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# UNIVERSITÀ DEGLI STUDI DI TORINO

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23 **Yeast dynamics during spontaneous fermentation of mawè and tchoukoutou, two**  
24 **traditional products from Benin**

25

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38

39 **Running title:** Yeast dynamics during African food fermentation

40

41

42 **Abstract**

43 Mawè and tchoukoutou are two traditional fermented foods largely consumed in Benin, West  
44 Africa. Their preparations remain as a house art and they are the result of spontaneous  
45 fermentation processes. In this study, dynamics of the yeast populations occurring during  
46 spontaneous fermentations of mawè and tchoukoutou were investigated using both culture-  
47 dependent and -independent approaches. For each product, two productions were followed.  
48 Samples were taken at different fermentation times and yeasts were isolated, resulting in  
49 collection of 177 isolates. They were identified by PCR-DGGE technique followed by  
50 sequencing of the D1/D2 domain of the 26S rRNA gene. The predominant yeast species  
51 identified were typed by rep-PCR. *Candida krusei* was the predominant yeast species in  
52 mawè fermentation followed by *Candida glabrata* and *Kluyveromyces marxianus*. Other  
53 yeast species were detected in lower numbers. The yeast successions that took place during  
54 mawè fermentation lead to a final population comprising *Saccharomyces cerevisiae*, *C. krusei*  
55 and *K. marxianus*. The yeast populations dominating fermentation of tchoukoutou were found  
56 to consist of *S. cerevisiae*, almost exclusively. Other yeast species were detected in the early  
57 stages of fermentation. For the predominant species a succession of biotypes was  
58 demonstrated by rep-PCR for the fermentation of both products. The direct analysis at DNA  
59 and RNA level in case of mawè did not reveal any other species but those already identified  
60 by culture-based analysis. On the other hand, for tchoukoutou, four species were identified  
61 that were not detected by culture-based approach. The spontaneous fermentation of mawè and  
62 tchoukoutou in the end were dominated by a few autochthonous species.

63

64 *Keywords:* yeasts, fermented foods, culture-dependent and -independent analysis, microbial  
65 successions, biotypes.

## 66 **1. Introduction**

67 Yeast has been reported to be involved in several types of indigenous African fermented  
68 foods and beverages (Hounhouigan et al. 1993d; Jespersen et al., 1994; Gadaga et al., 2000;  
69 Oyewole, 2001; Van der Aa Kuhle et al., 2001; Naumova et al., 2003; Jespersen et al., 2005;  
70 Omemu et al., 2007; N'guessan et al., 2011). However, the role of yeasts in these products and  
71 the dynamics of yeast populations are poorly studied. Possible roles are listed by Jespersen  
72 (2003). In general, yeasts contribute to the organoleptic properties of the final fermented  
73 products (Romano et al., 1997), they are capable of upgrading the nutritional value of the  
74 foods (Haefner et al., 2005; Hjortmo et al., 2005) and they are reported to have several  
75 probiotic effects (Gedek, 1999; Czerucka et al., 2000; Mumy et al., 2008; Pedersen et al.,  
76 2012) that can contribute to the improvement of human health, as reviewed by Moslehi-  
77 Jenabian et al. (2010). Detoxification of mycotoxins by yeast has also been reported (Moss et  
78 al., 2002; Shetty and Jespersen, 2006; Shetty et al., 2007). Considering the numerous roles of  
79 yeasts in terms of successful fermentations and impact on the quality of the final product,  
80 defining and understanding yeasts dynamics is important. Further, with an estimated 1 to 2  
81 billion women and children suffering from hunger or various forms of malnutrition and  
82 nutritional diseases, it is essential to study, improve, and expand the utilization of indigenous  
83 fermented foods in Africa and elsewhere.

84 Mawè and tchoukoutou are two traditional cereal-based fermented foods from Benin, West  
85 Africa. Mawè is a dehulled fermented maize dough used to prepare many cooked dishes  
86 including gels (*akassa*, *agidi*, *eko*), steam-cooked bread (*ablo*) and porridge (*koko*, *aklui*,  
87 *akluyonou*). The manufacturing processes have been described by Hounhouigan et al.  
88 (1993a). Tchoukoutou is the major opaque sorghum beer consumed in Benin. It has a sour  
89 taste, relatively high dry matter content (5-13 % w/v) and low alcohol content (2-3 % v/v),  
90 which makes it an appreciated beverage (Agu and Palmer, 1998; Briggs et al., 2004). In brief,

91 the manufacturing process consists of malting of red sorghum, milling, brewing and  
92 fermentation. For these traditional fermentations, Hounhouigan et al. (1993b, 1993c) and  
93 Kayodè et al. (2006), reported that lactic acid bacteria (LAB) and yeasts are the predominant  
94 microorganisms leading to a two steps fermentation process i.e. a lactic acid fermentation  
95 which confers acidity and storage longevity and an alcoholic fermentation respectively.  
96 However, these studies focused on the LAB populations and paid little attention to yeasts. To  
97 obtain detailed information on yeasts populations and to address up-to-date taxonomic  
98 databases, culture-independent techniques are needed e.g. by DGGE analysis. This technique  
99 has been widely applied for studying microbial dynamics in complex matrices (Silvestri et  
100 al., 2007; Nielsen et al., 2007; Bonetta et al., 2008; Ramos et al., 2010; Masoud et al., 2011)  
101 and to investigate yeast diversity in foods (Cocolin et al., 2000; Cocolin et al., 2002; Chang et  
102 al., 2008; Stringini et al. 2008) and wine (Prakitchaiwattansa et al., 2004; Rantsiou et al.,  
103 2005; Di Maro et al., 2007; Urso et al. 2008). The DGGE technique combined with cultural  
104 method has recently been applied to study the yeast ecology of mawè and tchoukoutou final  
105 products from Benin (Greppi et al., submitted).

106 In the present study, we investigated the yeast dynamics occurring during the fermentation of  
107 mawè and tchoukoutou using culture dependent and independent molecular-based techniques.  
108 The combination of both approaches allowed the quantification, identification and monitoring  
109 of the successions of yeast population actively involved in the fermentation of these two  
110 products. The results obtained represent the first step needed to select and study the  
111 functionality of yeasts able to enhance the quality of the final products in terms of safety,  
112 shelf life, organoleptic characteristics, nutritional properties and even health-promoting  
113 properties.

114

115 **2. Materials and methods**

116 *2.1 Sample collection and microbiological analysis*

117 The fermentations of both mawè and tchoukoutou were followed for two different local  
118 producers located at the University campus of Abomey-Calavi and at the Abomey-Calavi  
119 local market, respectively.

120 Samples of mawè were taken aseptically using sterile stomacher bags (Seward, West Sussex,  
121 UK) at 0, 6, 24, 48 and 72 hours. Time 0 was set when the milled grits were kneaded with  
122 water and left to ferment spontaneously. Samples were transported immediately to the  
123 laboratory for analyses, carried out not later than 30 minutes after sampling. Samples of  
124 tchoukoutou were collected at 0, 4, 8 and 12 hours. Time 0 was set when the cooked  
125 supernatant obtained by the first fermentation was filtered and the second fermentation started  
126 using material from previous fermentation i.e. back-slopping. In order not to change the  
127 natural production conditions the pots used by the producers were moved to the laboratory  
128 together with their content.

129 The pH measurements (inoLab pH 730, WTW GmbH, Weilheim, Germany; calibrated with  
130 buffer at pH 4.0 and 7.0) were made on each sample in duplicate.

131 Ten (10) g of mawè and 10 ml of tchoukoutou samples were diluted, homogenized and yeast  
132 enumerated on MYPG agar as previously described (Greppi et al., submitted). Results were  
133 expressed as log<sub>10</sub> colony forming units (cfu)/g (mawè) or /ml (tchoukoutou). From each  
134 sample 10 colonies were randomly selected and purified leading to a total of 177 isolates. All  
135 of them were maintained in glycerol (30%) at -20°C until identification.

136

137 *2.2. DNA extraction from pure cultures*

138 Yeast DNA of each isolate was extracted from 1 ml of 24 h MYPG pure culture and  
139 centrifuged at 14,000 × g for 10 min at 4°C. The pellet of yeast cells was subjected to DNA  
140 extraction according to procedures described by Cocolin et al. (2000). DNA was quantified by

141 using the Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific,  
142 Milan, Italy) and diluted to a concentration of 100 ng/ml.

143

### 144 *2.3. Direct extraction of nucleic acids from the samples*

145 Ten (10) g of mawè and 10 ml of tchoukoutou samples were separately homogenized with 40  
146 ml of Ringer solution in a Stomacher for 30 seconds at normal speed. For both RNA and  
147 DNA, the supernatant from 1 ml was collected and centrifuged at 13,200 rpm for 10 min. The  
148 nucleic acids were extracted from the pellet using a MasterPure™ Complete DNA and RNA  
149 Purification kit (Epicentre, Madison, WI, USA) following the supplier's instructions (Rantsiou  
150 et al., 2012). The RNA samples were treated with RNase-free DNase (Ambion, Milan, Italy)  
151 for 3 h at 37°C and checked for the presence of residual DNA by PCR amplification. When  
152 PCR products were obtained, the DNase treatment was repeated to eliminate DNA.

153

### 154 *2.4. PCR and RT-PCR*

155 One microlitre of the yeast DNA (100 ng) was used for the PCR assays as previously  
156 described (Greppi et al., submitted). The region amplified, using the primers NL1GC and a  
157 reverse primer LS2, was the D1 region of the 26S rRNA gene (Cocolin et al., 2000).

158 The reverse transcription (RT) reactions were performed using the M-MLV reverse  
159 transcriptase (Promega, Milan, Italy). Two hundred microgram of RNA were mixed with 1 µl  
160 of primer LS2 (100 µM) and sterile water to a final volume of 10 µL and incubated at 70°C  
161 for 5 min. The mix was placed on ice and a mixture containing 50 mM Tris-HCl (pH 8.3), 75  
162 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 2 mM of each dNTP, 1 µl of 200 U/l M-MLV and 0.96  
163 units of Rnasin ribonuclease inhibitor (Ambion) was transferred to the reaction tube. The  
164 reverse transcription was carried out at 42°C for 1 h. One µl of cDNA was amplified using the  
165 conditions described above.

166 *2.5 DGGE analysis*

167 Denaturing Gradient Gel Electrophoresis (DGGE) using the DCode apparatus (Bio-Rad,  
168 Hercules, CA, USA) was used to analyse the PCR products. They were electrophoresed in a  
169 0.8 mm polyacrylamide gel (8% [w/v]) acrylamide-bisacrylamide (37.5:1), as previously  
170 described (Cocolin et al., 2001; Greppi et al., submitted).

171

172 *2.6 Sequencing of DGGE bands and sequence analysis*

173 Selected DGGE bands were excised from the gels, checked by PCR-DGGE, amplified with  
174 yeast primers (NL1 without GC clamp and LS2) and sent for sequencing (MWG Biotech,  
175 Ebersberg, Germany) as described by Cocolin et al., 2001. Sequences were aligned in  
176 GenBank using the Blast Program (Altschul et al. 1997) for identification purposes.

177

178 *2.7 Identification of the isolates by PCR-DGGE*

179 Yeast isolates were identified by groupings based on their PCR-DGGE profiles and  
180 sequencing of representative isolates of each group. The DNA of the isolates was first  
181 amplified with primers NL1GC/LS2 and the products run on DGGE, according to Cocolin et  
182 al. (2000). Representatives of the different DGGE profile groups were identified by  
183 sequencing the partial 26S rRNA gene that was amplified with primers NL1/NL4, as  
184 previously described (Kurtzman and Robnett, 1998). The PCR products were sent to MWG  
185 Biotech for sequencing and the resultant sequences were aligned with those in GenBank using  
186 the Blast program, to determine the known relatives.

187

188 *2.8 Typing of the isolates by rep-PCR*

189 The predominant yeast species identified during the fermentations studied were subjected to  
190 rep-PCR analysis using primer (GTG)<sub>5</sub> (5'-GTGGTGGTGGTGGTG-3') according to Nielsen

191 et al. (2007). The rep-PCR was performed as previously described (Greppi et al., submitted).  
192 Amplicons were separated by 1.5 % agarose gel electrophoresis in 1X TBE (150min, 120 V)  
193 using a Generuler 1 kb DNA ladder as reference (Promega). The rep-PCR profiles were  
194 normalised and cluster analysis were performed using Bionumerics software (version 6.1,  
195 Applied Maths, Sint-Martens-Latem, Belgium). The dendograms were calculated on the basis  
196 of the Pearson's Coefficient of similarity with the Unweighted Pair Group Method using  
197 Arithmetic Averages (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992).

198

### 199 **3. Results**

#### 200 *3.1 Microbiological analysis of mawè and tchoukoutou*

201 The pH and yeast counts, reported in Table 1 and Table 2, are expressed as means and  
202 standard deviations for the two different fermentations. As seen in Table 1, during the 72  
203 hours of fermentation of mawè the pH decreased from  $5.14 \pm 0.64$  to  $3.44 \pm 0.11$ . At the  
204 beginning of the spontaneous mawè fermentations samples exhibited a viable yeast count of  
205  $2.93 \pm 0.03 \log_{10}$  cfu/g, at the end of the fermentation (72 hours), the number of yeast  
206 enumerated increased to  $5.64 \pm 0.16 \log_{10}$  cfu/g. During the 12 hours of fermentation of  
207 tchoukoutou, the pH decreased from  $3.98 \pm 0.16$  to  $3.61 \pm 0.11$  (Table 2). Yeast counts  
208 increased from  $4.97 \pm 0.12$  to  $6.47 \pm 0.07 \log_{10}$  cfu/ml. (Table 2).

209

#### 210 *3.2 Identification of isolates and species succession during fermentation*

211 According to the DGGE profiles obtained after amplification and DGGE analysis (data not  
212 shown), 9 and 6 species were identified during the mawè and tchoukoutou fermentations,  
213 respectively (Table 1 and 2).

214 In the case of mawè (Table 1), at the beginning of the fermentation, several yeast species were  
215 identified; after 24 and 48 hours *Candida glabrata* and *Candida krusei* dominated with

216 *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Clavispora lusitaniae* being  
217 present as well. At 72 hours the yeast populations mainly consisted of *C. krusei* and *S.*  
218 *cerevisiae*, together with some isolates of *K. marxianus*.

219 At the beginning of tchoukoutou fermentation (Table 2) several yeast species were present;  
220 the majority of the isolates were identified as *Cl. lusitaniae*. From 4 hours until the end of the  
221 fermentation almost all the isolates were identified as *S. cerevisiae*. However, as the number  
222 of *S. cerevisiae* decreased towards the fermentation, isolates of *Hanseniaspora guillermondii*,  
223 *C. krusei* and *Cl. clavispora* appeared. At 12 hours, *S. cerevisiae* and *Cl. lusitaniae* were the  
224 predominant yeast present.

225

### 226 3.3 Rep-PCR typing

227 Being the predominant species isolated during the mawè fermentation, *C. glabrata* and *C.*  
228 *krusei* isolates were typed by rep-PCR. The cluster analysis of the fingerprints obtained for 22  
229 isolates of *C. glabrata*, using a coefficient of similarity of 84%, resulted in 3 clusters (Figure  
230 1). Cluster I grouped isolates mainly found at 48 hours (T3) while isolates of cluster II were  
231 mainly detected at 6 hours of fermentation (T1). The third cluster (III) contained isolates  
232 detected at the beginning (T0), after 6 hours (T1) and 24 hours (T2). Neither cluster II nor  
233 cluster III contained isolates at 48 hours (T3). The analysis of the fingerprints of the *C. krusei*  
234 isolates (29), at a similarity coefficient of 80%, resulted in 2 main clusters (Figure 2). The  
235 composition of these clusters appeared to be independent of fermentation times.

236 For the tchoukoutou fermentation, 50 isolates of *S. cerevisiae* were grouped by rep-PCR  
237 (Figure 3). *S. cerevisiae* was the predominant species isolated during this fermentation. Using  
238 a coefficient of similarity of 87%, a differentiation of the isolates based on the fermentation  
239 time was observed. As shown in Fig. 3, cluster I and II contained isolates from throughout the  
240 fermentation. Cluster III was composed from isolates at T2 and T3 (8 and 12 hours) while

241 cluster IV from isolates at T1 and T2 (4 and 8 hours). Both cluster I and III did not have any  
242 isolate from T1 (6 hours). The only *S. cerevisiae* isolate at T0 was not included in the  
243 analysis.

244

#### 245 3.4 PCR-DGGE analysis of mawè and tchoukoutou samples at DNA and RNA level

246 DGGE fingerprints obtained from the total DNA and RNA extracted directly from mawè and  
247 tchoukoutou samples are shown in Figure 4 (panel A and B, respectively), and the results of  
248 the sequenced bands are reported as caption to the figure. As in both cases there were no  
249 differences in the results obtained between the two replicates, DGGE profiles for only one  
250 fermentation are reported. Considering mawè fermentation, the analysis on total DNA  
251 demonstrated how *K. marxianus* (band b) was present from the beginning of the fermentation  
252 until the end, *C. glabrata* (band c) was also detected at 6 hours and band d, corresponding to  
253 the closest relative *Pichia kudriavzevii* (formerly named as *I. orientalis*, anamorph *C. krusei*)  
254 was present from the 12 hours to the end of fermentation. At RNA level, *K. marxianus* (band  
255 b) and *C. glabrata* (band c) were detected during the whole fermentation while *P.*  
256 *kudriavzevii* was detected from 24 hours to the end. *Zea Mays* (band a) was also occasionally  
257 detected both at DNA and RNA level.

258 In the DGGE profiles obtained from tchoukoutou matrix (panel B), at both DNA and RNA  
259 level, band corresponding to *S. cerevisiae* (band e) was clearly detected during the whole  
260 fermentation process. At DNA level *K. marxianus* (band b) and *Hanseniaspora uvarum* (band  
261 f) were detected up to 6 hours of fermentation. On the other hand, at RNA level *K. marxianus*  
262 (band b) was always present while *H. guilliermondii* (band n) only at the beginning of the  
263 process.

264 Bands not marked on the DGGE gel were determined to be heteroduplex after cutting and  
265 sequencing (data not shown).

266

#### 267 **4. Discussion**

268 Microbial successions are often reported for spontaneously fermented products (Hounhouigan  
269 et al. 1993d; Jespersen et al., 1994; Jespersen, 2003). They are likely to be due to changes in  
270 nutrient availability, pH, temperature, presence and concentration of organic acids and oxygen  
271 availability. Since the overall quality of the final fermented products is strictly connected to  
272 the populations that are able to develop and to carry out the transformation process, and more  
273 specifically to certain biotypes within a species, understanding their dynamics is important.  
274 For mawè, no studies seem to be carried out on identification of yeasts successions during  
275 fermentation using molecular-based methods. In the present study, a significant yeast growth  
276 was registered. It increased about 1000-fold reaching the maximum population after 48h,  
277 while the pH was still decreasing during the fermentation of mawè. Six species were detected  
278 at the beginning and after 6 hours of mawè fermentation while from 24 hours until the end the  
279 fermentation was dominated by *C. krusei*, *C. glabrata*, *S. cerevisiae* and *K. marxianus*.  
280 Regarding *C. krusei* and *S. cerevisiae*, similar results were obtained by Jespersen et al. (1994)  
281 on kenkey, a maize-based dough from Ghana. The disappearance of some yeasts strains may  
282 be attributed to the increase in lactic acid concentration caused by the activity of the LAB. In  
283 general, *C. krusei* and *C. glabrata* dominated mawè fermentation. Candida species are  
284 ubiquitous organisms (Odds, 1998) and their ability for co-metabolism with lactic acid  
285 bacteria has been reported as desirable for adequate fermentation of traditional African food  
286 (Oguntoyinbo, 2008). Both species demonstrated a high stress tolerance to both acid and high  
287 temperature (Halm et al, 2004; Liu et al, 2005; Watanabe et al., 2010). The strong resistance  
288 to acidity and high environmental temperature can explain their dominance in mawè  
289 fermentation. The variations on yeast counts, yeast successions and on the identity of  
290 predominant yeast species during fermentation are expected to influence the quality of mawè,

291 including both the organoleptic quality and the nutritional and health related issues. In  
292 particular, *C. krusei* can have a positive impact on the organoleptic quality of African  
293 fermented maize dough, as reported by Annan et al. (2003) on kenkey. On the other hand, *C.*  
294 *glabrata* is of mounting importance in clinical microbiology. A review by Fidel et al. (1999)  
295 concludes that the species is emerging as a major pathogen that accounts for an increasing  
296 large population of nosocomial fungal infections. Therefore, it cannot be considered or  
297 included in starter culture preparation.

298 In the present study yeast diversity was also investigated by rep-PCR typing. This aspect is  
299 receiving strong attention in the field of food fermentation because it allows understanding  
300 dynamics during fermentation and it helps to understand if a particular culture inoculated as  
301 starter is able to dominate the fermentation (Cocolin et al., 2011). Our results revealed a  
302 succession of biotypes of *C. glabrata* during the fermentation of mawè. Some biotypes  
303 mainly present at the first 6 hours of fermentation were followed by others that dominated the  
304 remaining time of fermentation. Biotypes present during the entire fermentation were also  
305 seen. The cluster analysis of the *C. krusei* isolates indicated that a succession of biotypes  
306 during fermentation did not take place. In a previous study (Greppi et al., submitted) a variety  
307 of biotypes of *C. krusei* was reported for mawè from different sites in Benin as offered for  
308 sale. Such diversity and differences between production sites are likely to be explained by  
309 differences in the composition and microbiology of raw materials as well as fermentation  
310 conditions for the particular sites and operators (Jespersen et al., 2004).

311 The direct analysis on total DNA and RNA of mawè did not reveal any other species but  
312 those already identified by culture-based analysis. The detection limit of DGGE analysis for  
313 yeasts is about  $10^3$  cfu/g or ml (Cocolin et al., 2001), and if minor populations are present in  
314 the food samples analysed they may not be detected as DGGE bands. The results obtained  
315 indicated *K. marxianus*, *C. glabrata* and *C. krusei* as the species present and metabolically

316 active during the mawè fermentation. *K. marxianus* were clearly detected during the whole  
317 fermentation both from total DNA and RNA. *C. glabrata* and *C. krusei* were also detected  
318 both at DNA and RNA level indicating that they actively contribute to the fermentation.  
319 These results confirmed our cultural data except for the absence of *S. cerevisiae*, detected in  
320 high percentage in culture dependent analysis in the last stages of the fermentation. This could  
321 be due to PCR-bias in the food matrices where different yeast species are present at high level  
322 interfering with the specific binding of the primers to other species. A DGGE band that  
323 showed the closest relative in the GenBank database with *Z. mays* was detected in mawè  
324 samples at the first sampling points. This is assumed to be due to a lack of specificity of the  
325 set of primers used.

326 The other product investigated was tchoukoutou, the sorghum beer from Benin. Sorghum  
327 beers are traditional fermented products largely consumed in sub-Saharan Africa and several  
328 studies were performed on identification of yeast population associated with the fermentation  
329 (Demuyakor and Ohta, 1991; Sanni and Lonner, 1993; Konlani et al., 1996; Sefa-Dedeh et al.,  
330 1999; Van der Aa Kuhle et al., 2001; Glover et al., 2005; Maoura et al., 2005; Greppi et al.,  
331 submitted). Almost all of them focused on the yeasts in the final products. Only N'guessan et  
332 al. (2011) studied the mycobiota during the alcoholic fermentation of tchapalo, a sorghum  
333 beer from Cote d'Ivoire. In the present study, during tchoukoutou fermentation, an increase in  
334 the yeast counts was observed until the end of the fermentation accompanied by a decrease of  
335 pH. Considering the relatively short time of fermentation, the yeast growth reported was  
336 significant. The low values of pH measured at the beginning were due to a separate lactic acid  
337 fermentation that took place before the alcoholic fermentation. The fermentation was  
338 dominated by *S. cerevisiae*, however the non-*Saccharomyces* yeasts were detected during the  
339 early stages of fermentation. The preponderance of *Saccharomyces* species during the  
340 alcoholic fermentation of sorghum beers has been reported by several authors (Sefa-Dedeh et

341 al., 1999; Maoura et al., 2005; N'guessan et al., 2011). Isolates of *Cl. lusitaniae*, *C. krusei*, *D.*  
342 *nepalensis*, *H. guillermondii*, *S. cerevisiae* and *C. glabrata* were isolated at the beginning of  
343 the fermentation. Further, *H. guillermondii*, *C. krusei* and *Cl. lusitaniae* were isolated at lower  
344 percentage during the fermentation and can then be considered as sporadic.

345 Differences in yeast species in comparison to other African sorghum beers ecosystem studied,  
346 could contribute to the particular characteristic of tchoukoutou. Local differences in the  
347 production process (Van der Aa Kuhle et al., 2001; Jespersen, 2003) and different types of  
348 ingredients and of sorghum cultivars utilized can be a cause of the variation in the yeast biota  
349 as they have different biochemical characteristics, which influence substrates available for the  
350 yeast (Demuyakor and Ohta, 1991). The results from the characterization of the *S. cerevisiae*  
351 isolates demonstrated a succession of biotypes during the fermentation of tchoukoutou,  
352 despite the relatively short time of fermentation. Some appeared to be involved only in the  
353 early stages of fermentation followed by others that appeared after 8 hours until the end.  
354 Other biotypes were distributed homogeneously throughout the fermentation of tchoukoutou.  
355 The data obtained confirmed previous findings concerning the diversity of *S. cerevisiae*  
356 biotypes conducting the fermentation (Van der Aa Kuhle et al., 2001; Naumova et al. 2003;  
357 Glover et al, 2005). The occurrence and taxonomic characteristics of *S. cerevisiae* biotypes in  
358 African indigenous fermented foods and beverages have been reviewed by Jespersen (2003).  
359 The results obtained by the direct analysis on the fermentation of tchoukoutou revealed some  
360 yeast species not detected by the culture-based approach. This was the case for *H. uvarum* and  
361 *K. marxianus* at DNA level and of *H. guillermondii* and *K. marxianus* at RNA level. In case  
362 of DNA, these species may be present in the habitat as viable but not-cultivable cells, because  
363 of the cultivation conditions or their physiological state (Head et al. 1998; Ercolini, 2004) or  
364 they may be dead. *S. cerevisiae* were clearly detected during the whole fermentation of  
365 tchoukoutou both from total DNA, confirming the cultural data, and also on RNA level i.e.

366 metabolically active yeast cells. *K. marxianus* was also largely detected by culture-  
367 independent approach. This species was not found by culturing, instead *Cl. lusitaniae* was  
368 present at high percentage in plates but not detected by culture independent analysis. As  
369 already discussed, this could be due to PCR-bias.

370 As mentioned above, differences were seen between our results and those from previous  
371 studies. They may be related to differences between sample sites and in particular to the fact  
372 that the fermentations are the results of very heterogeneous processes depending on seasonal  
373 variations as well as differences in production methods. The variations are reflected both in  
374 maximum yeast cell counts, yeast successions and the identity of the predominant yeast  
375 species and they are expected to influence the quality of the final products.

376 The results obtained in this study clearly demonstrated that a significant yeast growth took  
377 place during mawè and tchoukoutou fermentations. Further changes in yeast species  
378 composition and successions at both species and biotype level within predominant species  
379 were found to take place during fermentations leading to a selection of a defined biota. The  
380 data obtained allowed to get, for the first time, a detailed picture of the ecological distribution  
381 of yeast populations during these traditional fermentations including information on  
382 populations metabolically active at the different stages by direct RNA analyses. These results  
383 have two main practical applications. The first concerns the decision of using of back-  
384 slopping in yeast fermentations, which only will include yeast viable at the end of  
385 fermentation eventually missing yeast contributing to the sensory quality of the product.  
386 Secondly, the information obtained on yeast populations is crucial as starting point in a  
387 perspective of defining the role of a defined mycobiota in the fermentation of mawè and  
388 tchoukoutou. For this reason, further studies are needed to clarify functional characteristics of  
389 the yeasts including effects on fermentation as well as on product quality and possibly human  
390 health.

391

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587

588 **Figure legends**

589 **Figure 1.** Cluster analysis of the rep-PCR fingerprints of *Candida glabrata* strains isolated  
590 during the fermentation of mawè. The first letter represents the sample, the second represents  
591 the replicate (a-b), the number represents the fermentation times [T0, T1 (6 h), T2 (24 h), T3  
592 (48 h)] and the progressive number of isolation.

593 **Figure 2.** Cluster analysis of the rep-PCR fingerprints of *Candida krusei* strains isolated  
594 during the fermentation of mawè. The first letter represents the sample, the second represents  
595 the replicate (a-b), the number represents the fermentation times [T0, T1 (6 h), T2 (24 h), T3  
596 (48 h, T4 (72h))] and the progressive number of isolation.

597 **Figure 3.** Cluster analysis of the rep-PCR fingerprints of *Saccharomyces cerevisiae* strains  
598 isolated during the fermentation of tchoukoutou. The first letter represents the sample, the  
599 second represents the replicate (a-b), the number represents the fermentation times [T0, T1 (4  
600 h), T2 (8 h), T3 (12 h)] and the progressive number of isolation.

601 **Figure 4.** DGGE profiles obtained by the amplification of total DNA and RNA extracted  
602 directly from mawè (panel A) and thoukoutou (panel B). Panel A, Lines 1-5, DNA from  
603 mawè fermentation (T0-T6-T24-T48-T72); lines 6-10, RNA from mawè fermentation. Panel  
604 B, Lines 1-4 DNA from tchoukoutou fermentation (T0-T4-T8-T12); lines 5-8 RNA from  
605 tchoukoutou fermentation. Identity of identified fragments (% identity, accession number):  
606 band a *Zea mays* (99%, BT088101), band b *Kluyveromyces marxianus* (100%, FJ896141),  
607 band c *Candida glabrata* (100%, HM591715), band d *Pichia kudriavzevii*, formerly named as  
608 *Issatchenkia orientalis*, anamorph of *Candida krusei* (100%, JQ585732); band e  
609 *Saccharomyces cerevisiae* (100%, JF427814); band f *Hanseniaspora uvarum* (100%,  
610 EU386753); band g *Hanseniaspora guillermondii* (100%, JQ707775).

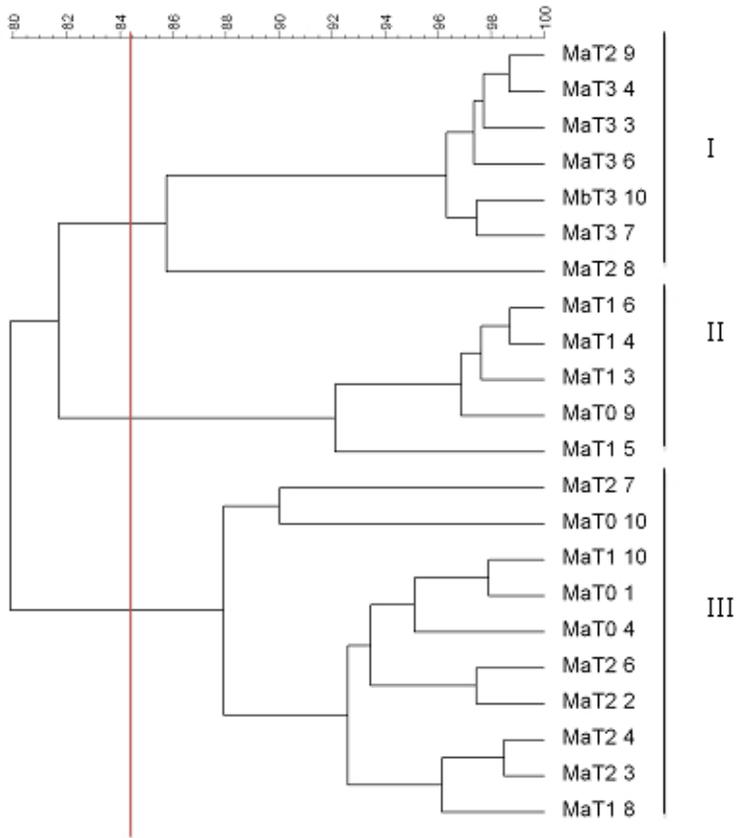
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614 **Figure 1**

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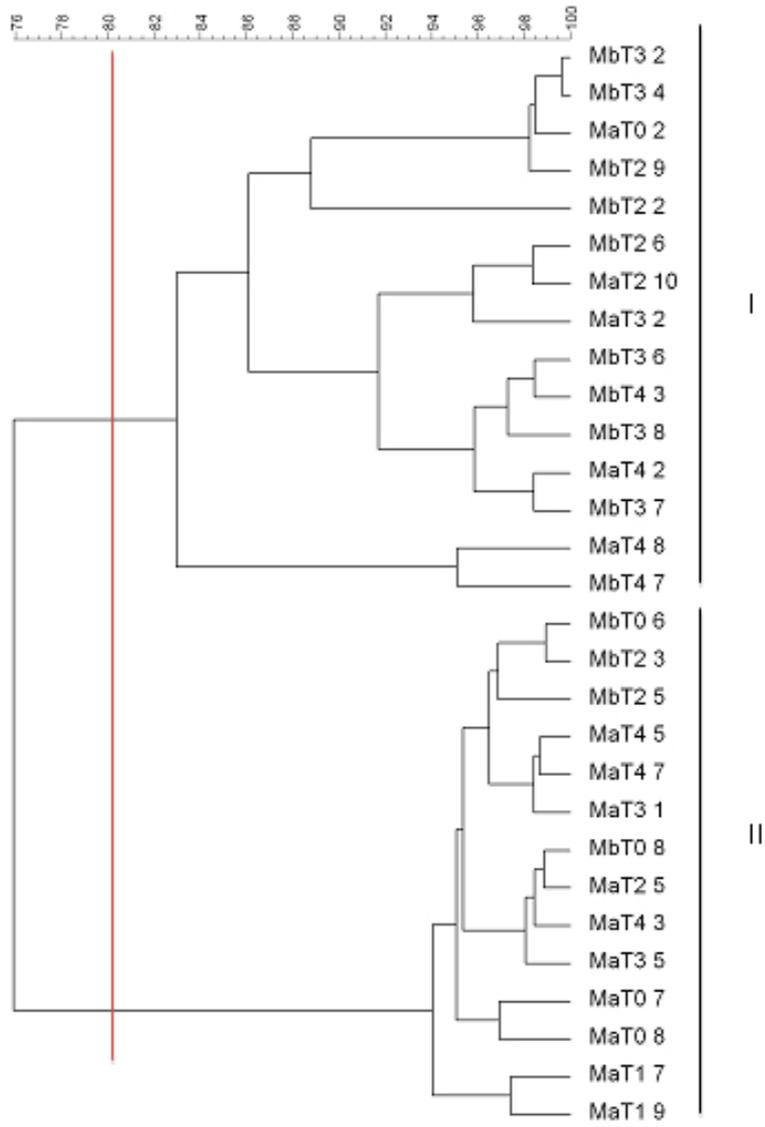
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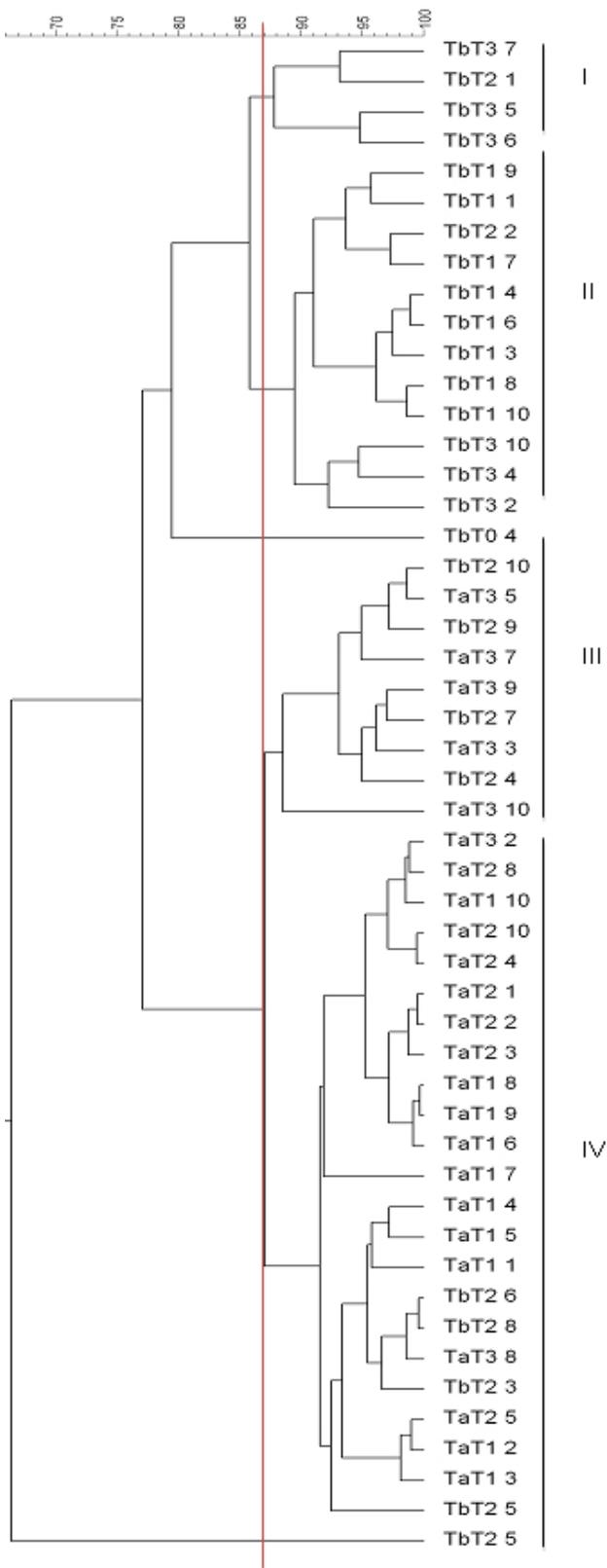
619 **Figure 2**

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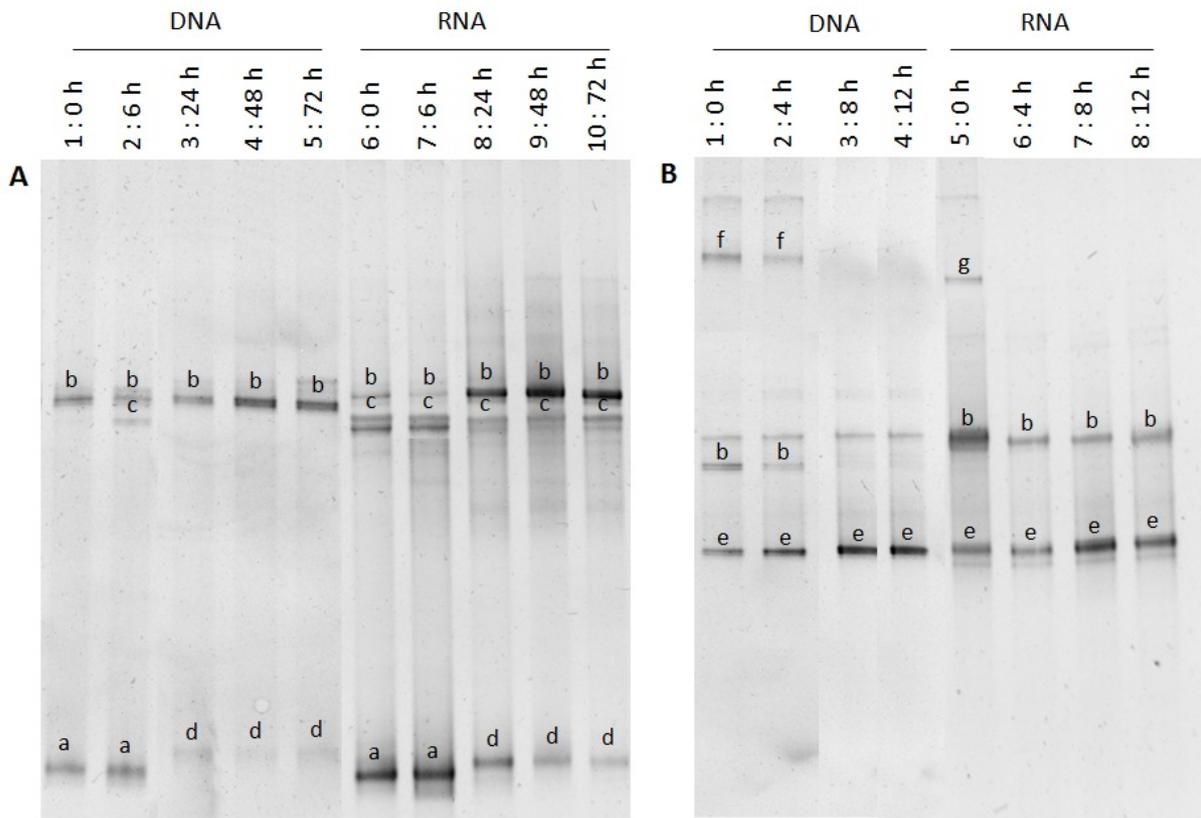
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625 **Figure 4**

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628 **Table 1.** pH measurements, yeast counts and identification of the isolated yeast during the  
 629 fermentation of mawè. Values of pH and CFU are mean  $\pm$  standard deviation for duplicate  
 630 analysis of two independent fermentations. Number of yeast isolates for each species,  
 631 percentage of isolations in brackets.

Mawè	Fermentation time (h)					Total
	0	6	24	48	72	
pH	5.14 $\pm$ 0.64	4.02 $\pm$ 0.24	3.61 $\pm$ 0.23	3.54 $\pm$ 0.18	3.44 $\pm$ 0.11	
Yeasts, log <sub>10</sub> CFU/g	2.93 $\pm$ 0.03	5.48 $\pm$ 0.02	5.63 $\pm$ 0.08	6.26 $\pm$ 0.56	5.64 $\pm$ 0.16	
Yeast population						
<i>Candida krusei</i>	5 (25)	2 (10)	7 (35)	8 (40)	7 (35)	29 (19.6)
<i>Candida glabrata</i>	5 (25)	6 (30)	7 (35)	6 (30)		22 (22.4)
<i>Saccharomyces cerevisiae</i>			1 (5)	5 (25)	11 (55)	17 (17.3)
<i>Kluyveromyces marxianus</i>	4 (20)		4 (20)	1 (5)	2 (10)	11 (11.2)
<i>Candida tropicalis</i>	1 (5)	7 (35)				8 (8.2)
<i>Clavispora lusitaniae</i>	2 (10)	1 (5)	1 (5)			4 (4.1)
<i>Wickerhamomyces anomalas</i>		3 (15)				3 (3.1)
<i>Pichia farinosa</i>	3 (15)					3 (3.1)
<i>Rhodotorula mucilaginosa</i>		1 (5)				1 (1)

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636 **Table 2.** pH measurements, yeast counts and identification of the isolated yeast during the  
 637 fermentation of tchoukoutou. Values of pH and CFU are mean  $\pm$  standard deviation for  
 638 duplicate analysis of two independent fermentations. Number of yeast isolates for each  
 639 species, percentage of isolations in brackets.  
 640

Tchoukoutou	Fermentation time (h)				
	0	4	8	12	Total
pH	3.98 $\pm$ 0.13	3.93 $\pm$ 0.05	3.80 $\pm$ 0.10	3.61 $\pm$ 0.11	
Yeasts, log <sub>10</sub> CFU/ml	4.97 $\pm$ 0.12	5.16 $\pm$ 0.00	5.62 $\pm$ 0.34	6.47 $\pm$ 0.07	
Yeast population					
<i>Saccharomyces cerevisiae</i>	1 (5)	19 (95)	17 (85)	14 (70)	51 (64.6)
<i>Clavispora lusitaniae</i>	8 (42)			5 (25)	13 (16.5)
<i>Candida krusei</i>	4 (21)	1 (5)	2 (10)		7 (8.9)
<i>Hanseniaspora guilliermondii</i>	2 (11)		1 (5)	1 (5)	4 (5.1)
<i>Debaryomyces nepalensis</i>	2 (11)				2 (2.5)
<i>Candida glabrata</i>	2 (11)				2 (2.5)

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