UNIVERSITÀ DEGLI STUDI DI TORINO

22

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

25

26 Anna Greppi[a], Kalliopi Rantisou[a], Wilfrid Padonou[b], Joseph Hounhouigan[b], Lene Jespersen[c],

27 Mogens Jakobsen[c], Luca Cocolin[a]*

28

29 [a]Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Via Leonardo

30 da Vinci 44, 10095, Grugliasco, Torino, Italy

31 [b]Département de Nutrition et Sciences Alimentaires, Faculté des Sciences Agronomiques,

32 Université d'Abomey-Calavi, 01 BP 526, Cotonou, Benin

33 [c]Department of Food Science, Food Microbiology, Faculty of Life Sciences, University of

34 Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark

35

36 *Author for correspondence: Via Leonardo da Vinci 44, 10095, Grugliasco, Torino. Phone

37 0039 011 670-8553, Fax 0039 011 670-8549, email: lucasimone.cocolin@unito.it

38

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.

1. Introduction

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

Mawè and tchoukoutou are two traditional cereal-based fermented foods from Benin, West Africa. Mawè is a dehulled fermented maize dough used to prepare many cooked dishes including gels (*akassa, agidi, eko*), steam-cooked bread (*ablo*) and porridge (*koko, aklui, akluiyonou*). The manufacturing processes have been described by Hounhouigan et al. (1993a). Tchoukoutou is the major opaque sorghum beer consumed in Benin. It has a sour taste, relatively high dry matter content (5-13 % w/v) and low alcohol content (2-3 % v/v), 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 $\log_{10}$ 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^{TM} 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$_2$, 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)$_5$ (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 ± 0.64 to 3.44 ± 0.11. At the

204 beginning of the spontaneous mawè fermentations samples exhibited a viable yeast count of

205 2.93 ± 0.03 $\log_{10}$ cfu/g, at the end of the fermentation (72 hours), the number of yeast

206 enumerated increased to 5.64 ± 0.16 $\log_{10}$ cfu/g. During the 12 hours of fermentation of

207 tchoukoutou, the pH decreased from 3.98 ± 0.16 to 3.61 ± 0.11 (Table 2). Yeast counts

208 increased from 4.97 ± 0.12 to 6.47 ± 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. guillermondii* (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

4. Discussion

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

Acknowledgments

396   **References**

397   Agu, R.C., Palmer, G.H., 1998. A reassessment of sorghum for lager-beer brewing.

398   Bioresource Technology 66, 253-261.

399   Altschul, S.F., Madden, T.L., Shaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman,

400   D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search

401   programs. Nucleic Acids Research 25, 3389–3402.

402   Annan, N. T., Poll, L., Sefa-Dedeh, S., Plahar, W. A., Jakobsen, M., 2003. Volatile

403   compounds produced by *Lactobacillus fermentum*, *Saccharomyces cerevisiae* and *Candida*

404   *krusei* in single starter culture fermentations of Ghanaian maize dough. Journal of Applied

405   Microbiology 94, 462-474.

406   Bonetta, S., Bonetta. S., Carraro, E., Rantsiou. K., Cocolin. L., 2008 Microbiological

407   characterisation of Robiola di Roccaverano cheese using PCR–DGGE. Food Microbiology 25

408   (6), 786–792.

409   Briggs, D.E., Boulton, C.A., Brookes, P.A., Stevens, R., 2004. Native African beers.

410   In Brewing: Science and practice (pp. 589-605). Woodhead publishing Ltd, Cambridge, UK.

411   Chang, H.W., Kim, K.H., Nam, H.D., Roh, S.W., Ki, M.-S., Jeon, C.-O., Oh, H.-M.,

412   Bae, J.-W., 2008. Analysis of yeast and archaeal population dynamics in kimchi using

413   denaturing gradient gel electrophoresis. International Journal of Food Microbiology 126, 159–

414   166.

415   Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in

416   wine fermentations. FEMS Microbiology Letters 189, 81–87.

417   Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel

418   electrophoresis analysis of the 16 S rRNA gene V1 region to monitor dynamic changes in the

419   bacterial population during fermentation of Italian sausages. Applied and Environmental

420   Microbiology 67, 5113–5121.

421   Cocolin, L., Aggio, D., Manzano, M., Cantoni, C., Comi, G., 2002. An application of
422   PCR-DGGE analysis to profile the yeast populations in raw milk. International Dairy Journal
423   12, 407–411.

424   Cocolin, L., Dolci, P., Rantsiou, K., 2011. Biodiversity and dynamics of meat
425   fermentations: The contribution of molecular methods for a better comprehension of a
426   complex ecosystem. Meat Science 89, 296–302

427   Czerucka, D., Dahan, S., Mograbi, B., Rossi, B., Rampal, P. 2000. *Saccharomyces*
428   *boulardii* preserves the barrier function and modulates the signal transduction pathway
429   induced in enteropathogenic *Escherichia coli*-infected T84 cells. Infection and Immunity 68,
430   5998-6004.

431   Demuyakor, B., Ohta, Y., 1991. Characteristics of pito yeasts from Ghana. Food
432   Microbiology 8, 183–193.

433   Di Maro, E., Ercolini, D., Coppola, S., 2007. Yeast dynamics during spontaneous
434   wine fermentation of the Catalanesca grape. International Journal of Food Microbiology 117,
435   201–210.

436   Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of
437   microbes in food. Journal of Microbiological Methods 56, 297–314.

438   Fidel, P.L.J.R., Vazquez, J.A., Sobel, J.D., 1999. *Candida glabrata*: Review of
439   Epidemiology, Pathogenesis and Clinical Disease with Comparison to *C*. *albicans*. Clinical
440   Microbiology Reviews, 80-96.

441   Gadaga, T.H., Mutukumira A.N., Narvhus J.A., 2000. Enumeration and identification
442   of yeasts isolated from Zimbabwean traditional fermented milk. International Diary Journal
443   10, 459-466.

444   Gedek, B.R. 1999. Adherence of *Escherichia coli* serogroup 0 157 and the *Salmonella*

445 Typhimurium mutant DT 104 to the surface of *Saccharomyces boulardii*. Mycoses 42, 261-

446 264.

447   Glover, R.L.K., Abaidoo, R.C., Jakobsen, M., Jespersen, L., 2005. Biodiversity of

448 Saccharomyces cerevisiae isolated from a survey of pito production sites in various parts of

449 Ghana. Systematic and Applied Microbiology 28, 755–761.

450   Greppi, A., Rantsiou, K., Padonou, W., Hounhouigan, J., Jespersen, L., Jakobsen, M.,

451 Cocolin, L., Determination of yeast diversity in ogi, mawè, gowé and tchoukoutou, four

452 traditional fermented products from Benin, using culture-dependent and -independent

453 methods. Submitted.

454   Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M., Zelder, O., 2005.

455 Biotechnological production and applications of phytases. Applied Microbiology and

456 Biotechnology *68*, 588-597.

457   Halm, M., Hornbaek, T., Arneborg, N., Sefa-Dedeh, S., Jespersen, L., 2004. Lactic

458 acid tolerance determined by measurment of intracellular pH of single cells of *Candida krusei*

459 and *Saccharomyces cerevisiae* isolated from fermented maize dough. International Journal of

460 Food Microbiology 94, 97-103.

461   Head, I.M., Saunders, J.R. and Pickup, R.W., 1998. Microbial evolution, diversity,

462 and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial

463 Ecology 35, 1-21.

464   Hjortmo, S., Patring, J., Jastrebova, J., Andlid, T., 2005. Inherent biodiversity of folate

465 content and composition in yeasts. Trends Food Science and Technology 16, 311-316.

466   Hounhouigan, D.J., Nout, M. J. R., Nago, C.M., Houben, J.H.M., Rombouts, F.M.,

467 1993a. Changes in the Physico-chemical Properties of Maize During Natural Fermentation of

468 Mawè. Journal of Cereal Science 17, 291-300.

469 Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H. and Rombouts, F.M.

470 1993b. Characterization and frequency distribution of species of lactic acid bacteria involved

471 in the processing of mawe`, a fermented maize dough from Benin. International Journal of

472 Food Microbiology 18, 279–287.

473 Hounhouigan, D.J., Nout, M.J.R, Nago, C.M., Houben, J.H. and Rombouts, F.M ,

474 1993c. Composition and microbiological and physical attributes of mawè, a fermented maize

475 dough from Benin. International Journal of Food Science and Technology 28, 513-517.

476 Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombouts, F.M. 1993d

477 Microbiological changes in mawè during natural fermentation. World Journal of

478 Microbiology and Biotechnology 10, 410-413.

479 Jespersen L., Halm M., Kpodo K., Jakobsen M., 1994. Significanze of yeasts and

480 moulds occurring in maize dough fermentation for "kenkey" production. International Journal

481 of Food Microbiology 24, 239-248.

482 Jespersen, L., 2003. Occurrence and taxonomic characterisation of strains of

483 *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages.

484 FEMS Yeast Research 3, 191–200.

485 Jespersen, L., Nielsen, D.S., Honholt, S., Jakobsen, M., 2005. Occurrence and

486 diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast

487 Research 5, 441-453.

488 Kayodé, A.P.P., Hounhouigan, D.J., Nout, M.J.R., Niehof, A., 2006. Household

489 production of sorghum beer in Benin: technological and socio-economic aspects. International

490 Journal of Consumer Studies, 1470-6431.

491 Konlani, S., Delgenes, J.P., Moletta, R., Traore, A., Doh, A., 1996. Isolation and

492 physiological characterization of yeasts involved in sorghum beer production. Food

493 Biotechnology 10, 29–40.

494   Kurtzman, C.P., Robnett, C.J., 1998. Identification and phylogeny of ascomycetous

495   yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences.

496   Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 73,

497   331–371.

498   Liu, H-J., Liu, D-H, Zhong, J-J, 2005. Interesting physiological response of the

499   osmophilic yeast *Candida krusei* to heat schock. Enzyme and Microbial Technology 36, 409-

500   416.

501   Maoura, N., Mbaiguinam, M., Nguyen, H.V., Gaillardin, C., Pourquie, J., 2005.

502   Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer

503   of Tchad. African Journal of Biotechnology 4, 646–656.

504   Masoud, W., Takamiya, M., Vogensen, F.K., Lillevang, S., Al-Soud, W.A., Sørensen,

505   S.J., Jakobsen, M., 2011. Characterization of bacterial populations in Danish raw milk

506   cheeses made with different starter cultures by denaturating gradient gel electrophoresis and

507   pyrosequencing. International Dairy Journal 21, 142-148.

508   Moslehi-Jenabian, S., Pedersen, L.L., Jespersen, L., 2010. Beneficial Effects of

509   Probiotic and Food Borne Yeasts on Human Health. Nutrients 2, 449-473.

510   Moss, M.O., Long, M.T. 2002. Fate of patulin in the presence of the yeast

511   *Saccharomyces cerevisiae*. Food Additives and Contaminants 19, 387-399.

512   Mumy, K.L., Chen, X.H., Kelly, C.P., McCormick, B.A., 2008. *Saccharomyces*

513   *boulardii* interferes with *Shigella* pathogenesis by post invasion signaling events. American

514   Journal Gastrointestinal Liver Physiology 294, G599-G609.

515   N' guessan, K.F., Brou, K., Jacques, N., Casaregola, S., Dje, K.M., 2011.

516   Identification of yeasts during alcoholic fermentation of *tchapalo*, a traditional sorghum beer

517   from Côte d'Ivoire. Antonie Van Leeuwenhoek. 99, 855-864.

518   Naumova, E.S., Korshunova, I.V., Jespersen, L., MNaumov, G.I., 2003. Molecular

519 genetic identification of *Saccharomyces* sensu stricto strains from African sorghum beer.

520 FEMS Yeast Research 3, 177-185

521   Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel,

522 W.H., 2007. The microbiology of Ghanaian cocoa fermentations analyzed using culture-

523 dependent and culture-independent methods. International Journal of Food Microbiology 114,

524 168–186.

525   Odds, F. C. 1988. Ecology and epidemiology of candidiasis, p. 89. In F. C. Odds (ed.)

526 Candida and candidosis. A review and bibliography, 2nd ed. Balliere Tindall, London, United

527 Kingdom.

528   Oguntoyinbo, F. A., 2008. Evaluation of diversity of *Candida* species isolated from

529 fermented cassava during traditional small scale *gari* production in Nigeria. Food Control 19,

530 465-469.

531   Omemu, A.M., Oyewole, O.B., Bankole, M.O., 2007. Significance of yeasts in the

532 fermentation of maize for Ogi production. Food Microbiology 24, 571–576.

533   Oyewole, O.B., 2001. Characteristics and significance of yeasts' involvement in

534 cassava fermentation for "fufu" production. International Journal of Food Microbiology 65,

535 213-218.

536   Pedersen, L.L., Owusu-Kwarteng, J., Thorsen, L. and Jespersen, L., 2012.

537 Biodiversity and probiotic potential of yeasts isolated from *Fura*, a West African

538 spontaneously fermented cereal. International Journal of Food Microbiology 159, 144-151.

539   Prakitchaiwattana, C.J., Fleet, G.H., Heard, G.M., 2004. Application and evaluation of

540 denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS

541 Yeast Research 4, 865–877.

542   Ramos, C.L., Gonzaga de Almeida, E., Vinícius de Melo Pereira, G., Gomes Cardoso,

543   P., Souza Dias, E., Freitas Schwan, E., 2010. Determination of dynamic characteristics of

544   microbiota in a fermented beverage produced by Brazilian Amerindians using culture-

545   dependent and culture-independent methods. International Journal of Food Microbiology 140,

546   225–231.

547   Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L.,

548   2005. Culture-dependent and -independent methods to investigate the microbial ecology of

549   Italian fermented sausages. Applied and Environmental Microbiology 71, 1977–1986.

550   Rantsiou, K., Greppi, A., Garosi, M., Acquadro, A., Mataragas, M., Cocolin, L., 2012.

551   Strain dependent expression of stress response and virulence genes of *Listeria monocytogenes*

552   in meat juices as determined by micro-array. International Journal of Food Microbiology 152,

553   116-122.

554   Romano, P., Suzzi, G., Domizio, P., Fatichenti, F., 1997. Secondary products

555   formation as a tool for discriminating non-*Saccharomyces* wine strains. Antonie van

556   Leeuwenhoek 71, 239–242.

557   Sanni, A.I., Lonner, C., 1993. Identification of yeasts isolated from Nigerian

558   traditional alcoholic beverages. Food Microbiology 10, 517–523.

559   Sefa-Deheh, S., Sanni, A.I., Tetteh, G. and Sakyi-Dawson, E. 1999. Yeasts in the

560   traditional brewing of pito in Ghana. World Journal of Microbiology and Biotechnology 15,

561   593-597.

562   Shetty, P.H. and Jespersen, L., 2006. *Saccharomyces cerevisiae* and lactic acid

563   bacteria as potential mycotoxin decontaminating agents. Trends in Food Science and

564   Technology 17, 48-55.

565   Shetty, P.H., Hald, B., Jespersen, L., 2007. Surface binding of aflatoxin B_1 by

566   *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous

567   fermented foods. International Journal of Food Microbiology 113, 41-46.

568   Silvestri, G., Santarelli, S., Aquilanti, L., Beccaceci, A., Osimani, A., Tonucci, F.,

569   Clementi, F., 2007. Investigation of the microbial ecology of Ciasculo, a traditional Italian

570   Salami, by culture-dependent techniques and PCR-DGGE. Meat Science 77, 413-423.

571   Stringini, M., Comitini, F., Taccari, M., Ciani M., 2008. Yeast diversity in crop-

572   growing environments in Cameroon. International Journal of Food Microbiology 127, 184-

573   189.

574   Urso, R., Rantsiou, K., Dolci, P., Rolle, L., Comi, G., Cocolin, L., 2008. Yeast

575   biodiversity and dynamics during sweet wine production as determined by molecular

576   methods. FEMS Yeast Resource 8, 1053–1062.

577   Van der Aa Kuhle, A., Jespersen, L., Glover, R.L.K., Diawara, B. and Jakobsen, M.,

578   2001. Identification and characterization of *Saccharomyces cerevisiae* isolated from West

579   African sorghum beer. Yeast 18, 1069–1079.

580   Vauterin, L., Vauterin, P., 1992. Computer-aided objective comparison of

581   electrophoretic patterns for grouping and identification of microorganisms. European Journal

582   of Clinical Microbiology 33, 633–641.

583   Watanabe, I., Nakamura, T., Shima, J., 2010. Strategy for simultaneous

584   saccharification and fermentation using a respiratory-deficient mutant of *Candida glabrata*

585   for bioethanol production. Journal of Bioscience and Bioengineering 110, 176-179.

586

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*, anomorph of *Candida krusei* (100%, JQ585732); band e

609   *Saccharomyces cerevisiae* (100%, JF427814); band f *Hanseniaspora uvarum* (100%,

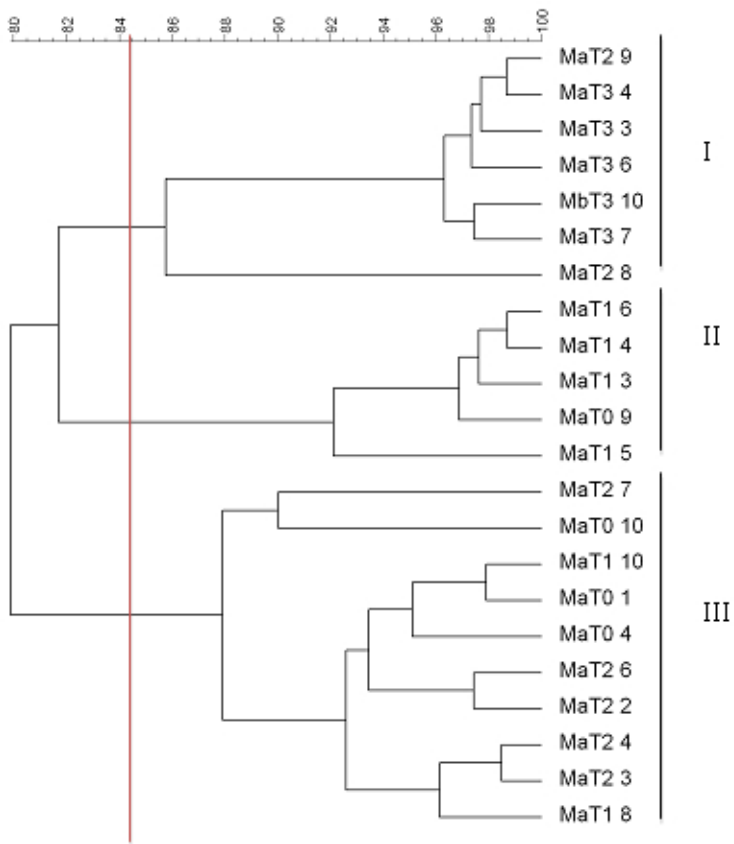610   EU386753); band g *Hanseniaspora guillermondii* (100%, JQ707775).
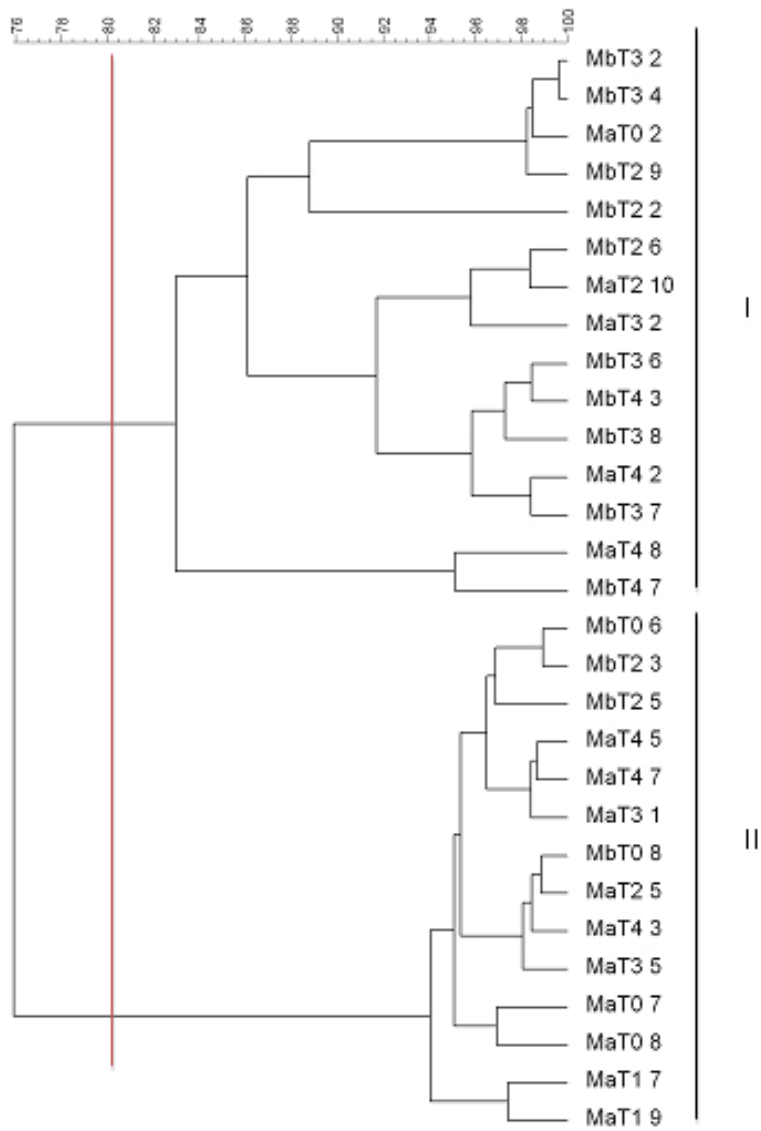
611

612

613

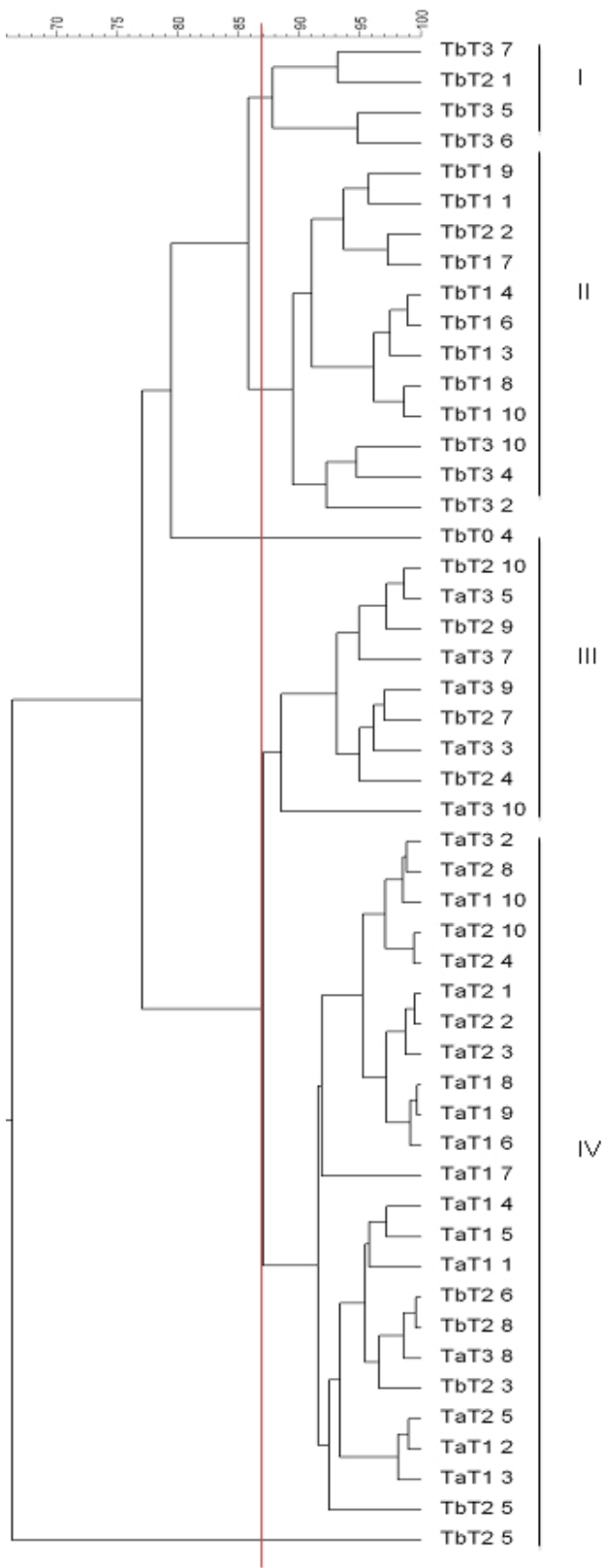614   **Figure 1**

615

616

617

618

619   **Figure 2**

620

621

622

623 **Figure 3**
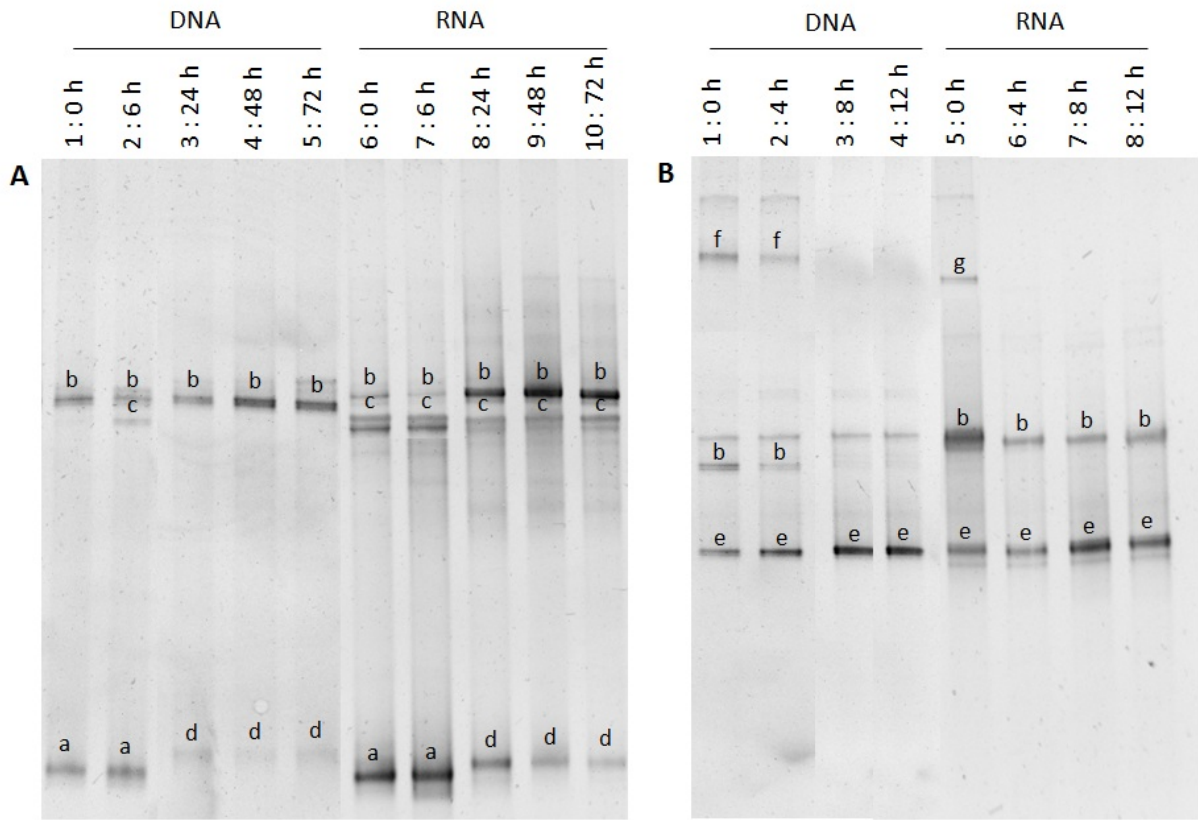

624

625 **Figure 4**

626

627

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

632
633
634
635

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 ± 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 ± 0.13	3.93 ± 0.05	3.80 ± 0.10	3.61 ± 0.11	
Yeasts, $\log_{10}$ CFU/ml	4.97 ± 0.12	5.16 ± 0.00	5.62 ± 0.34	6.47 ± 0.07	
Yeast population					
Saccharomyces cerevisiae	1 (5)	19 (95)	17 (85)	14 (70)	51 (64.6)
Clavispora lusitaniae	8 (42)			5 (25)	13 (16.5)
Candida krusei	4 (21)	1 (5)	2 (10)		7 (8.9)
Hanseniaspora guilliermondii	2 (11)		1 (5)	1 (5)	4 (5.1)
Debaryomyces nepalensis	2 (11)				2 (2.5)
Candida glabrata	2 (11)				2 (2.5)

641

642