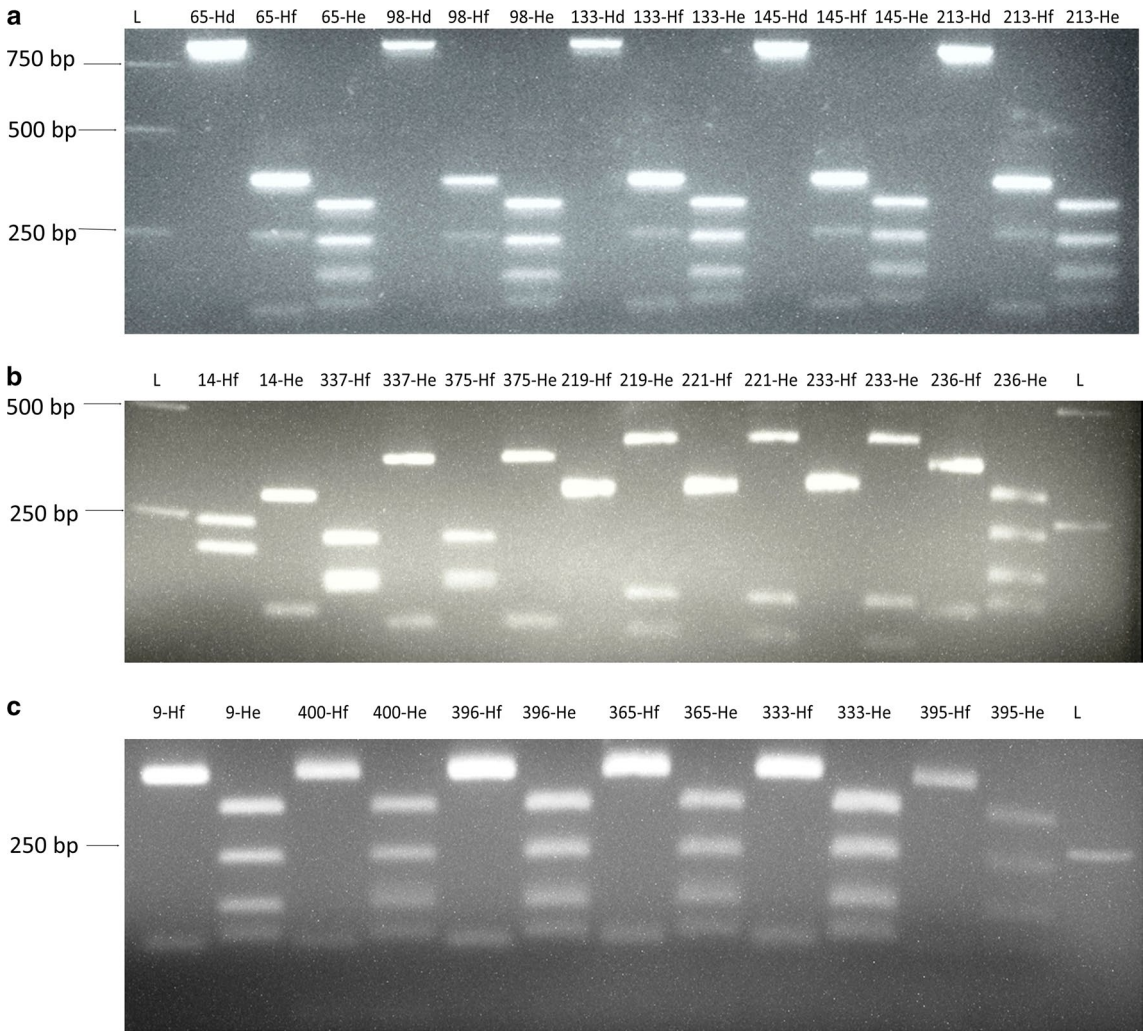

Fig. 4 Restriction patterns obtained after digestion of the amplified PCR product of the ITS1-5.8S-ITS4 domain of selected yeasts with the restriction endonuclease *HindIII* (Hd), *HinfI* (Hf) and *HaeIII* (He). All the restriction patterns of **a** and **b** belong to *S. cerevisiae*. *C. ethanolica* (14), *P. kudriavzevii* (337, 375) and *D. hansenii* (233, 219, 221). The 250-bp ladder (L) was used during this analysis

Table 2 Restriction patterns of the isolated non-Saccharomyces yeasts obtained with the application of endonucleases *HinfI* and *HaeIII* for the digestion of the 5.8S-ITS region and the corresponding yeast species in the Spanish Type Culture Collection (CECT) database

Strains	Amplified DNA (bp)	Restriction patterns (bp)		Identification	Match (%)
		HinfI	*HaeIII*		
14	450	200 + 250	110 + 310	*C. ethanolica*	100
219, 221, 233	650	325 + 325	90 + 150 + 420	*D. hansenii*	100

nutritive value. The co-presence of *S. cerevisiae* with *P. kudriavzevii* is observed in the starters collected from Toucountouna (26.67 %), N'dali (45 %) and from Natitingou where *P. kudriavzevii* is the dominant yeast (61.11 %). *P. kudriavzevii* has been identified from several West African traditional beverages made from fermented cereals. N'guessan et al. [38] observed its co-presence with *S. cerevisiae* in *Tchapolo*, the Ivory Coast traditional sorghum beer. *P. kudriavzevii* is also the dominant yeast species isolated from *Dolo*, traditional sorghum beer produced in several localities of Burkina Faso [39]. Annan et al. [40] used *P. kudriavzevii* in combination with *S. cerevisiae* to enhance

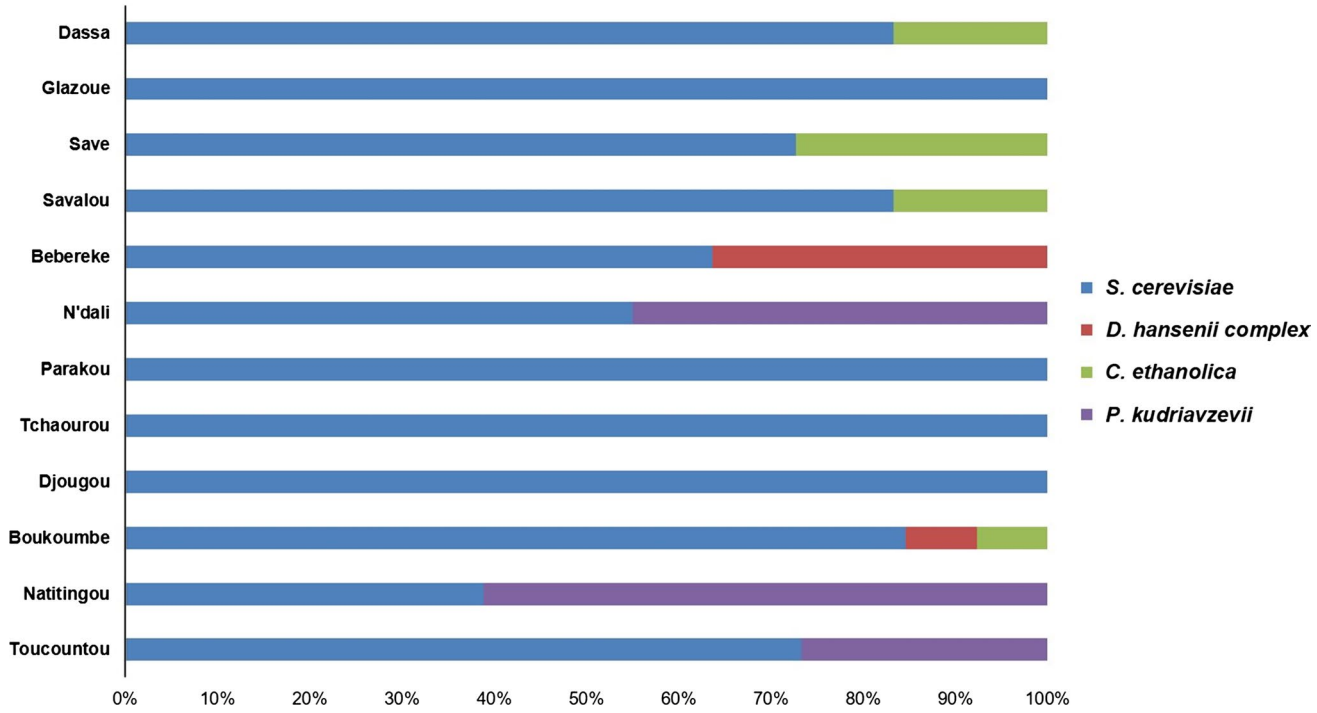

Fig. 5 Diversity of yeast population of the traditional starter collected in 12 localities of Beninese sorghum beer production

the taste and flavor of *Koko*, a Nigerian cereal-based African fermented food. *C. ethanolica* and *D. hansenii* complex have never been identified from West African sorghum beer. Recently, Visintin et al. [41] observed that *C. ethanolica* occurs during fermentation of West African cocoa beans and indexed this yeast species to possess several enzymes that may impact the quality of the final product. *S. cerevisiae* and *C. ethanolica* were the two different yeast species isolated from the starter collected in Dassa, Savalou and Save. The beer produced in central Benin is also different from that produced in the north of Benin by it organoleptic quality (taste and flavor) and lower alcohol content. The non-*Saccharomyces D. hansenii* complex yeasts were identified in the starter collected in two localities in northeast Benin. Some strains of *D. hansenii* may have a great importance and cooperate with *S. cerevisiae* for the specific aroma compound profile of the Beninese sorghum beer. In fact, the strains BE-219 and BE-225 identified as *D. hansenii* express cellobiase (β-glucosidase) and may be a potential candidate for beer flavor enhancement. Specific β-glucosidase is able to hydrolyze the aryl-β-D-glucosides from the non-reducing terminus and generates a good precursor from the secondary gluco-conjugate metabolite sequestrated in the cereal grain for beer bioflavouring. *D. hansenii* is present in many habitats, but has been most isolated from a traditional cheese. Indexed by Romero et al. [42] as a contaminant of intermediate moisture foods, Gori et al. [43] observed the heterogeneity of this yeast species with regard to the cheese flavoring. Indeed, Gori et al. [43] isolated from a cheese, *D. hansenii* producing high alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, 3-methyl-3-buten-1-ol) and aldehydes (2-methylpropanal, 3-methylbutanal). Even if spontaneous fermentations are ubiquitous in many developed countries in Africa, Asia and South America and constitute the only way of food preservation, the controlled fermentation with the selected performant yeast and LAB from the African traditional starter should be a good alternative to solve African sorghum beer safety problems, increase its shelf life and guarantee the same organoleptic quality for the consumers. The selection of performant yeast and LAB from this traditional starter still remains a challenge for the industrial production of *Tchoukoutou* as opposed to the existing household production.

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Compliance with ethical standards

Conflict of interest None.

Compliance with ethics requirements This article does not contain any studies with human and animals subjects.

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