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# UNIVERSITÀ DEGLI STUDI DI TORINO

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

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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*, *akluyonou*). 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,

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

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

## **2. Materials and methods**

## *2.1 Sample collection and microbiological analysis*

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

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

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

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

## *2.2. DNA extraction from pure cultures*

Yeast DNA of each isolate was extracted from 1 ml of 24 h MYPG pure culture and centrifuged at  $14,000 \times g$  for 10 min at 4°C. The pellet of yeast cells was subjected to DNA 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™ 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<sub>2</sub>, 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.



## 2.5 DGGE analysis

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

## 2.6 Sequencing of DGGE bands and sequence analysis

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

## 2.7 Identification of the isolates by PCR-DGGE

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

## 2.8 Typing of the isolates by rep-PCR

The predominant yeast species identified during the fermentations studied were subjected to rep-PCR analysis using primer (GTG)<sub>5</sub> (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  $5.14 \pm 0.64$  to  $3.44 \pm 0.11$ . At the beginning of the spontaneous mawè fermentations samples exhibited a viable yeast count of  $2.93 \pm 0.03 \log_{10} \text{ cfu/g}$ , at the end of the fermentation (72 hours), the number of yeast enumerated increased to  $5.64 \pm 0.16 \log_{10} \text{ cfu/g}$ . During the 12 hours of fermentation of tchoukoutou, the pH decreased from  $3.98 \pm 0.16$  to  $3.61 \pm 0.11$  (Table 2). Yeast counts increased from  $4.97 \pm 0.12$  to  $6.47 \pm 0.07 \log_{10} \text{ cfu/ml}$ . (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 guillermoidii*, *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

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

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

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

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

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

266

#### 267 4. Discussion

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

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

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

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

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

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



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

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

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

391

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## Figure legends

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

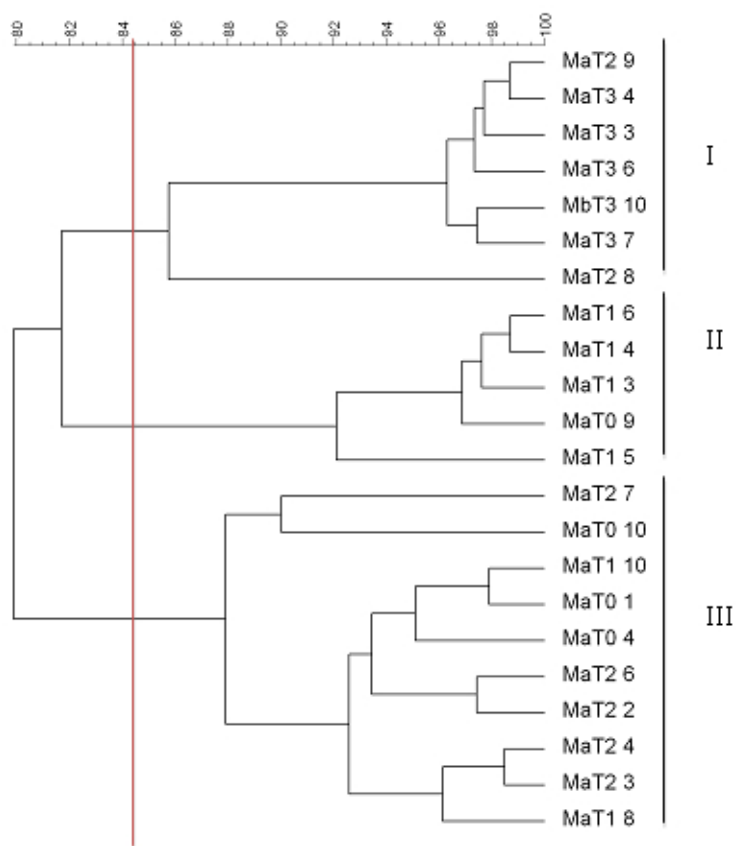
**Figure 2.** Cluster analysis of the rep-PCR fingerprints of *Candida krusei* strains isolated during the fermentation of mawè. The first letter represents the sample, the second represents the replicate (a-b), the number represents the fermentation times [T0, T1 (6 h), T2 (24 h), T3 (48 h), T4 (72h)]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

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

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

614 **Figure 1**

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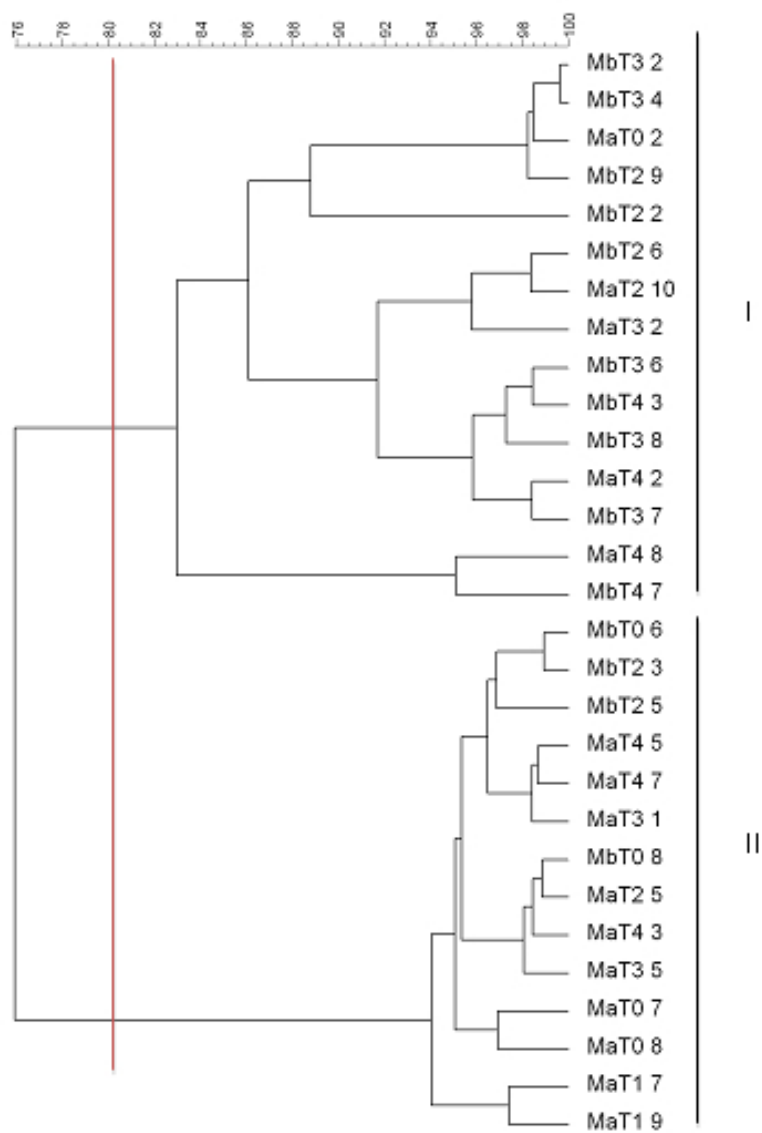


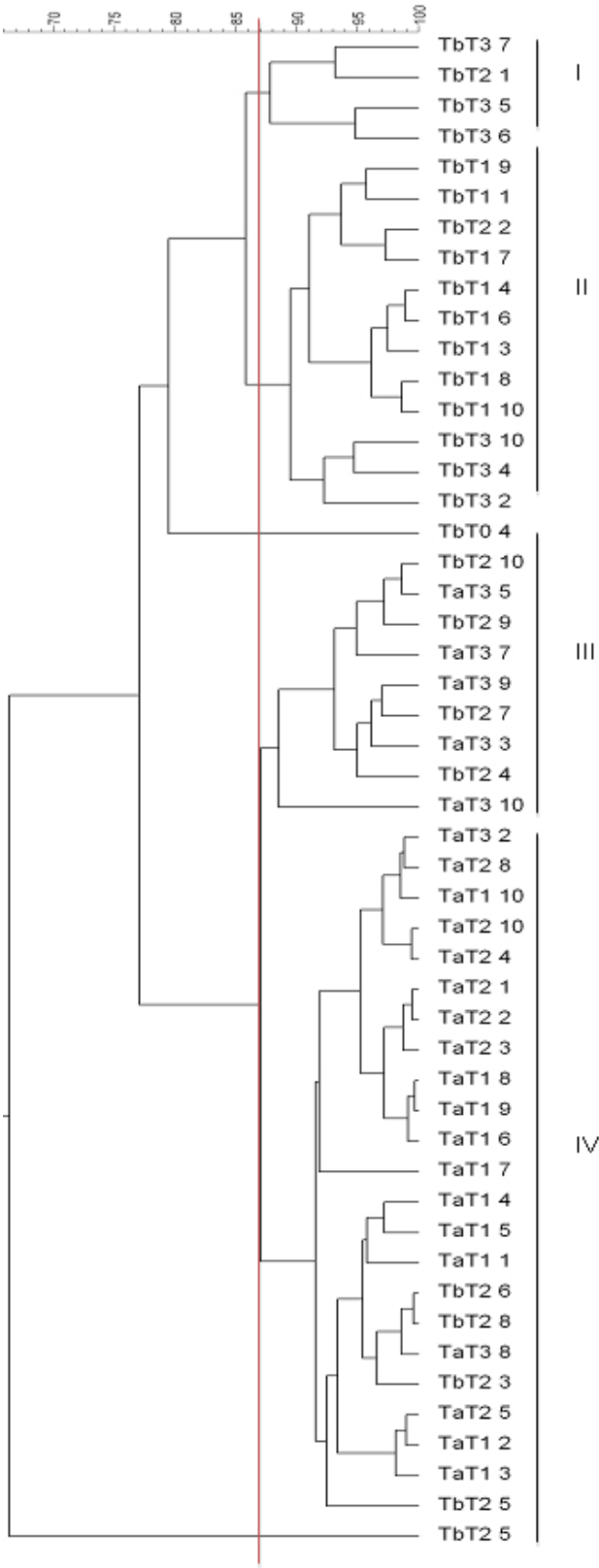
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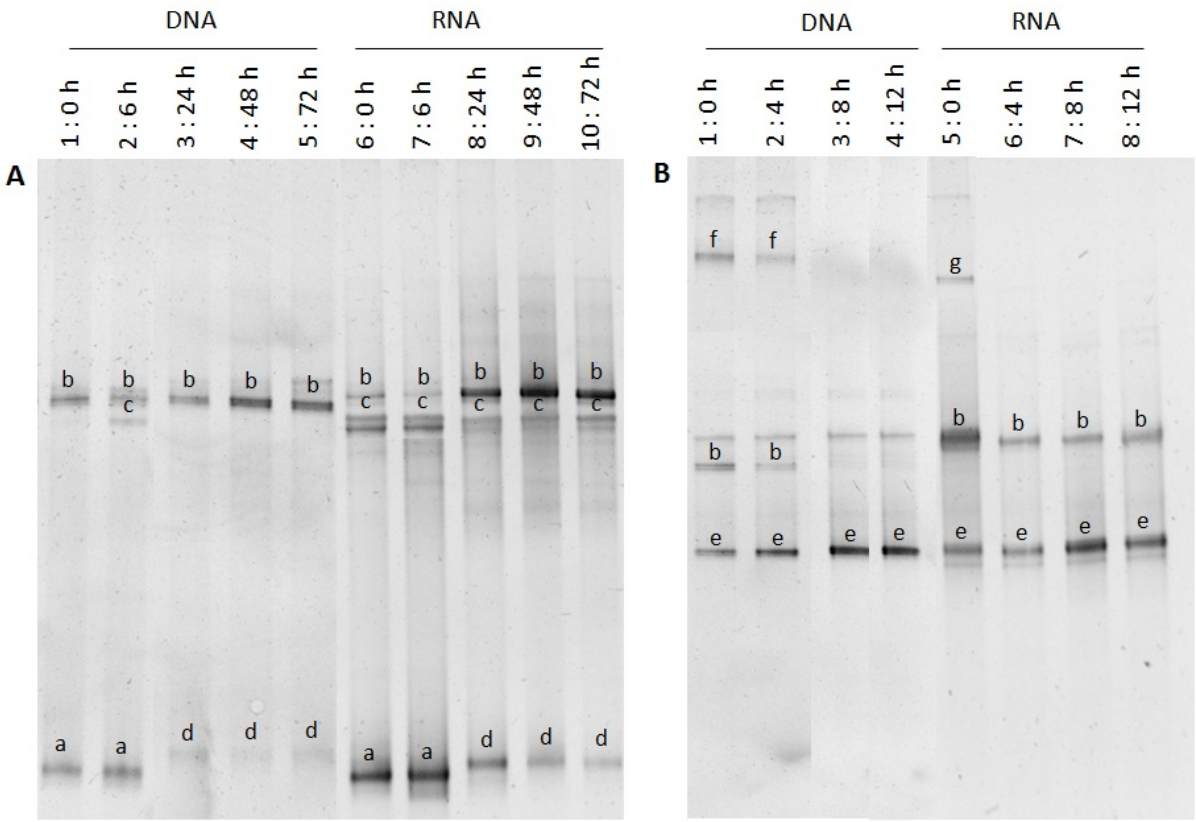
Figure 2





625 **Figure 4**

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

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



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

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