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

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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 the collection of 177 isolates. They were identified by the PCR-DGGE technique followed by the 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 the 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 levels in the case of mawè did not reveal any other species except those already identified by culture-based analysis. On the other hand, for tchoukoutou, four species were identified that were not detected by the culture-based approach. The spontaneous fermentation of mawè and tchoukoutou in the end were dominated by a few autochthonous species.

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 and Long, 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 yeast dynamics is important. Furthermore, 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

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brief, the manufacturing process consists of the malting of red sorghum, milling, brewing and fermentation. For these traditional fermentations, Hounhouigan et al. (1993b, 1993c) and Kayodé et al. (2006) reported that lactic acid bacteria (LAB) and yeasts are the predominant microorganisms leading to a two step 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 yeast 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, 2002; Chang et al., 2008; Stringini et al. 2008) and wine (Prakitchaiwattana et al., 2004; Rantsiou et al., 2005; Di Maro et al., 2007; Urso et al. 2008). The DGGE technique combined with cultural methods has recently been applied to study the yeast ecology of mawè and tchoukoutou final products from Benin (Greppi et al., 2013).

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 populations 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 from two different local producers located at the university campus of Abomey-Calavi and at the Abomey-Calavi local market, respectively were followed.

Samples of mawè were taken aseptically using sterile stomacher bags (Seward, West Sussex, UK) at 0, 6, 24, 48 and 72 h. 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 min after sampling. Samples of tchoukoutou were collected at 0, 4, 8 and 12 h. 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) grams of mawè and 10 ml of tchoukoutou samples were diluted, homogenized and yeast enumerated on MYPG agar as previously described (Greppi et al., 2013). 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 ×*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) grams of mawè and 10 ml of tchoukoutou samples were separately homogenized with 40 ml of Ringer solution in a Stomacher for 30 s 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 microliter of the yeast DNA (100 ng) was used for the PCR assays as previously described (Greppi et al., 2013). 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 micrograms 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 microliter 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 analyze 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., 2013).

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 $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) according to Nielsen et al. (2007). The rep-PCR was performed as previously described (Greppi et al., 2013). Amplicons were separated by 1.5% agarose gel electrophoresis in 1× TBE (150 min, 120 V) using a Generuler 1 kb DNA ladder as reference (Promega). The rep-PCR profiles were normalized and cluster analysis was 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 and Vauterin, 1992).

3. Results

3.1. Microbiological analysis of mawè and tchoukoutou

The pH and yeast counts, reported in Tables 1 and 2, are expressed as means and standard deviations for the two different fermentations. As seen in Table 1, during the 72 h 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, and at the end of the fermentation (72 h), the number of yeast enumerated increased to 5.64 ± 0.16 $\log_{10}$ cfu/g. During the 12 h 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 h *Candida glabrata* and *Candida krusei* dominated with *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Clavispora lusitaniae* being present as well. At 72 h 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 h 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 h, *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 (Fig. 1). Cluster I grouped isolates were mainly found at 48 h (T3) while isolates of cluster II were mainly detected at 6 h of fermentation (T1). The third cluster (III) contained isolates detected at the beginning (T0), after 6 h (T1) and 24 h (T2). Neither cluster II nor cluster III contained isolates at 48 h (T3). The analysis of the fingerprints of the *C. krusei* isolates (29), at a similarity coefficient of 80%, resulted in 2 main clusters (Fig. 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 (Fig. 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 h) while cluster IV from isolates at T1 and T2 (4 and 8 h). Both cluster I and III did not have any isolate from T1 (6 h). 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 levels

DGGE fingerprints obtained from the total DNA and RNA extracted directly from mawè and tchoukoutou samples are shown in Fig. 4 (panels 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 h and band d, corresponding to the closest relative *Pichia kudriavzevii* (formerly named as *I. orientalis*, anamorph *C. krusei*) was present from 12 h 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 h to the end. *Zea Mays* (band a) was also occasionally detected both at DNA and RNA levels.

Table 1
pH measurements, yeast counts and identification of the isolated yeast during the fermentation of mawè. Values of pH and CFU are mean ± 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)					
	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)

Table 2
pH measurements, yeast counts and identification of the isolated yeast during the fermentation of tchoukoutou. Values of pH and CFU are mean ± 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 ± 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 guillermondii	2 (11)		1 (5)	1 (5)	4 (5.1)
Debaryomyces nepalensis	2 (11)				2 (2.5)
Candida glabrata	2 (11)				2 (2.5)

In the DGGE profiles obtained from tchoukoutou matrix (panel B), at both DNA and RNA levels, the 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 h of fermentation. On the other hand, at RNA level *K. marxianus* (band b) was always present while *H. guillermondii* (band g) was detected 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).

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 carry out the transformation process, and more specifically to certain biotypes within a species, understanding their dynamics is important.

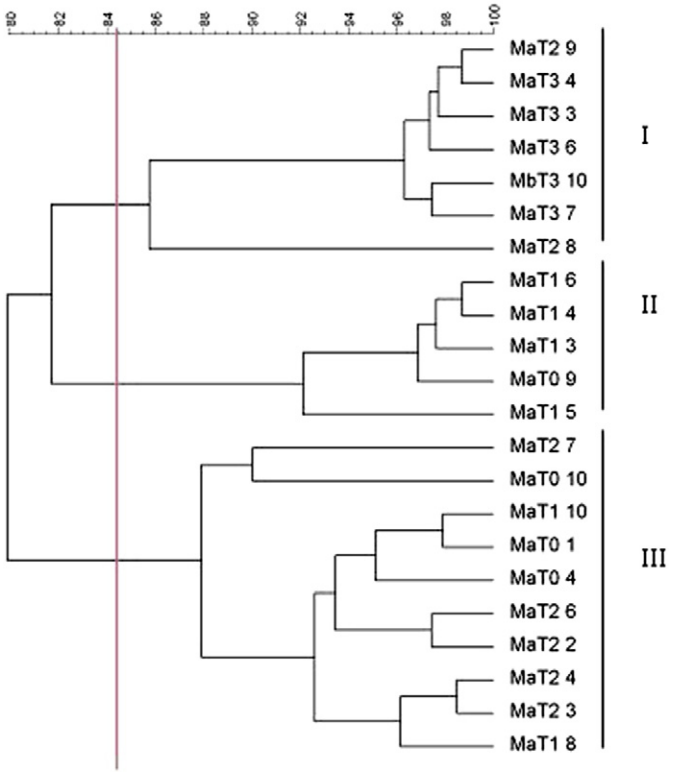

Fig. 1. Cluster analysis of the rep-PCR fingerprints of *Candida glabrata* strains isolated during the fermentation of mawè. The first letter represents the sample, the second represents the replicate (a–b), the number represents the fermentation times [T0, T1 (6 h), T2 (24 h), T3 (48 h)] and the progressive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

For mawè, no studies seem to have been carried out on identification of yeast 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 48 h, while the pH was still decreasing during the fermentation of mawè. Six species were detected at the beginning and after 6 h of mawè fermentation while from 24 h 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 yeast 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, 1988) 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, 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 increasingly 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 that mainly present at the first 6 h 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., 2013) 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

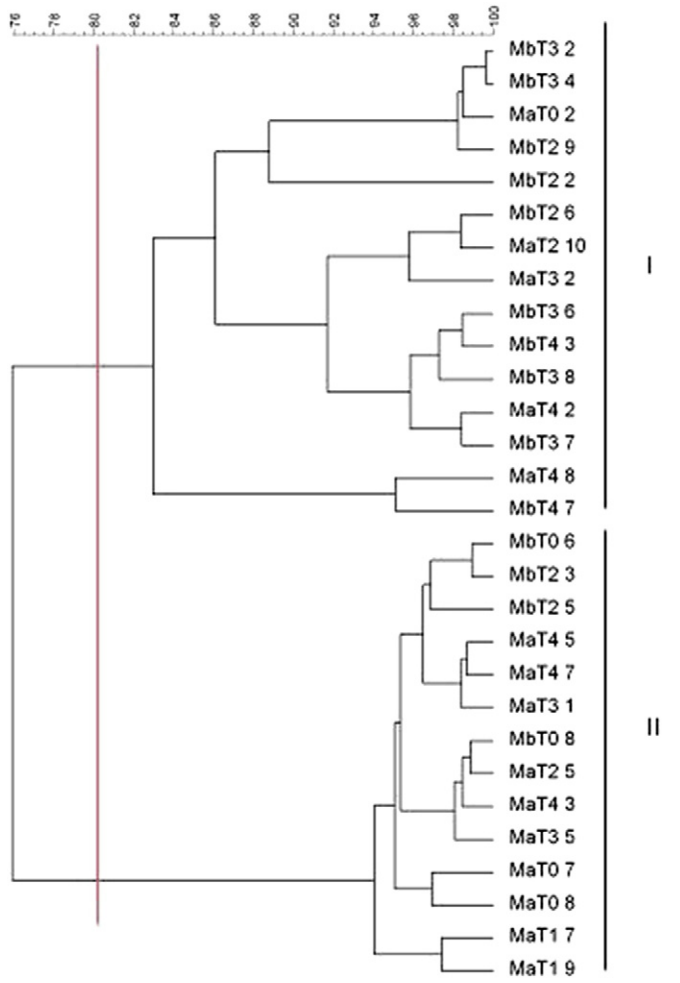

Fig. 2. Cluster analysis of the rep-PCR fingerprints of *Candida krusei* strains isolated during the fermentation of mawè. The first letter represents the sample, the second represents the replicate (a–b), the number represents the fermentation times [T0, T1 (6 h), T2 (24 h), T3 (48 h, T4 (72 h)] and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

as fermentation conditions for the particular sites and operators (Jespersen et al., 2005).

The direct analysis on total DNA and RNA of mawè did not reveal any other species except 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 analyzed 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 levels 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 a 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 the identification of the yeast population associated

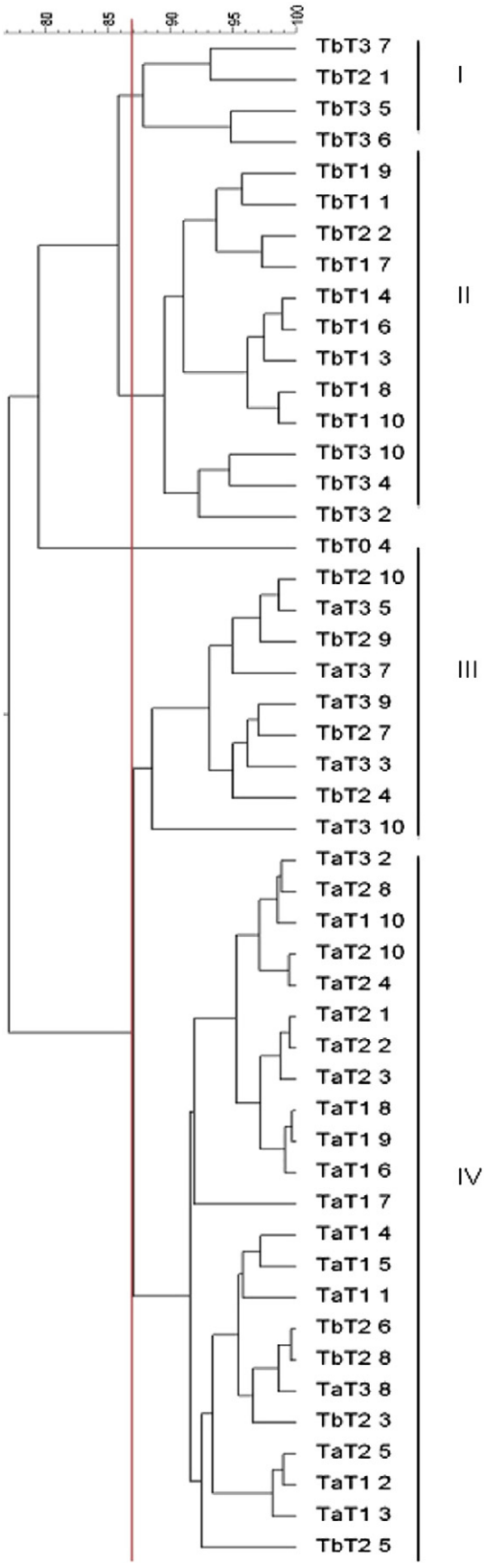

Fig. 3. Cluster analysis of the rep-PCR fingerprints of *Saccharomyces cerevisiae* strains isolated during the fermentation of tchoukoutou. The first letter represents the sample, the second represents the replicate (a–b), the number represents the fermentation times [T0, T1 (4 h), T2 (8 h), T3 (12 h)]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

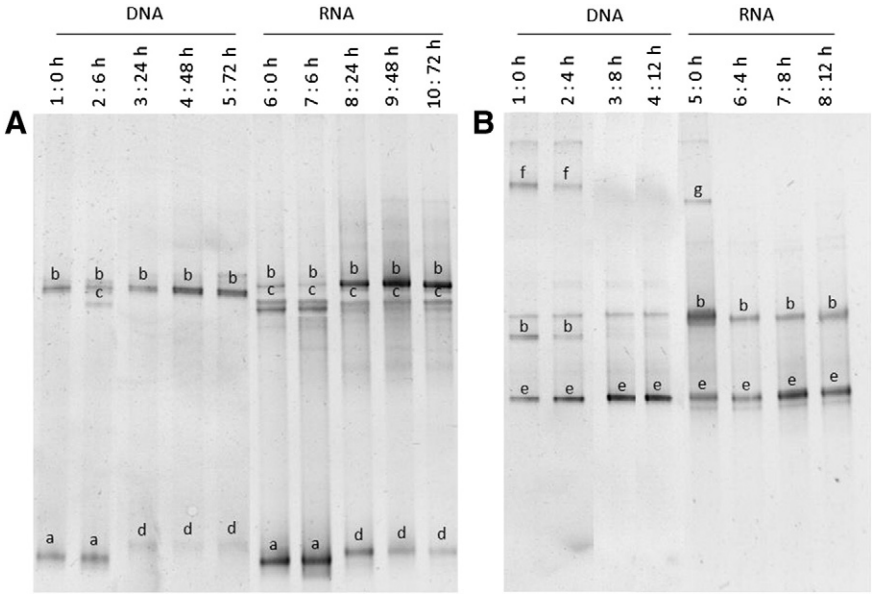

Fig. 4. DGGE profiles obtained by the amplification of total DNA and RNA extracted directly from mawè (panel A) and tchoukoutou (panel B). Panel A, lines 1–5, DNA from mawè fermentation (T0–T6–T24–T48–T72); lines 6–10, RNA from mawè fermentation. Panel B, lines 1–4 DNA from tchoukoutou fermentation (T0–T4–T8–T12); 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*, anomorph of *Candida krusei* (100%, JQ585732); band e *Saccharomyces cerevisiae* (100%, JF427814); band f *Hanseniaspora uvarum* (100%, EU386753); band g *Hanseniaspora guillermondii* (100%, JQ707775).

with the fermentation (Demuyakor and Ohta, 1991; Sanni and Lonner, 1993; Konlani et al., 1996; Sefa-Deheh et al., 1999; Van der Aa Kuhle et al., 2001; Glover et al., 2005; Maoura et al., 2005; Greppi et al., 2013). 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-Deheh 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 a lower percentage during the fermentation and can then be considered as sporadic.

Differences in yeast species in comparison to other African sorghum beer ecosystems 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 h 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 the DNA level and of *H. guillermondii* and *K. marxianus* at the RNA level. In the 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 the RNA level i.e. metabolically active yeast cells. *K. marxianus* was also largely detected by a culture-independent approach. This species was not found by culturing, instead *Cl. lusitaniae* was present at a 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 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 a

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.

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References

Agu, R.C., Palmer, G.H., 1998. A reassessment of sorghum for lager-beer brewing. Bioresource Technology 66, 253–261.
Altschul, S.F., Madden, T.L., Shaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.
Annan, N.T., Poll, L., Sefa-Dedeh, S., Plahar, W.A., Jakobsen, M., 2003. Volatile compounds produced by *Lactobacillus fermentum*, *Saccharomyces cerevisiae* and *Candida krusei* in single starter culture fermentations of Ghanaian maize dough. Journal of Applied Microbiology 94, 462–474.
Bonetta, S., Bonetta, S., Carraro, E., Rantsiou, K., Cocolin, L., 2008. Microbiological characterisation of Robiola di Roccaverano cheese using PCR–DGGE. Food Microbiology 25 (6), 786–792.
Briggs, D.E., Boulton, C.A., Brookes, P.A., Stevens, R., 2004. Native African beers. Brewing: Science and Practice. Woodhead Publishing Ltd., Cambridge, UK, pp. 589–605.
Chang, H.W., Kim, K.H., Nam, H.D., Roh, S.W., Ki, M.-S., Jeon, C.-O., Oh, H.-M., Bae, J.-W., 2008. Analysis of yeast and archaeal population dynamics in kimchi using denaturing gradient gel electrophoresis. International Journal of Food Microbiology 126, 159–166.
Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology Letters 189, 81–87.
Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113–5121.
Cocolin, L., Aggio, D., Manzano, M., Cantoni, C., Comi, G., 2002. An application of PCR-DGGE analysis to profile the yeast populations in raw milk. International Dairy Journal 12, 407–411.
Cocolin, L., Dolci, P., Rantsiou, K., 2011. Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. Meat Science 89, 296–302.
Czerucka, D., Dahan, S., Mograbi, B., Rossi, B., Rampal, P., 2000. *Saccharomyces boulardii* preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic *Escherichia coli*-infected T84 cells. Infection and Immunity 68, 5998–6004.
Demuyakor, B., Ohta, Y., 1991. Characteristics of pito yeasts from Ghana. Food Microbiology 8, 183–193.
Di Maro, E., Ercolini, D., Coppola, S., 2007. Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. International Journal of Food Microbiology 117, 201–210.
Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56, 297–314.
Fidel, P.L.J.R., Vazquez, J.A., Sobel, J.D., 1999. *Candida glabrata*: review of epidemiology, pathogenesis and clinical disease with comparison to *C. albicans*. Clinical Microbiology Reviews 80–96.
Gadaga, T.H., Mutukumira, A.N., Narvhus, J.A., 2000. Enumeration and identification of yeasts isolated from Zimbabwean traditional fermented milk. International Diary Journal 10, 459–466.
Gedek, B.R., 1999. Adherence of *Escherichia coli* serogroup 0 157 and the *Salmonella* Typhimurium mutant DT 104 to the surface of *Saccharomyces boulardii*. Mycoses 42, 261–264.
Glover, R.L.K., Abaidoo, R.C., Jakobsen, M., Jespersen, L., 2005. Biodiversity of *Saccharomyces cerevisiae* isolated from a survey of pito production sites in various parts of Ghana. Systematic and Applied Microbiology 28, 755–761.
Greppi, A., Rantsiou, K., Padonou, W., Hounhouigan, J., Jespersen, L., Jakobsen, M., Cocolin, L., 2013. Determination of yeast diversity in ogi, mawè, gowé and tchoukoutou by using culture-dependent and -independent methods. International Journal of Food Microbiology 165, 84–88.
Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M., Zelder, O., 2005. Biotechnological production and applications of phytases. Applied Microbiology and Biotechnology 68, 588–597.
Halm, M., Hornbaek, T., Arneborg, N., Sefa-Dedeh, S., Jespersen, L., 2004. Lactic acid tolerance determined by measurement of intracellular pH of single cells of *Candida krusei* and *Saccharomyces cerevisiae* isolated from fermented maize dough. International Journal of Food Microbiology 94, 97–103.
Head, I.M., Saunders, J.R., Pickup, R.W., 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial Ecology 35, 1–21.
Hjortmo, S., Patring, J., Jastrebova, J., Andlid, T., 2005. Inherent biodiversity of folate content and composition in yeasts. Trends in Food Science & Technology 16, 311–316.
Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H.M., Rombouts, F.M., 1993. Changes in the physico-chemical properties of maize during natural fermentation of mawè. Journal of Cereal Science 17, 291–300.
Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombouts, F.M., 1993. Characterization and frequency distribution of species of lactic acid bacteria involved in the processing of mawè, a fermented maize dough from Benin. International Journal of Food Microbiology 18, 279–287.
Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombouts, F.M., 1993. Composition and microbiological and physical attributes of mawè, a fermented maize dough from Benin. International Journal of Food Science and Technology 28, 513–517.
Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombouts, F.M., 1993. Microbiological changes in mawè during natural fermentation. World Journal of Microbiology and Biotechnology 10, 410–413.
Jespersen, L., Halm, M., Kpodo, K., Jakobsen, M., 1994. Significance of yeasts and moulds occurring in maize dough fermentation for “kenkey” production. International Journal of Food Microbiology 24, 239–248.
Jespersen, L., 2003. Occurrence and taxonomic characterisation of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. FEMS Yeast Research 3, 191–200.
Jespersen, L., Nielsen, D.S., Honholt, S., Jakobsen, M., 2005. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast Research 5, 441–453.
Kayodé, A.P.P., Hounhouigan, D.J., Nout, M.J.R., Niehof, A., 2006. Household production of sorghum beer in Benin: technological and socio-economic aspects. International Journal of Consumer Studies 1470–6431.
Konlani, S., Delgenes, J.P., Moletta, R., Traore, A., Doh, A., 1996. Isolation and physiological characterization of yeasts involved in sorghum beer production. Food Biotechnology 10, 29–40.
Kurtzman, C.P., Robnett, C.J., 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 73, 331–371.
Liu, H.-J., Liu, D.-H., Zhong, J.-J., 2005. Interesting physiological response of the osmophilic yeast *Candida krusei* to heat shock. Enzyme and Microbial Technology 36, 409–416.
Maoura, N., Mbaiguinam, M., Nguyen, H.V., Gaillardin, C., Pourquie, J., 2005. Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of Chad. African Journal of Biotechnology 4, 646–656.
Masoud, W., Takamiya, M., Vogensen, F.K., Lillevang, S., Al-Soud, W.A., Sørensen, S.J., Jakobsen, M., 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. International Dairy Journal 21, 142–148.
Moslehi-Jenabian, S., Pedersen, L.L., Jespersen, L., 2010. Beneficial effects of probiotic and food borne yeasts on human health. Nutrients 2, 449–473.
Moss, M.O., Long, M.T., 2002. Fate of patulin in the presence of the yeast *Saccharomyces cerevisiae*. Food Additives and Contaminants 19, 387–399.
Mumy, K.L., Chen, X.H., Kelly, C.P., McCormick, B.A., 2008. *Saccharomyces boulardii* interferes with *Shigella* pathogenesis by post invasion signaling events. American Journal Gastrointestinal Liver Physiology 294, G599–G609.
N' guessan, K.F., Brou, K., Jacques, N., Casaregola, S., Dje, K.M., 2011. Identification of yeasts during alcoholic fermentation of *tchapalo*, a traditional sorghum beer from Côte d'Ivoire. Antonie Van Leeuwenhoek 99, 855–864.
Naumova, E.S., Korshunova, I.V., Jespersen, L., MNaumov, G.I., 2003. Molecular genetic identification of *Saccharomyces* sensu stricto strains from African sorghum beer. FEMS Yeast Research 3, 177–185.
Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H., 2007. The microbiology of Ghanaian cocoa fermentations analyzed using culture-dependent and culture-independent methods. International Journal of Food Microbiology 114, 168–186.
Odds, F.C., 1988. Ecology and epidemiology of candidiasis, Candida and Candidosis. A Review and Bibliography, 2nd ed. Balliere Tindall, London, United Kingdom, p. 89.
Oguntoyinbo, F.A., 2008. Evaluation of diversity of *Candida* species isolated from fermented cassava during traditional small scale *gari* production in Nigeria. Food Control 19, 465–469.
Omemu, A.M., Oyewole, O.B., Bankole, M.O., 2007. Significance of yeasts in the fermentation of maize for Ogi production. Food Microbiology 24, 571–576.
Oyewole, O.B., 2001. Characteristics and significance of yeasts' involvement in cassava fermentation for “fufu” production. International Journal of Food Microbiology 65, 213–218.
Pedersen, L.L., Owusu-Kwarteng, J., Thorsen, L., Jespersen, L., 2012. Biodiversity and probiotic potential of yeasts isolated from *Fura*, a West African spontaneously fermented cereal. International Journal of Food Microbiology 159, 144–151.
Prakitchaiwattana, C.J., Fleet, G.H., Heard, G.M., 2004. Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS Yeast Research 4, 865–877.
Ramos, C.L., Gonzaga de Almeida, E., de Melo, Vinícius, Pereira, G., Gomes Cardoso, P., Souza Dias, E., Freitas Schwan, E., 2010. Determination of dynamic characteristics of microbiota in a fermented beverage produced by Brazilian Amerindians using culture-dependent and culture-independent methods. International Journal of Food Microbiology 140, 225–231.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L., 2005. Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. Applied and Environmental Microbiology 71, 1977–1986.

Rantsiou, K., Greppi, A., Garosi, M., Acquadro, A., Mataragas, M., Cocolin, L., 2012. Strain dependent expression of stress response and virulence genes of *Listeria monocytogenes* in meat juices as determined by micro-array. International Journal of Food Microbiology 152, 116–122.

Romano, P., Suzzi, G., Domizio, P., Fatichenti, F., 1997. Secondary products formation as a tool for discriminating non-*Saccharomyces* wine strains. Antonie Van Leeuwenhoek 71, 239–242.

Sanni, A.I., Lonner, C., 1993. Identification of yeasts isolated from Nigerian traditional alcoholic beverages. Food Microbiology 10, 517–523.

Sefa-Deheh, S., Sanni, A.I., Tetteh, G., Sakyi-Dawson, E., 1999. Yeasts in the traditional brewing of pito in Ghana. World Journal of Microbiology and Biotechnology 15, 593–597.

Shetty, P.H., Jespersen, L., 2006. *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends in Food Science & Technology 17, 48–55.

Shetty, P.H., Hald, B., Jespersen, L., 2007. Surface binding of aflatoxin B_1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. International Journal of Food Microbiology 113, 41–46.

Silvestri, G., Santarelli, S., Aquilanti, L., Beccaceci, A., Osimani, A., Tonucci, F., Clementi, F., 2007. Investigation of the microbial ecology of Ciasculo, a traditional Italian Salami, by culture-dependent techniques and PCR-DGGE. Meat Science 77, 413–423.

Stringini, M., Comitini, F., Taccari, M., Ciani, M., 2008. Yeast diversity in crop-growing environments in Cameroon. International Journal of Food Microbiology 127, 184–189.

Urso, R., Rantsiou, K., Dolci, P., Rolle, L., Comi, G., Cocolin, L., 2008. Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. FEMS Yeast Resource 8, 1053–1062.

Van der Aa Kuhle, A., Jespersen, L., Glover, R.L.K., Diawara, B., Jakobsen, M., 2001. Identification and characterization of *Saccharomyces cerevisiae* isolated from West African sorghum beer. Yeast 18, 1069–1079.

Vauterin, L., Vauterin, P., 1992. Computer-aided objective comparison of electrophoretic patterns for grouping and identification of microorganisms. European Journal of Clinical Microbiology 33, 633–641.

Watanabe, I., Nakamura, T., Shima, J., 2010. Strategy for simultaneous saccharification and fermentation using a respiratory-deficient mutant of *Candida glabrata* for bioethanol production. Journal of Bioscience and Bioengineering 110, 176–179.