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Running title: Yeast dynamics during African food fermentation

Abstract

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

Keywords: yeasts, fermented foods, culture-dependent and -independent analysis, microbial 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

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

2.3. Direct extraction of nucleic acids from the samples

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

2.4. PCR and RT-PCR

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

The reverse transcription (RT) reactions were performed using the M-MLV reverse transcriptase (Promega, Milan, Italy). Two hundred microgram of RNA were mixed with 1 µl of primer LS2 (100 µM) and sterile water to a final volume of 10 µL and incubated at 70°C for 5 min. The mix was placed on ice and a mixture containing 50 mM Tris-HCl (pH 8.3), 75 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 units of Rnasin ribonuclease inhibitor (Ambion) was transferred to the reaction tube. The reverse transcription was carried out at 42°C for 1 h. One µl of cDNA was amplified using the 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

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

3. Results

3.1 Microbiological analysis of mawè and tchoukoutou

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

3.2 Identification of isolates and species succession during fermentation

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

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

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

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

3.3 Rep-PCR typing

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

For the tchoukoutou fermentation, 50 isolates of *S. cerevisiae* were grouped by rep-PCR (Figure 3). *S. cerevisiae* was the predominant species isolated during this fermentation. Using a coefficient of similarity of 87%, a differentiation of the isolates based on the fermentation time was observed. As shown in Fig. 3, cluster I and II contained isolates from throughout the 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).

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

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

Differences in yeast species in comparison to other African sorghum beers ecosystem studied, could contribute to the particular characteristic of tchoukoutou. Local differences in the production process (Van der Aa Kuhle et al., 2001; Jespersen, 2003) and different types of ingredients and of sorghum cultivars utilized can be a cause of the variation in the yeast biota as they have different biochemical characteristics, which influence substrates available for the yeast (Demuyakor and Ohta, 1991). The results from the characterization of the *S. cerevisiae* isolates demonstrated a succession of biotypes during the fermentation of tchoukoutou, despite the relatively short time of fermentation. Some appeared to be involved only in the early stages of fermentation followed by others that appeared after 8 hours until the end. Other biotypes were distributed homogeneously throughout the fermentation of tchoukoutou. The data obtained confirmed previous findings concerning the diversity of *S. cerevisiae* biotypes conducting the fermentation (Van der Aa Kuhle et al., 2001; Naumova et al. 2003; Glover et al, 2005). The occurrence and taxonomic characteristics of *S. cerevisiae* biotypes in African indigenous fermented foods and beverages have been reviewed by Jespersen (2003).

The results obtained by the direct analysis on the fermentation of tchoukoutou revealed some yeast species not detected by the culture-based approach. This was the case for *H. uvarum* and *K. marxianus* at DNA level and of *H. guillermondii* and *K. marxianus* at RNA level. In case of DNA, these species may be present in the habitat as viable but not-cultivable cells, because of the cultivation conditions or their physiological state (Head et al. 1998; Ercolini, 2004) or they may be dead. *S. cerevisiae* were clearly detected during the whole fermentation of 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.

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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.
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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).

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Figure 1

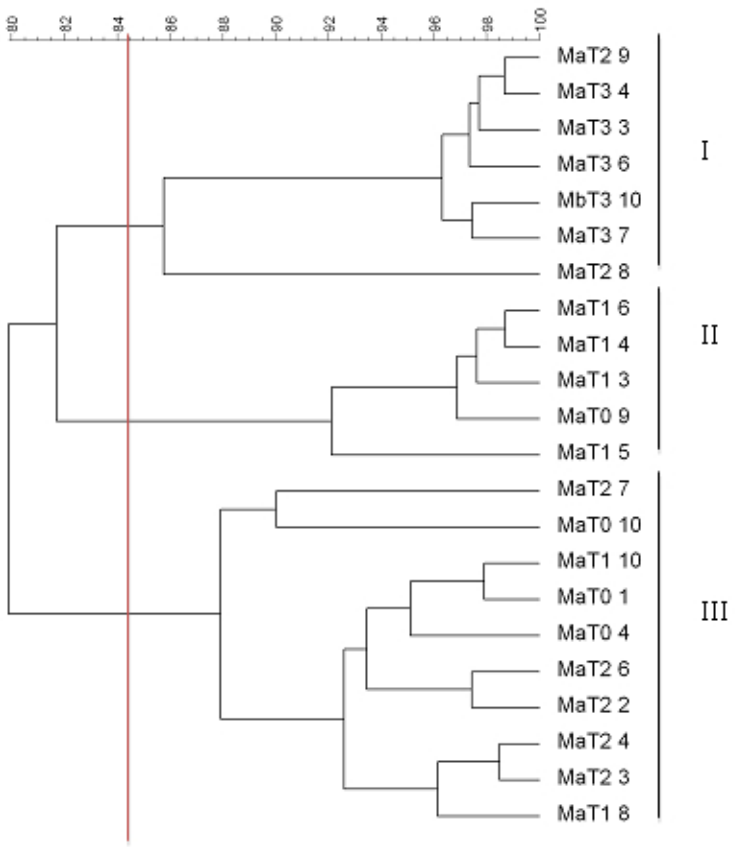
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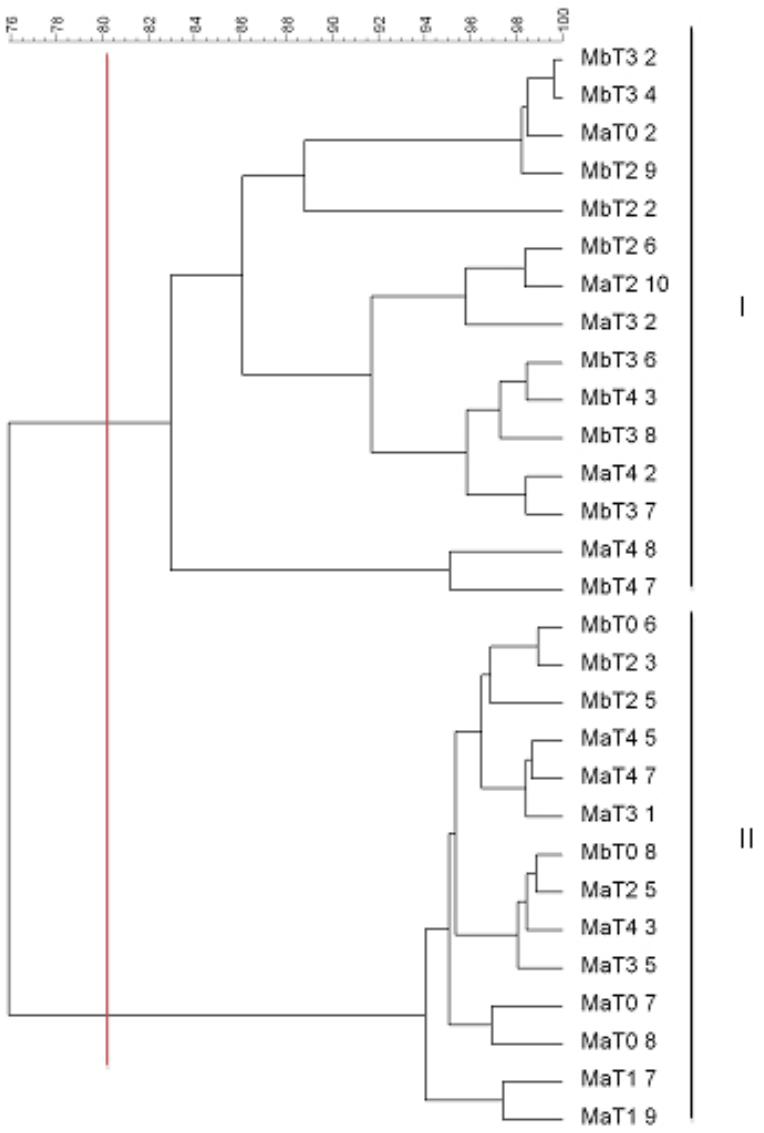
Figure 2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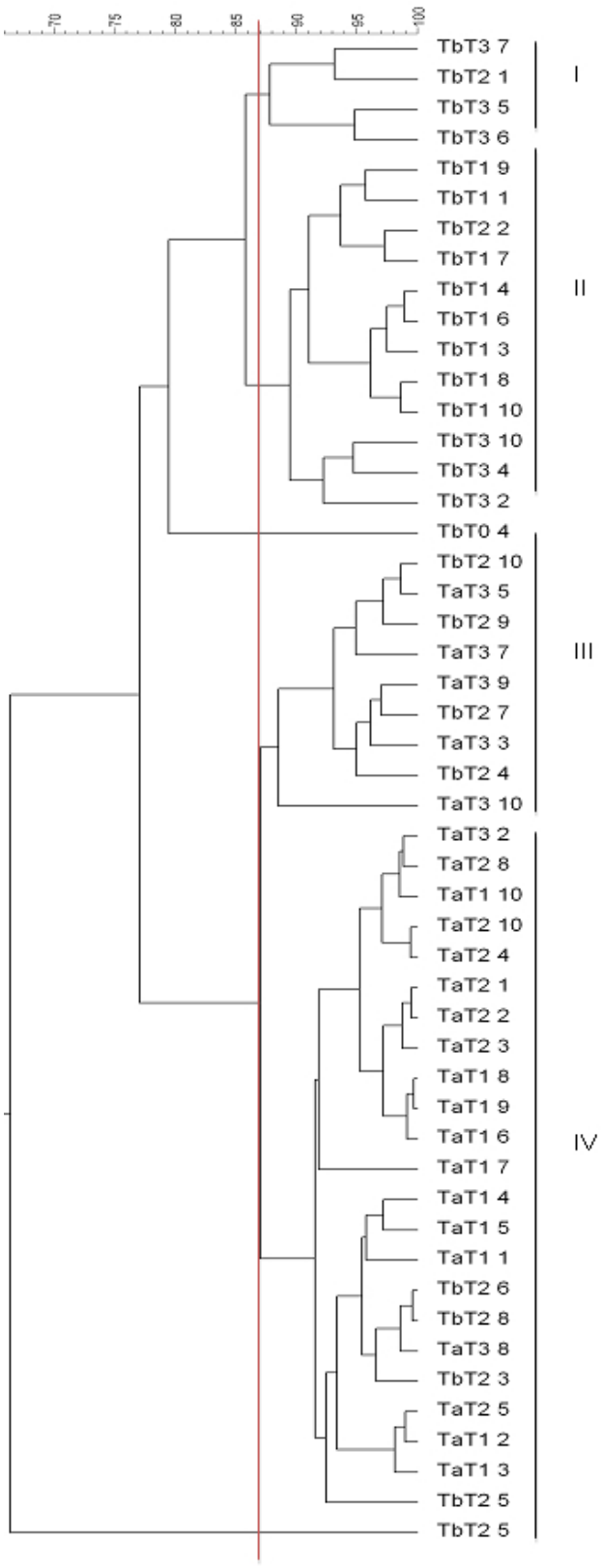
623 **Figure 3**



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625 **Figure 4**

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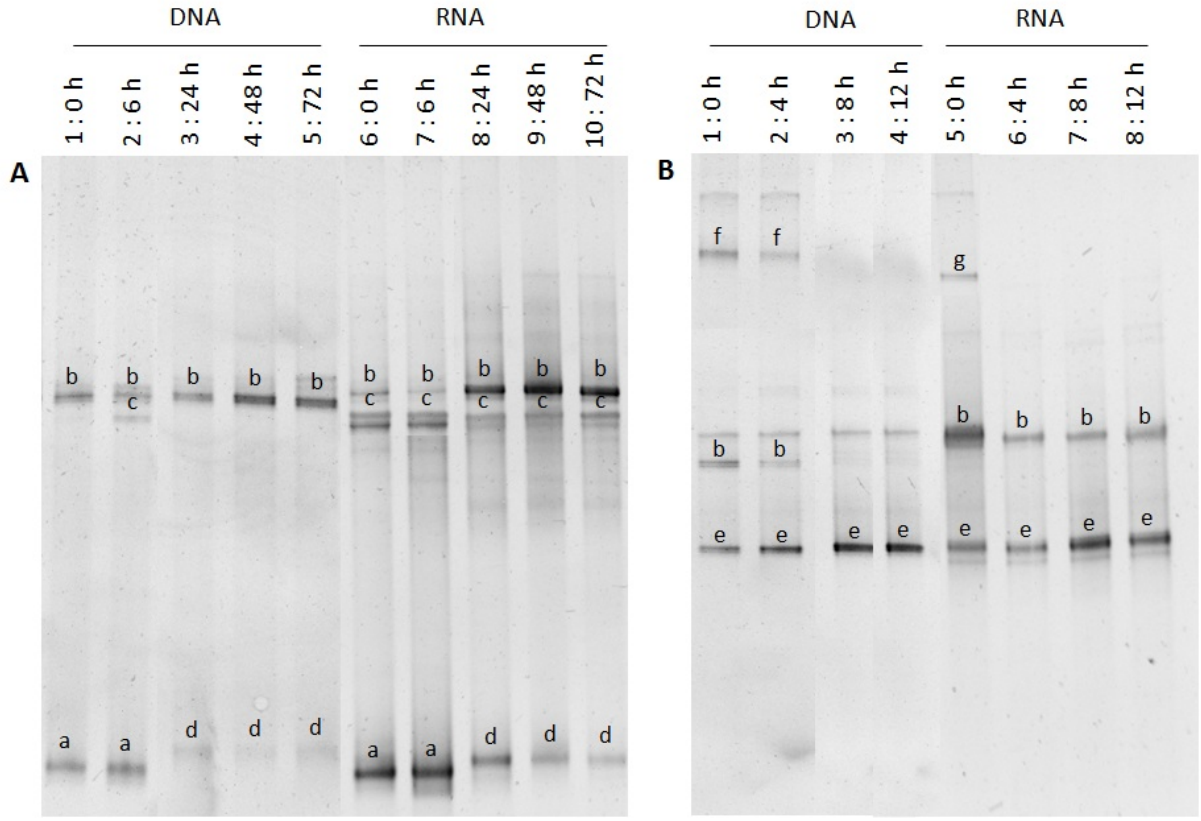

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628 **Table 1.** pH measurements, yeast counts and identification of the isolated yeast during the
629 fermentation of mawè. Values of pH and CFU are mean ± standard deviation for duplicate
630 analysis of two independent fermentations. Number of yeast 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)

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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 ± 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)

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