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Determination of yeast diversity in ogi, mawè, gowé and tchoukoutou, four traditional fermented products from Benin, using culture-dependent and -independent methods

Anna Greppi[a], Kalliopi Rantsiou[a], Wilfrid Padonou[b], Joseph Hounhouigan[b], Lene Jespersen[c], Mogens Jakobsen[c], Luca Cocolin[a]*

[a]Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Via Leonardo da Vinci 44, 10095, Grugliasco, Torino, Italy

[b]Département de Nutrition et Sciences Alimentaires, Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, Benin

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

*Author for correspondence: Via Leonardo da Vinci 44, 10095, Grugliasco, Torino. Phone 0039 011 670-8553, Fax 0039 011 670-8549, email: lucasimone.cocolin@unito.it

Running title: Yeast ecology in fermented products from Benin

Abstract

The maize based ogi and mawè and the sorghum based gowé and tchoukoutou are spontaneously traditional fermented products widely consumed by the population of Benin (West Africa). Yeast occurrence in the products, as sold on local markets at different locations, was studied using a combination of culture-dependent and independent methods. Number of yeasts varied from 3.75 $\log_{10}$ colony forming units (cfu)/g for ogi to 5.60 $\log_{10}$ cfu/g for tchoukoutou. Isolated yeasts (236) were identified based on different migration profiles on Denaturing Gradient Gel Electrophoresis (DGGE) and 26S rRNA gene sequencing. *Candida krusei* was the yeast most frequently isolated with strongest predominance in the maize based products. Other predominant yeast present at equal or lower incidence were *Clavispora lusitaniae* and *Saccharomyces cerevisiae* in ogi and mawè, *Cl. lustianiae*, *Candida tropicalis* and *Kluyveromyces marxianus in* gowè and *Cl. lusitaniae*, *S. cerevisiae* and *Candida rugosa* in tchoukoutou. Grouping of *C. krusei* isolates (164) by rep-PCR analysis indicated that several biotypes were involved in fermentation of the four products. The DGGE analysis on the DNA directly extracted from the food matrices demonstrated the presence of *Dekkera bruxellensis* and *Debaryomyces hansenii*, not detected by the culture-based approach. This is the first study combining culture-dependent and independent methods to reveal predominant yeast species and biotypes in traditional foods from Benin.

Key words: yeasts, predominant species, *Candida krusei*, biodiversity, fermented foods, culture dependent and independent analysis.

1. Introduction

Yeast occur in significant numbers ranging from 10^5 to 10^8 cfu/g in several traditional African fermented food constituting a major dietary component in different regions of Africa, including West Africa (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). A mixed mycobiota comprising the genera *Candida*, *Debaryomyces, Dekkera, Geotrichum, Hanseniaspora, Kodamaea, Kluyveromyces, Meyerozyma*, *Pichia, Rhodotorula, Saccharomyces*, *Trichosporum* and *Zygosaccharomyces* species has been reported. In general the role of yeast in these foods is not understood. The same applies for the potential interactions of such yeast with the human host, although some theories have been proposed as reviewed Moslehi-Jenabian et al. (2010).

The four products sampled at different locations in Benin and included in the present study are ogi, a gruel obtained by fermentation of a suspension of maize in water (Oke, 1967; Akinrele 1970; Dada and Muller, 1983); mawè, a fermented maize dough (Hounhouigan et al, 1993a); gowé, a malted and fermented maize or sorghum-based food (Michodjèhoun-Mestres et al., 2005; Vieira-Dalodé et al., 2007) and tchoukoutou, an opaque sorghum beer largely produced in the North of Benin (Kayodé et al. 2006). Related food products are called in different ways in other countries of West Africa e.g kenkey is a maize-dough produced from whole maize in Ghana similar to mawè for which predominant yeast were studied by Jespersen et al. (1994); tchapalo is a sorghum beer from Cote d'Ivoire similar to tchoukoutou, studied by N'guessan et al. (2011). Updated and molecular based studies of the yeast communities in traditional African food based upon fermentation of important raw materials like maize and sorghum are not available except for local beers (Van der Aa Kuhle et al., 2001; N' guessan et al., 2011). According to information available lactic acid bacteria (LAB) in high number are associated with yeasts in such products (Akinrele 1970; Odunfa and

Adeyele, 1985; Ampe and Miambi, 2000; Teniola and Odunfa 2001; Omemu et al. 2007; Oguntoyinbo et al. 2011 (ogi); Hounhouigan et al. 1993b, 1993c (mawè); Vieira-Dalodé et al., 2007 (gowé); Kayodé et al. 2006 (tchoukoutou)). However, the majority of these studies focused on LAB and yeast identifications were mainly based on morphological, physiological and biochemical characteristics of isolates. These methods have draw-backs in the way that they cannot differentiate isolates at a level suitable in biodiversity studies and they do not allow detection of viable but non-culturable microorganisms (Head et al., 1998). Further, they are no more accepted as the sole basis of taxonomic classification. The present work compares yeast populations in marketed ogi, mawè, gowé and tchoukoutou using a combination of culture-dependent and -independent molecular methods to create a new and better-suited platform for selection of cultures for further studies of specific yeast characteristics to understand their significance for fermentation and product quality.

2. Materials and methods

2.1 Samples collection

Samples of ogi, mawè, gowé and tchoukoutou were collected aseptically in sterile stomacher bags (Seward, West Sussex, UK) from local villages and urban markets in Benin. For each product, three different sites were chosen. Ogi samples were collected in two villages in Bohicon and Abomey-Calavi, respectively, and at the local market of Porto-Novo; mawè samples came from local markets in Cotonou, Godomey and Porto-Novo; gowé samples were obtained from a producer's house in Cotonou, a street seller of Cotonou and the local market of Godomey; tchoukoutou samples were collected at the local market in Cotonou, a producer's house in Abomey-Calavi and a street producer at Cotonou. All samples were stored at 4°C and subjected to microbiological analysis within 48 hours.

2.2 Determination of pH

To determine the pH, each sample was diluted five times in sterile water. The pH determinations for each diluted sample were performed in duplicate, using a digital pH meter (inoLab pH 730, WTW GmbH, Weilheim, Germany), calibrated with buffer at pH 4.0 and 7.0. For each product, mean and standard deviation of the three sample sites were calculated.

2.3 Enumeration and isolation of yeasts

Ten (10) g of each ogi, mawè and gowé samples and 10 ml of tchoukoutou samples were suspended in 40 ml of sterile diluent [0.1% bactopeptone (Oxoid, Milan, Italy), 0.85% NaCl (Merck, Darmstadt, Germany), all w/v, pH adjusted to 7.0] and homogenized for 30 seconds at 230 rpm with a Stomacher (Lab Blender, Model 400, Seward Medical, London, England). From appropriate 10-fold dilutions, yeasts were enumerated on MYGP agar [3 g yeast extract (Oxoid), 3 g malt extract (Oxoid), 5 g bactopeptone (Oxoid), 10 g glucose (Sigma, Milan, Italy) and 20 g agar (Oxoid) per litre of distilled water, all w/v, added of 50 mg chloramphenicol (Fisher chemicals, Milan, Italy) and 25 mg of chlortetracycline (Sigma)] and incubated at 24°C for 3 days. Plates showing between 30 and 300 colonies forming units (cfu)/g were counted and results were expressed as log_{10}. For each product, mean and standard deviation between the three sample sites were calculated. From the appropriate dilution, a sector of the agar plate was selected, from which 20 yeast colonies were sub-cultured on new plates and purified by repeated streaking. Colonies on MYPG were examined by microscopy (HBO 50/AC 100x/1.25 oil pH3, Zeiss, Jena, Germany). A total of 236 isolates were collected. All of them were maintained in glycerol (30 v/v) at -20°C.

2.4. DNA extraction from pure cultures

Genomic DNA of each isolate was extracted from 1 ml of 24 h MYPG culture, 30°C, 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 then quantified by using the Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy) and diluted to a concentration of 100 ng/ml.

2.5 Direct extraction of DNA from samples

Total DNA from the fermented samples was extracted using the MasterPure™ Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA) following the supplier's instructions (Rantsiou et al., 2012).

2.6 PCR-DGGE protocol

One microlitre of the yeast DNA and of the DNA extracted directly from the samples (100 ng) was used for the PCR assays in a total volume of 25 µl containing 1X PCR Buffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphospates (dNTP), 0.75 U of *Taq* Polymerase (Sigma), and 0.2 µM of each primer. The D1 region of the 26S rRNA gene was amplified using the primers NL1GC and LS2 (Cocolin et al., 2000). The amplification cycle of denaturation at 95°C for 1 min, annealing at 40°C for 45 s and extension at 72°C for 2 min was repeated 35 times. The cycle was preceded by an initial denaturation at 95°C for 5 min and followed by a final extension at 72°C for 7 min. The amplicons were analysed on 2% (w/v) agarose gels using a GeneRuler DNA Ladder of 100 bp as molecular weight standard (Promega, Milan, Italy). The gels were run at 100 V/cm (constant voltage) in 1X TAE (45 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8) and stained with ethidium bromide. Digital images of the gels were captured using UVI pro platinun 1.1 Gel Software (Eppendorf, Hamburg, Germany).

The PCR products were analysed by DGGE using the DCode apparatus (Bio-Rad, Hercules, CA, USA). They were electrophoresed in a 0.8 mm polyacrilamide gel (8% [w/v]) acrylamide-bisacrylamide (37.5:1), as described by Cocolin et al. (2001), using a denaturing gradient from 30 to 60%.

2.7 Identification by sequencing

DGGE profiles of the isolates were visually grouped and the partial 26S rRNA gene of representatives of each group was amplified with primers NL1/NL4 (Kurtzman and Robnett, 1998). The PCR products were sent to MWG Biotech (Edersberg, Germany) for sequencing.

For the culture-independent 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 as described by Cocolin at al (2001).

Alignments in GenBank with the BLAST program (Altschul et al., 1997) were performed to determine the closest known relatives of the partial 26S rDNA sequences obtained.

2.8 Grouping of Candida krusei *isolates by rep-PCR*

For the most abundant species *C. krusei* 164 isolates were grouped by rep-PCR. One hundred nanograms of the DNA extracted from pure cultures of the isolates was subjected to rep-PCR analysis using primer $(GTG)_5$ according to Nielsen et al. (2007). Reactions were carried out in a final volume of 25 μL containing: 1X PCR Gold Buffer, 1.5 mM MgCl2, 0.2 mM of each of dNTP, 2 μM primer $(GTG)_5$ and 1.25 U/μL *Taq* polymerase (Applied Biosystem, Milan, Italy). The PCR reaction consisted of 30 cycles of denaturation at 90°C for 30s, annealing at 40°C for 60s and extension at 65°C for 8 min. The initial denaturation was at 95°C for 5 min and the final extension at 65°C for 16 min. Amplicons were separated by 1.5 % agarose gel electrophoresis in 1X TBE (150 min, 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 and Vauterin, 1992).

3. Results

3.1 Yeast counts and pH

The number of yeast expressed as $\log_{10}$ cfu/g ± standard deviation for ogi, mawè, gowé and tchoukoutou samples were 3.75 ± 1.07, 5.52 ± 0.41, 4.04 ± 1.03 and 5.60 ± 0.88, respectively. The corresponding pH values were 3.76 ± 0.10, 5.04 ± 1.46, 3.68 ±0.32 and 3.32 ± 0.47.

3.2 Identification of yeast isolates by PCR-DGGE

As shown in Table 1, a total of six different species were identified. All of them at 100% of identity with sequences in GenBank database. *C. krusei* was the species most frequently isolated in all the products analysed. In the maize-based products (ogi and mawè) it represented more than 90% of the isolates. Concerning other yeast species, ogi and mawè were almost identical, with *Clavispora lusitaniae* and *Saccharomyces cerevisiae* detected at lower incidence. The biota of tchoukoutou samples showed less isolates of *C. krusei* but higher levels of *Cl. lusitaniae* and *S. cerevisiae* and presence of *Candida rugosa.* Gowé samples differed from the others with the presence of *Candida tropicalis* and *Kluyveromyces marxianus* occurring in similar levels as *C. krusei* (Tab. 1). Sample sites for ogi and mawè were very similar, minor differences were observed among tchoukoutou sample sites whereas pronounced differences were observed among the gowé sample sites (Tab. 1).

3.3 Direct food matrix DGGE analysis

The DGGE fingerprints obtained are shown in Figure 2. A total of 6 bands (a-f) were sequenced. The GenBank database allowed the identification of the bands with 100% identity and the species name and accession number are shown in Figure 2. Bands in the middle of the DGGE gel were also cut and sequenced and they were determined to be heteroduplex (data not shown). For ogi, *Pichia kudriavzevii*, teleomorph of *C. krusei* (band a) and *Cl. lusitaniae* (c) were detected, both species were also detected by culturing. *Dekkera bruxellensis* (b) was detected but not by culturing. *S. cerevisiae* (e) was not detected by direct DGGE and so was *C. krusei* (a) in two samples of ogi. For mawè, *C. krusei* (a), *Cl. lusitaniae* (c) and *S. cerevisiae* (e) were detected and the corresponding species were detected by culturing as well. Instead, *K. marxianus* (d) was detected by direct DGGE but not by culturing. Contrary to cultural data, *C. krusei* (a) was absent in one mawè sample (lane 5). For gowé *K. marxianus* (d) was detected by direct DGGE and by culturing whereas band *Debaryomyces hansenii* (f) was detected only by direct DGGE. *C. krusei, Cl. lustianiae* and *C. tropicalis*, were not detected by direct DGGE, contrary to culturing. In tchoukoutou samples only *S. cerevisiae* (e) was detected according to culturing. *C. krusei, Cl. lusitaniae* and *C. rugosa* were not detected by direct DGGE but by culturing.

It was noticed that no samples contained band a together with bands c, f or e indicating an apparent disagreement of the DGGE analysis with the cultural data as shall be discussed below.

3.4 *Grouping of* C. krusei *isolates*

All 164 isolates of *C. krusei* were analysed by rep-PCR for grouping and product specificity. The dendrogram obtained after cluster analysis of rep-PCR patterns is shown in Figure 1. Using a coefficient of similarity of 80%, eight different clusters were observed. Cluster IV

and VIII were common to all products and included a total of 89 (54%) of the isolates; cluster IV was the biggest one, containing a total of 76 isolates (46%) (Fig.1). For each product this cluster accounted for roughly 50% of the isolates except for tchoukoutou (30%). Ogi isolates were distributed in all the clusters. Cluster II and III only contained ogi isolates. In general, in gowè and tchoukoutou samples less biodiversity was observed.

4. Discussion

Samples of ogi, mawè, gowé and tchoukoutou were collected at different locations in Benin including markets, villages or street sellers. Details were not known with regards to the way the products were processed, time of fermentation, when and how the women decided to sell them and how they were transported and stored. Even though the oral transmission of the techniques appears to be quite precise, every producer creates her own suitable technique and decides to sell the product at her preferred time. This can explain the high value of pH obtained for the mawè samples (5.04 ± 1.46). Hounhouigan et al. (1993a) reported pH values of approximately 3.5 in commercial mawè. The same range of pH was reported by Jespersen et al. (1994) on kenkey. On the other hand, the pH measured for ogi, gowé and tchoukoutou samples were in accordance with the results found in literature (Omemu et al. 2007 (ogi); Vieira-Dalodé et al. 2007 (gowé); Kayodé et al., 2006 (tchoukoutou)). The yeast counts did not always match with those previously reported for ogi (Omemu et al, 2007), mawè (Hounhouigan et al., 1993b; Jespersen et al., 1994), gowé (Vieira-Dalodé et al., 2007) and tchoukoutou (Kayodé et al., 2006) and this is consistent with a high inter-product variability, but the order of magnitude was similar. By comparing concentrations of yeast with reported counts of LAB (Teniola et al., 2001 (ogi); Hounhouigan et al, 1993c (mawè); Vieira-Dalodé et al. 2007 (gowé); Kayodé et al., 2006 (tchoukoutou)) it seems that, in terms of cfu/g, LAB

occur in 3-4 tenfold higher numbers. However, the larger size of yeast cells i.e. the total yeast biomass indicate a significant bioactivity despite the much lower cfu/g.

In the four products studied identification of yeast isolates revealed that *C. krusei* was the predominant culturable species. In ogi and mawè samples, this species was almost the only one isolated. These products are maize-based fermented products and it can be speculated that the composition of the food matrix supports growth and survival of this specific species. The predominance of *C. krusei* in the later stages of maize dough African fermentation has also been reported by Jespersen et al. (1994). To find a possible explanation for its predominance, Halm et al. (2004) measured the intracellular pH of single cells of *C. krusei* and *S. cerevisiae* isolated from fermented maize dough. The authors wanted to investigate if the high predominance of the yeast in the late stages of the fermentation was due to a particular tolerance to high lactic acid concentration. Their results showed how *C. krusei* is more resistant to short-term pH changes caused by lactic acid than *S. cerevisiae*. Concerning the tchoukoutou, the fermentation is sometimes interrupted by the producers after approximately 12 hours to avoid a final product with too high level of ethanol as consumers in Cotonou and Abomey-Calavi often prefer lighter i.e. a lower ethanol beverage contrarily to the traditional recipe from the North of Benin. This could explain the predominance of *C. krusei* instead of *S. cerevisiae*, normally reported as being predominant in local beers with higher level of ethanol (Demuyakor and Ohta, 1991; Sefa-Deheh et al., 1999; N'guessan et al., 2011). Concerning the overall predominant yeast *C. krusei*, cluster analysis based on rep-PCR profiles did not indicate an apparent link between the four products and the clustering. Apart from two clusters specific for ogi isolates from the four different matrices clustered together underlining a homogeneous distribution of the *C. krusei* biotypes in the products. However, the usefulness of the rep-PCR was demonstrated by the presence of eight distinct clusters. It is assumed that isolates of different clusters have different roles in the fermentation. It is

realized that other typing techniques could have indicated different clustering and relationships with products. For bacteria, rep-PCR is a powerful, rapid, reproducible and highly discriminatory tool (Versalovic et al., 1994). So far with limited use in typing of yeast but Redkar et al. (1996) was successful in biotyping *Candida* spp. by use of rep-PCR.

The presence of *C. krusei* was also detected in previous studies on ogi from Nigeria (Omemu et al., 2007), kenkey from Ghana (Jespersen et al., 1994), gowé from Benin (Vieira-Dalodé et al., 2007) and sorghum beers from Togo and Burkina Faso (Konlani et al., 1996).

The culture-independent approach identified *D. bruxellensis* and *D. hansenii,* not detected by the culture-dependent. They may be present in the habitat in viable but not-culturable states (Head et al. 1998; Ercolini, 2004) or as dead cells. On the other hand, *C. tropicalis* and *C. rugosa* were only detected by culture-dependent analysis and this can be explained by the detection limit of 10^3 cfu/ml for the DGGE analysis (Cocolin et al., 2000). Unexpectedly, the band identified as *C. krusei* (the anamorph of *P. kudriavzevii*), was absent in the direct DGGE analysis for two samples of ogi, one of mawè and all gowé and tchoukoutou samples despite its presence according to the cultural data. This could be due to PCR-bias in the food matrices where different yeast species are present at high level and interfering with the specific binding to *C. krusei* of the primers applied. The same could apply for *Cl. lusitaniae* in ogi and mawè samples, *D. hansenii* in gowé sample and *S. cerevisiae* in tchoukoutou samples. In all cases these species were clearly amplified by the primer set, possibly masking the presence of *C. krusei*. As support of this explanation it was noticed that none of the samples showed a profile with the simultaneous amplification of *C. krusei* and *Cl. lustianiae*, *D. hansenii* or *S. cerevisiae*. Such results indicate the need for simultaneous use of culture-dependent and –independent techniques for studying yeast populations in traditional fermented products with unknown mycobiota.

It is realized that few samples of the same products were analyzed. However, this work is

considered as a first attempt to compare the mycobiota between different African fermented foods that have not been investigated by the combined use of culture dependent and – independent methods before. Further studies are required to investigate the dynamics of yeast population during fermentation to assess whether the dominance of *C. krusei* was preceded by other yeast species in the previous stages of fermentation and with the purpose of selecting starter cultures with specific functional characteristics for controlled fermentations.

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Table 1. Identification of yeasts from marketed samples of ogi, mawè, gowé and thoukoutou by PCR-DGGE. For each product, three different sample sites in Benin were chosen, indicated in roman numbers. The number of isolates for each species and the total number (in %in brackets) are shown for each product.

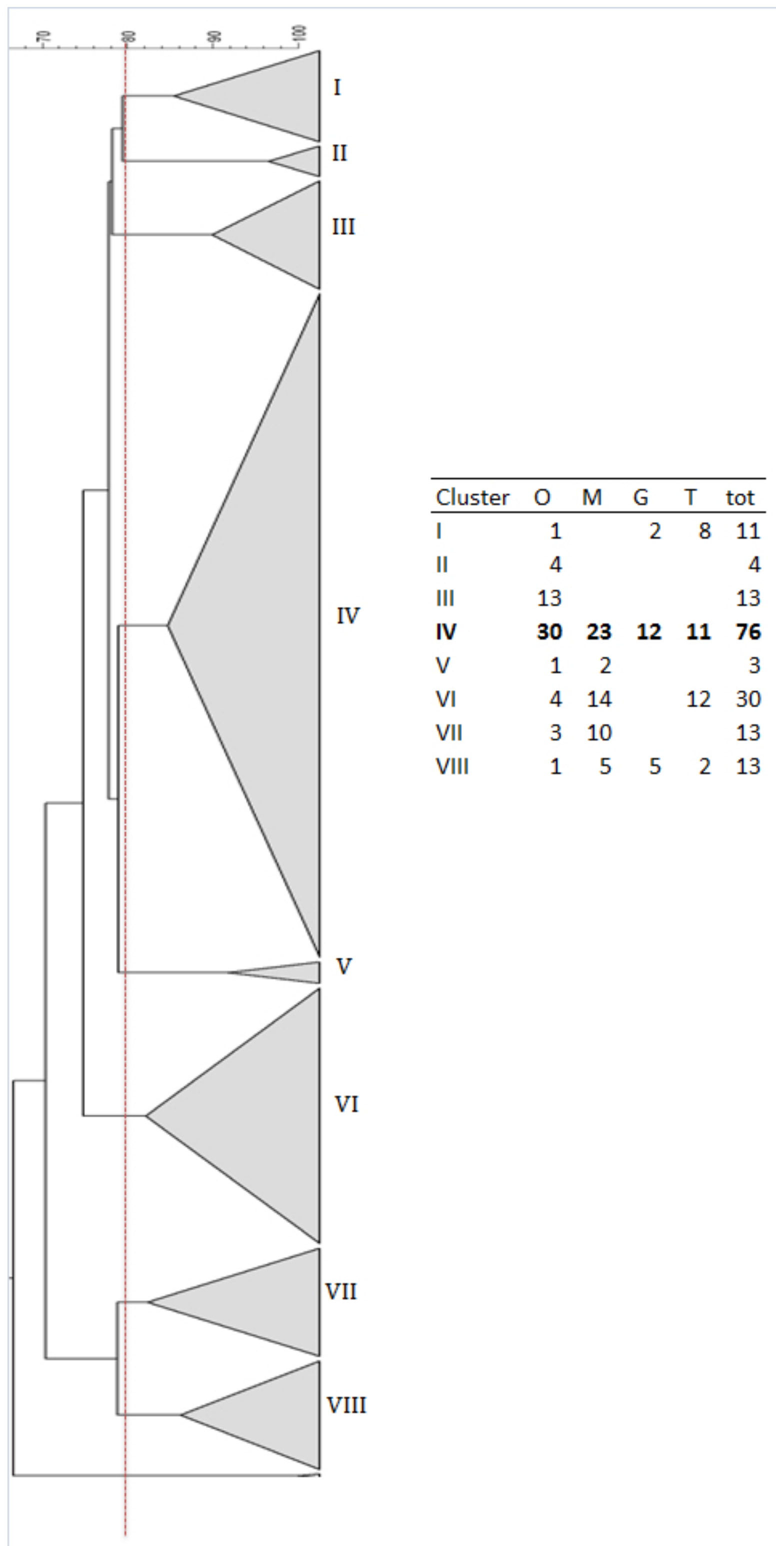
Yeast species	**Ogi**				**Mawè**				**Gowé**				**Tchoukoutou**				**All products**
	I	**II**	**III**	**tot**	**I**	**II**	**III**	**tot**	**I**	**II**	**III**	**tot**	**I**	**II**	**III**	**tot**	**tot**
Candida krusei	19	19	19	57 (96)	20	16	18	54 (92)	19			19 (32)	10	18	6	34 (59)	164 (69)
Clavispora lusitaniae			1	1 (2)			1	1 (2)	1		3	4 (6)		1	11	12 (20)	18 (8)
Saccharomyces cerevisiae	1			1 (2)		4		4 (6)					8	1		9 (15)	14 (6)
Candida tropicalis										3	16	19 (32)					19 (8)
Kluyveromyces marxianus										17	1	18 (30)					18 (8)
Candida rugosa															3	3 (5)	3 (1)
Tot	**20**	**19**	**20**	**59**	**20**	**20**	**19**	**59**	**20**	**20**	**20**	**60**	**18**	**20**	**20**	**58**	**236**

Figure legends

Figure 1. Cluster analysis of rep-PCR fingerprints of *Candida krusei* isolates (164). The dendrogram was generated after cluster analysis of the digitized fingerprints and was derived from UPGMA linkage of Pearson correlation coefficients. A similarity coefficient of 80% was chosen to guarantee differentiation. The origin of the isolates is shown as O = ogi samples, M= mawè samples, G= gowé samples and T= tchoukoutou samples. In bold, the bigger common cluster.

Figure 2. DGGE profiles of the partial 26S rRNA gene fragment of the yeasts present in the food matrices. M: marker: K.a.: *Kloekera apiculata*, C.a.: *Candida zemplinina;* lines 1-2-3*:* ogi samples; lines 4-5-6: mawè samples; lines 7-8-9 gowé samples; lines 10-11-12*:* thoukoutou samples. O = ogi samples, M= mawè samples, G= gowé samples and T= tchoukoutou samples. Band a *Pichia kudriavzevii* (formerly named as *Issatchenkia orientalis*, anamorph of *Candida krusei.* Accession No. JQ585732), band b *Dekkera bruxellensis* (FJ805785), band c *Clavispora lusitaniae* (EF694616), band d *Kluyveromyces marxianus* (FJ896141), band e *Saccharomyces cerevisiae* (JF427814), band f *Debaryomyces hansenii* (HQ641266).

Figure 1



Cluster	O	M	G	T	tot
I	1		2	8	11
II	4				4
III	13				13
IV	**30**	**23**	**12**	**11**	**76**
V	1	2			3
VI	4	14		12	30
VII	3	10			13
VIII	1	5	5	2	13

Figure 2