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Original Article

Molecular identification of yeasts involved in the alcoholic fermentation of Tchoukoutou and Atan in Benin using sequencing

Chimene N. M. Nanoukon[1*], Celestin. K. C. Tchekessi[2], Marius E. Badoussi[1,3], Berenice M. Y. Accrombessi[2], Brice M. Ohin[2], Christine N'tcha[4], Amed S. Havivi[1], Laurinzo Déguénon[1], Pamela Loumèdjinon[1], Farid A. K. Baba-Moussa[2], L. Gustave Djèdatin[5]

[1] Laboratory of Genetics, Biotechnology and Applied Botany (GeBBA), National School of Applied Biosciences and Biotechnologies (ENSBBA), National University of Science, Technology, Engineering and Mathematics(UNSTIM), Dassa-Zoume, Benin

[2] Laboratory of Microbiology, Food Technology and Phytopathology (LA.MI.T.A. P), Department of Plant Biology, Faculty of Sciences and Technology (FAST) of the University of Abomey-Calavi (UAC), Benin

[3] Laboratory of Food Sciences and Technologies, Faculty of Agricultural Sciences, University of Abomey-Calavi, Cotonou, Benin

[4] Laboratory of Biology and Molecular Typing in Microbiology, Faculty of Technical Sciences, University of Abomey Calavi, Cotonou, Benin

[5] Laboratory of Pluridisciplinary Research of Technical Education (LaRPET), High Normal School of Technical Education (ENSET) of Lokossa, National University of Sciences, Technologies, Engineering and Mathematics (UNSTIM), Benin

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Abstract

In Benin, the consumption of local beverages is increasing, particularly "Tchoukoutou" and "Atan". However, they face numerous challenges that hinder their recognition as fermented drinks with potential medical and cultural benefits. This study aims to determine the biodiversity of yeasts responsible for the fermentation of "Tchoukoutou" and "Atan" produced in Benin to promote their production and commercialization. To achieve this, beverage samples were randomly collected from six (06) municipalities in Benin and subjected to physicochemical and microbiological analysis. Yeast identification was performed through sequencing of the D1/D2 region of the 26S ribosomal RNA (26S rRNA) gene. Physicochemical analysis revealed that the pH values ranged between 3.65 and 3.92 for "Tchoukoutou" and between 3.71 and 4.04 for "Atan." Regarding sugar content (Brix degree), "Tchoukoutou" values ranged from 1 to 12° Brix, while those of "Atan" ranged from 1° to 14° Brix. Microbiological analysis showed that the yeast count in "Tchoukoutou" and "Atan" was 1.57×10^3 and 5.29×10^3 CFU/mL, respectively. A total of five (05) different yeast species were identified in "Tchoukoutou" and six (06) in "Atan". *Schizosaccharomyces pombe* (44.44%) was the predominant yeast species in the fermentation of "Tchoukoutou", followed by *Saccharomyces cerevisiae* (22.22%). In contrast, for "Atan", *Saccharomyces cerevisiae* (53.33%) was the most dominant species, followed by *Saccharomyces boulardii* (13.33%), and *Candida parapsilosis* (13.33%). Other yeast species present in lower percentages included *Candida parapsilosis* (11.11%), *Pichia sp. Feni* (11.11%) and *Pichia manshurica* (11.11%) in "Tchoukoutou" and *Pichia kudriavzevii* (6.66%), *Pichia ethanolica* (6.66%) and *Schizosaccharomyces osmophilus* (6.66%) in "Atan". These results indicate that Beninese "Tchoukoutou" and "Atan" fermentation is primarily driven by *Schizosaccharomyces pombe,* a yeast not commonly found in traditional Benin beverages, *and Saccharomyces cerevisiae*, respectively. This information is valuable for developing genetic engineering strategies to control their alcoholic fermentation, improve packaging, and extend shelf life.

Keywords: Tchoukoutou, Atan, Fermentation, Yeast, Sequencing, Benin.

1. Introduction

The trends in African traditional beverages continue to grow and are becoming increasingly important across the continent. These beverages are produced from various raw materials such as cereals, palm trees, etc. The *Poaceae* (*Gramineae*) family, the Leguminosae (Fabaceae) family, and the Palmaceae (Arecaceae) family are the three plant families that stand out in terms of global utility [1]. In Benin, the production and consumption of traditional beverages have grown significantly due to their importance in terms of nutrition and their contribution to the economy and the sustainability of cultural values [1, 2]. Among these beverages are the traditional sorghum beer "Tchoukoutou" and palm wine "Atan". These traditional drinks are closely linked to traditions and cultural values. They are fermented and alcoholic beverages produced from Sorghum (*Sorghum bicolor*) for Tchoukoutou [3], mainly in northern Benin, and from the sap of the oil palm (*Elaeis*

* Corresponding author.
E-mail address: chimnanouk85@yahoo.fr (C. N. M. Nanoukon).
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guineensis) [4] for palm wine "Atan" in southern Benin.

Indeed, "Tchoukoutou" is a highly appreciated beverage in Benin and is consumed throughout the country. It is obtained through the alcoholic fermentation of sweet sorghum wort, involving various yeasts and lactic acid bacteria (LAB) [5, 6]. The production process of this local beer includes malting (soaking, germination, sun drying), brewing (mashing, boiling, filtration), and fermentation [7, 8], which consists of an initial lactic fermentation followed by an alcoholic fermentation, both of which are uncontrolled [9]. Lactic acid bacteria play a role during the souring stage, acidifying the wort and creating favourable conditions for yeast growth [10]. Yeasts, in turn, carry out alcoholic fermentation, which is the final stage of the production process, significantly determining the characteristics of the finished product [9].

As for palm wine "Atan", it is a natural beverage derived from palm sap, initially sweet upon collection and later alcoholic under the influence of wild yeasts present in the sap of Elaeis [1] following spontaneous fermentation [4]. The extraction technique of this beverage varies from one locality to another in Africa, with several descriptions provided by researchers [11-16]. However, two main methods are used in West Africa. The first is non-destructive and is performed on a standing live palm tree by excising the apical bud to collect the sap. The second, which is lethal, is practiced on a felled palm tree, where the sap is collected by incising the apical bud. In this case, palm wine is extracted by felling the tree and making daily cuts on the apical bud to keep it "bleeding" [4].

The fermentation process is complex and rapid, starting immediately upon sap collection in the receiving container. Yeast proliferation quickly increases the turbidity of "Atan", and fermentation ceases after about twelve hours when all sugars have been converted [17]. Essentially, it is a suspension of bacteria and yeasts in the more or less fermented sap of the palm's phloem. The microflora of palm wine is highly diverse, consisting of lactic acid bacteria, acetic acid bacteria, *Zymomonas spp*., micrococci, and yeasts [1]. During fermentation, the unfermented sugars in the sap are transformed into organic acids and alcohol, with their concentrations being inversely proportional to the sugar content. Additionally, during fermentation, protein levels increase due to the proliferation of microorganisms [17]. Among the yeasts, *Saccharomyces cerevisiae* is of industrial importance due to its ability to convert sugars (such as glucose and maltose) into ethanol and carbon dioxide, which is essential in baking, brewing, distillation, and biofuel industries. *Saccharomyces cerevisiae* breaks down glucose through aerobic respiration in the presence of oxygen. In the absence of oxygen, the yeast undergoes anaerobic fermentation, producing two molecules of adenosine triphosphate (ATP) along with two by-products: carbon dioxide and ethanol [18].

The production of traditional beverages has significant socio-economic importance as they are widely consumed across almost all regions of Benin and are affordable compared to industrial alcoholic beers. Due to their relatively low cost, they serve as an essential source of income for local producers [5]. According to [19], traditional beverages also have therapeutic properties and contain antioxidants that may help prevent cardiovascular diseases. They provide energy and help combat fatigue from agricultural labor and even malaria.

The main factors influencing the quality of these traditional beverages include the quality of spontaneous fermentation, the production process, and the proportions of ingredients. Improving these factors requires knowledge of their production's physical, chemical, and microbiological processes. However, traditional beverages, often consumed during active fermentation, have a short shelf life. Fermentation begins spontaneously at ambient temperature, significantly altering the taste of these traditional drinks, making them unpleasant for consumption [20, 21]. For instance, if "Atan" is not consumed within two days after fermentation, it starts developing a vinegar-like taste, which is unacceptable to consumers [22]. Therefore, the uncontrolled fermentation of palm sap is a major barrier to promoting palm wine "Atan" produced in Benin. Studies have been conducted in Benin on the isolation and phenotypic identification of potential yeasts from sorghum beer "Tchoukoutou" and palm wine "Atan", but no molecular-level research has yet been reported. Given their importance to local producers and consumers in Benin, finding solutions to control their fermentation and extend their shelf life is crucial. To achieve this, understanding the microbiology of their fermentation process is essential. This information is valuable for developing genetic engineering strategies to control their alcoholic fermentation, improve packaging, and extend shelf life. The main objective of this study is to identify the yeast species involved in the fermentation of "Tchoukoutou" and "Atan" produced in Benin.

2. Materials and Methods

2.1. Materials

2.1.1. Study framework

The study was conducted in six (06) communes in Benin, selected to represent key zones of production, marketing, and consumption of Tchoukoutou and Atan. These include Natitingou (Atacora Department) and Parakou (Borgou Department) in northern Benin; Glazoué (Collines Department) in central Benin; and Sèmè-Kpodji (Ouémé Department), Zè (Atlantique Department), and Comè (Mono Department) in southern Benin (Fig. 1). Natitingou, located in the northwest, spans 3,045 km², representing 12.8% of Atacora. It lies between 10°18'46.48"

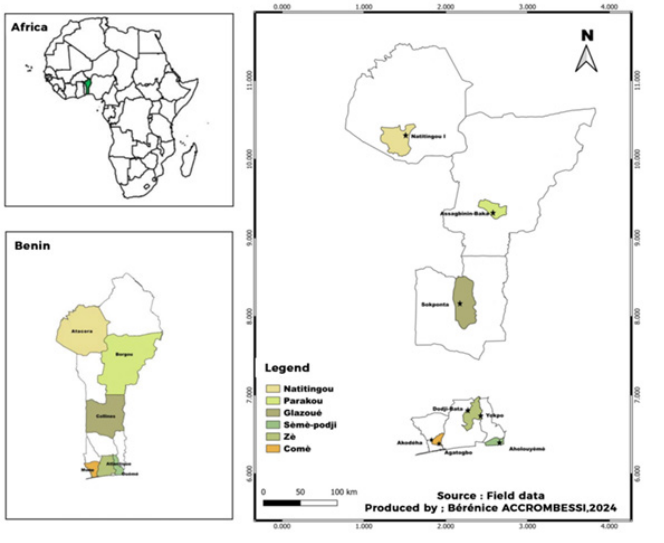

Fig. 1. Geographical location of the municipalities in the six (06) departments.

N and 1°23'19.02" E. Parakou, further north, extends over approximately 30 km², with a district area of about 300 km², situated at 9°21'00" N and 2°37'00" E. In central Benin, Glazoué is a rural commune 234 km from Cotonou, located at 7°58'14.99" N and 2°14'14.40" E. In the southeast, Sèmè-Kpodji covers 250 km², positioned at 6°22'00" N and 2°37'00" E. Zè, also in the south, spans 543 km² and lies at 6°46'60" N and 2°18'00" E. Comè, approximately 100 km west of Cotonou, covers 163 km² and is located at 6°25'32" N and 1°55'46" E.

The study sites and participants were selected using a non-probabilistic, purposive sampling method, following a preliminary survey. Selection criteria included: cultivation of Sorghum bicolor and Elaeis guineensis in the target areas; the predominance of these crops; technical expertise in Tchoukoutou and Atan production; the economic importance of these beverages for local populations; the centrality of sodabi production in the producers' livelihoods; and the producers' experience and longstanding involvement in the field.

2.1.2. Plant material

The biological material used consisted of "Tchoukoutou" and "Atan", obtained respectively from sorghum (*Sorghum bicolor*) and oil palm (*Elaeis guineensis*), which are grown and consumed in Benin.

2.2. Methods

2.2.1. Sampling

Sampling was carried out randomly at three production and sales sites located in the municipalities of Natitingou, Parakou, Glazoué, Sèmè-Podji, Zè, and Comè. Eighteen samples were collected in sterile 50 ml bottles, each sealed with a screw cap, with 3 samples from each municipality. The samples were then stored in a thermally insulated cooler to prevent contamination and were immediately transported to the laboratory for analysis.

2.2.2. Physicochemical analysis

Physicochemical analyses were conducted to evaluate several key parameters of the collected beverage samples, such as pH and Brix degree. Measuring these parameters helps characterize the composition and physicochemical properties of the beverages and ensures their quality.

2.2.2.1. Determination of hydrogen potential (pH)

The beverage samples, taken out of the freezer and left for at least one hour to return to room temperature (30 – 32°C), were measured every 12 hours over 72 hours for "Tchoukoutou" and every 4 hours over 48 hours for "Atan" throughout the fermentation, using a pH meter calibrated with buffer solutions of pH 4 and pH 7, equilibrated at a temperature of 28°C. Each measurement was repeated three times and the average value was considered [23].

2.2.2.2. Determination of Brix degree

The determination of sugar content, expressed in Brix degrees, was carried out according to the method of [24]. This measurement is essential to determine the quantity of dissolved solids in the solution, including sugars, acids and other non-volatile compounds. The Brix degree was also measured every 12 hours over a period of 72 hours for "Tchoukoutou" and every 4 hours over a period of 48 hours for "Atan" throughout fermentation, using a RoHS brand refractometer.

2.2.3. Microbiological analysis

The microbiological analysis performed consisted of yeast enumeration. For this, 1 mL of each sample was aseptically transferred into a test tube containing 9 mL of sterilized Tryptone Salt (TS) broth. The mixture was homogenized using a stomacher, resulting in a 10^{-1} dilution. Subsequently, 1 mL of the 10^{-1} dilution was aseptically transferred, near a Bunsen burner flame, using a sterile pipette, into another sterile test tube containing 9 mL of sterilized TS broth and homogenized, yielding a 10^{-2} dilution. This serial decimal dilution process was continued as needed. Yeast detection and enumeration were carried out on Sabouraud agar supplemented with chloramphenicol. A 0.1 mL aliquot of each dilution (10^{-1}, 10^{-2}, and 10^{-3}) was aseptically plated onto pre-poured Petri dishes, followed by uniform spreading using a sterile spreader. The inoculated plates were incubated at 25°C for 4 days. Yeast colonies appeared milky and whitish in color. The results obtained from the detection and enumeration of microbial organisms were expressed in CFU/mL using the following formula:

$$N = \sum c\,/(n1 + 0{,}1n2)dv$$

Where:
N = concentration in colony-forming units per milliliter (CFU/mL)
$\sum$**c** = total number of colonies counted on the selected plates
$\mathbf{n_1}$ = number of plates from the first selected dilution
$\mathbf{n_2}$ = number of plates from the second selected dilution
v = volume of inoculum plated on each Petri dish (in milliliters)
d = dilution factor corresponding to the first selected dilution (i.e., the least diluted sample)
For low colony counts (between 1 and 14 colonies), only one dilution was considered, and microbial load was calculated using the following formula:

$$N = C/vnd$$

Where:
N = number of CFUs observed in the selected plates
C = total number of colonies counted on the plates from the selected dilution
v = volume of inoculum plated (in milliliters)
n = number of usable plates
d = dilution factor corresponding to the selected dilution

2.2.4. Yeast isolation

Yeast identification was carried out based on macroscopic and microscopic characteristics observed from pure colony plates, following the methodology described by Bessan (2023). A strain conformity test was performed using microscopic observation to assess cell morphology, typically round to oval, and reproductive modes, primarily budding and occasionally fission. A decimal dilution method was employed using the standard [25]. The diluted samples were inoculated on Sabouraud agar with chloramphenicol and incubated at 37°C for 3 days. Individual yeast colonies with different morphologies were selected for purification on Sabouraud agar plates with chloram-

phenicol. The yeast isolates were preserved in a 2 mL cryotube containing 75% Sabouraud broth and 25% glycerol for long-term storage at -20°C.

2.2.5. Molecular characterization of Yeasts isolated from "Tchoukoutou" and "Atan"

2.2.5.1. Genomic DNA Extraction

Yeast DNA was extracted from colonies grown on Sabouraud medium. Colonies were cultured overnight in 10 mL of liquid YPD medium, centrifuged for 5 min, and the supernatant was removed. The pellet was resuspended in 1 mL of sterile distilled water and transferred to a 1.5 mL Eppendorf tube, followed by centrifugation at 6000 rpm for 6 sec. After discarding the supernatant, 200 µL of lysis buffer, 200-300 µL of glass beads, and 200 µL of phenol/chloroform/IAA were added. The mixture was vortexed for 5 min, followed by the addition of 200 µL TE buffer (pH 8) and vortexing for 1 min. Subsequently, centrifugation at 15000 rpm for 5 min was performed, and the aqueous layer was transferred to a new 1.5 mL Eppendorf tube. The supernatant was mixed with 1 mL of 100% ethanol and stored at -20°C for 1 to 24 hours. After centrifugation at 15000 rpm for 5 min, the supernatant was discarded, and the pellet was air-dried at 37°C for 5 min. It was resuspended in 400 µL TE buffer (pH 8), and 1 µL of RNase A (1 mg/mL) was added, vortexed, and incubated at 37°C for 1 hour. The mixture was then frozen at -20°C for 1 hour after adding 50 µL of 4M LiCl, 45 µL of free water, and 1 mL of 100% ethanol. After centrifugation at 15000 rpm for 5 min, the liquid was removed, and 1 mL of 70% ethanol was added and vortexed (this step was repeated twice). The mixture was centrifuged again at 15000 rpm for 5 min, ethanol was discarded, and the pellet was air-dried at 37°C for 5 min. Finally, it was suspended in 50 µL of free water and stored at -20°C for long-term preservation [26].

2.2.5.2. Verification of the quality of extracted genomic DNA

The quality and presence of extracted genomic DNA were verified using 1% agarose gel electrophoresis. The gel was prepared by dissolving agarose in TBE buffer, adding BET stain, and allowing it to solidify before loading the DNA samples mixed with loading dye. Electrophoresis was conducted at 100V for 30 minutes, and DNA bands were visualized under UV light to assess their intensity and quality [26].

2.2.5.3. Sequencing of 26S rDNA D1/D2 region

In the present study, Yeast isolate species were identified by sequencing the D1/D2 region of the 26S rRNA. Studies have shown that the sequence of the D1/D2 region can reliably identify most yeast species with taxonomic accuracy equivalent to that obtained by combined phenotypic methods [27]. Indeed, this region balances conserved areas (allowing universal amplification with standard primers) and variable areas (sufficiently divergent to allow discrimination between species).The D1/D2 region of the 26S rDNA gene was amplified using specific primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) of the 26S rDNA described by [27]. The reaction mixture used for the amplification of this gene consisted of 8 µL of buffer, 7 µL of 2x Mytag H3Mα, 1 µL of DNA, and 8 µL of sterile distilled water, for a final volume of 20 µL. The amplification program involved an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 59 seconds, hybridization at 53°C for 30 seconds, elongation at 72°C for 59 seconds, final elongation at 72°C for 7 minutes, and cooling of the amplified product to -20°C. PCR products were visualized on a 1.5% agarose gel. The gel was prepared with 1.5 g of agarose in 100 ml of 0.5X TBE, boiled, cooled, and poured into a mold. After solidifying, the gel was placed in an electrophoresis chamber, and 8 µL of DNA mixed with 2 µL of loading dye was loaded. Migration was done at 100 V for 30 minutes, and bands were visualized under UV light. The amplicons were purified using the QIAquick PCR Purification kit (Qiagen 28104, Crawley, UK) and sequenced using primers NL-1 and NL-4. The sequencing was done by Genewiz using the Sanger sequencing method [28].

2.2.5.4. BLAST sequence analysis of 26S rDNA D1/D2 region

The interpretable FASTA format sequences from Genewiz were selected for comparison with those from databases. The identity of a sequence is determined through a homology search in databases such as GENBANK from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The sequences were aligned with those known and present in the GENBANK database. Searches conducted using BLAST (Basic Local Alignment and Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) at NCBI provide a list of probable results corresponding to our sequence, along with sequence similarity values. Sequence comparison programs aim to identify regions of identity or close similarity between two sequences and deduce those that are biologically significant, based on what is observed in international databases. BLAST detects short segments (eleven identical nucleotides or two similar tripeptides) that are locally homologous to the unknown sequence [29]. The results of sequence comparisons are presented as the homology ratio between the obtained sequences and the closest reference sequences, along with the species name.

2.2.5.5. Phylogenetic analysis

Searches were performed in the NCBI GENBANK nucleotide database to identify the closest known relatives of the obtained partial 26S rDNA sequences. The sequences were then processed using multiple sequence alignment tools, specifically the MAFFT v6.864 DNA alignment program, to determine the evolutionary relationship between the yeast strains using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method [30].

2.2.6. Statistical data analysis

The results of the physicochemical and microbiological analyses were organized and processed using Microsoft Excel 2013. This software was used to analyse the data statistically (mean values and standard deviation) and create graphs.

3. Results

3.1. Physicochemical characteristics of traditional beverages

Figures 2a and 3a illustrate the correlation between pH and Brix degree according to the fermentation duration of the traditional beverages. A progressive drop in pH was

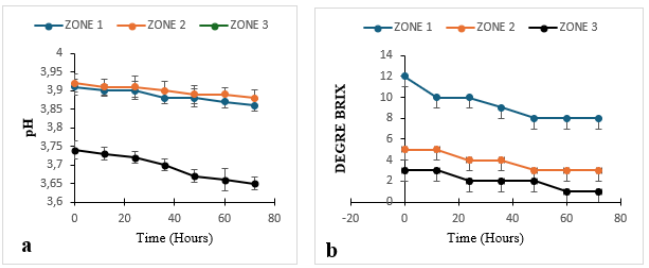

Fig. 2. Evolution of pH (a) and Brix degree (b) as a function of fermentation time of ‘’Tchoukoutou’’.

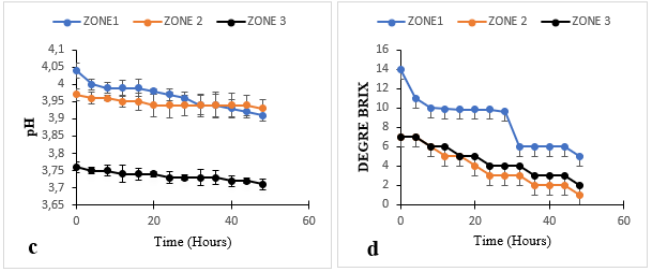

Fig. 3. Evolution of pH (c) and Brix degree (d) as a function of fermentation time of "Atan".

observed during fermentation, from 3.91 ± 0,02 at the start of fermentation to 3.86 ± 0,01 at the end of fermentation in the first zone, from 3.92 ± 0,02 at the start of fermentation to 3.88 ± 0,02 at the end of fermentation in the second zone, and from 3.74 ± 0,02 at the start of fermentation to 3.65 ± 0,01 at the end of fermentation in the third zone. This led to a drop in Brix from 12° ± 0,02 at the start of fermentation to 8° ± 0,02 at the end in the first zone, from 5° ± 0,04 at the start to 3° ± 0,04 at the end in the second zone, and from 3° ± 0,01 at the start to 1° ± 0,03 at the end in the third zone (Figure 2).

A gradual decrease in pH was observed during fermentation of "Atan": from 4.04 ± 0,02 at the beginning to 3.91 ± 0,01 at the end of fermentation in the first zone; from 3.97 ± 0,01 at the start to 3.93± 0,02 at the end in the second zone; and from 3.76 ± 0,01 at the beginning to 3.71 ± 0,01 at the end in the third zone (Figure 3c). This resulted in a decline in Brix from 14° ± 0,01 at the start to 5° ± 0,02 at the end of fermentation in the first zone; from 7° ± 0,02 at the beginning to 1° ± 0,03 at the end in the second zone; and from 7° ± 0,02 at the start to 2° ± 0,02 at the end in the third zone (Figure 3d).

3.2. Microbiological characteristics of traditional beverages

The results obtained from Fig. 4 provide insights into the microbiological characteristics of beverages. The yeast load in the traditional beverage Tchoukoutou across the three municipalities (Natitingou, Parakou, and Glazoué) ranges from 0.1×10³ to 3.04×10³ CFU/mL. For the traditional beverage Atan, this range is from 1.32×10³ to 9.27×10³ CFU/mL in the municipalities (Sèmè-podji, Zê, and Comè). A total of 25 yeast isolates were obtained from samples of Tchoukoutou and Atan.

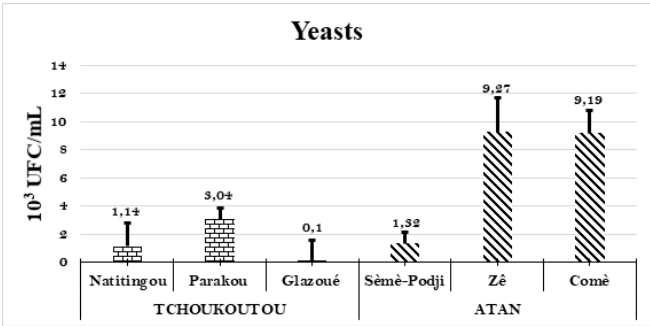

Fig. 4. Microbiological characteristics of various samples from Tchoukoutou and Atan.

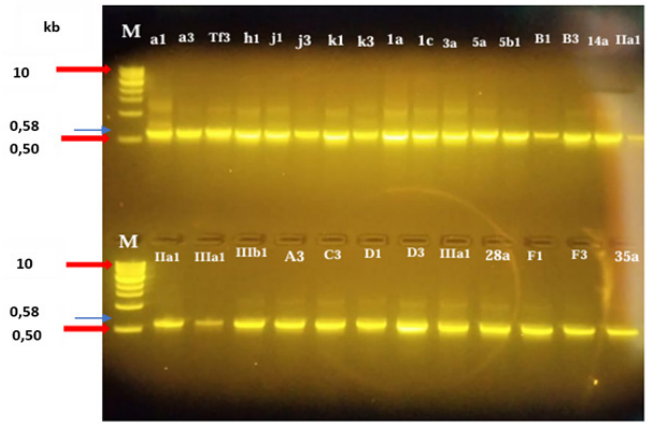

Fig. 5. Agarose gel electrophoresis of PCR-amplified D1/D2 domain of the 26S rRNA gene from yeast isolates. M: 1 kb molecular weight marker; lanes a1-35a: amplified fragment of D1/D2 domain of isolated yeasts.

3.3. Molecular characterization of yeasts isolated from “Tchoukoutou” and “Atan during alcoholic fermentation

The 26S rRNA gene amplification after agarose gel electrophoresis was positive for all presumptive yeast isolates. The results suggest that the target gene of 500-600 bp has been obtained (Fig. 5). Based on 26S rRNA gene identification and sequencing, the indigenous yeast isolates from “Tchoukoutou” and “Atan” were classified into four major genera: Saccharomyces, Schizosaccharomyces, Candida, and Pichia. Table 1 shows the results for yeast species identification in Tchoukoutou and Atan. In total, five (05) yeast species were identified in the “Tchoukoutou” beverage: Schizosaccharomyces pombe (44.44%), Saccharomyces cerevisiae (22.22%), Candida parapsilosis (11.11%), Pichia sp. *Feni* (11.11%) and Pichia manshurica (11.11%). Six (06) species were identified in the “Atan” beverage: Saccharomyces cerevisiae (53.33%), Saccharomyces boulardii (13.33%), Candida parapsilosis (13.33%), Pichia kudriavzevii (6.66%), Pichia ethanolica (6.66%), and Schizosaccharomyces osmophilus (6.66%). Schizosaccharomyces pombe was found to be the dominant species among all yeast strains isolated from “Tchoukoutou”, whereas Saccharomyces cerevisiae was predominant in the “Atan” samples (Table S1, supplementary data).

3.4. Phylogenetic Analysis

A phylogenetic analysis was conducted to characterize the evolutionary relationships among the isolated yeast species. The isolates' 26S rDNA D1/D2 region and that of other species in NCBI GenBank showed 97-100 % similarity (Table 1). The analysis revealed a close relationship between isolates from Tchoukoutou to *Schizosaccharomy-*

Table 1. Yeast species isolated from ‘’Tchoukoutou’’ and ‘’Atan’’ using sequencing.

Samples	Codes	Identification by 26S rDNA sequencing/ BLAST searching	Identity Percentage (100-97%)
TCHOUKOUTOU	a1	*Schizosaccharomyces pombe*	97
	a3	*Schizosaccharomyces pombe*	99
	Tf3	*Saccharomyces cerevisiae*	99
	h1	*Saccharomyces cerevisiae*	96
	j1	*Candida parapsilosis*	100
	j3	*Schizosaccharomyces pombe*	99,6
	k1	*Pichia sp. Feni* *Pichia manshurica*	99,12 98,95
	k3	*Schizosaccharomyces pombe*	98
ATAN	1a	*Pichia kudriavzevii*	93
	1c	*Saccharomyces cerevisiae*	99
	5a	*Candida parapsilosis*	94
	5b1	*Candida parapsilosis*	99
	B1	*Saccharomyces cerevisiae*	97
	B3	*Saccharomyces cerevisiae*	99
	IIb1	*Saccharomyces cerevisiae*	98
	C3	*Saccharomyces cerevisiae*	99
	D3	*Saccharomyces boulardii*	96
	IIIb1	*Saccharomyces cerevisiae*	96
	IIIa1	*Pichia ethanolica*	98
	28a	*Schizosaccharomyces osmophilus*	95
	F1	*Saccharomyces cerevisiae*	95
	F3	*Saccharomyces cerevisiae* *Saccharomyces boulardii*	97,75 99

ces pombe, *Saccharomyces cerevisiae*, *Candida parapsilosis*, *Pichia sp. Feni* and *Pichia manshurica*. The isolates from atan were very closely related to *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Candida parapsilosis*, *Pichia kudriavzevii*, *Pichia ethanolica*, and *Schizosaccharomyces osmophilus* (Table 1). The phylogenetic trees (Figures 6 and 7) revealed a structuring of the strains into two distinct clades (Groups I and II) for the beverage "Tchoukoutou," and into four distinct clades (Groups I, II, III, and IV) for the beverage "Atan." This classification was based on comparing the D1/D2 regions of the rDNA, which were widely recognized for their strong discriminatory power at the species level, allowing for the identification of both phylogenetic proximity and evolutionary divergence among the different taxa.

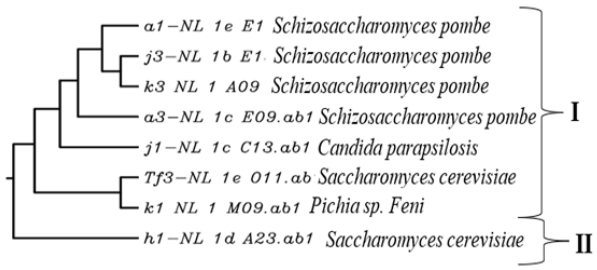

Fig. 6. Phylogenetic tree of yeast strains isolated from « Tchoukoutou » samples based on D1/D2 rDNA sequence analysis.

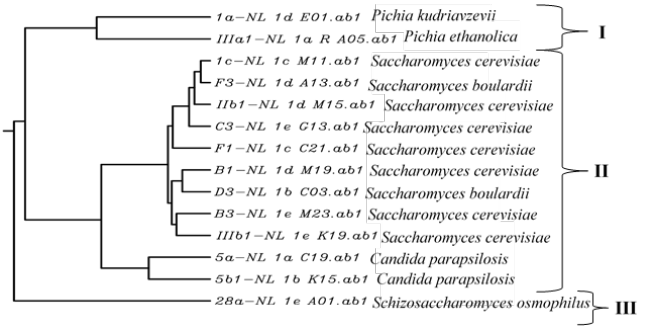

Fig. 7. Phylogenetic tree of yeast strains isolated from "Atan" samples based on D1/D2 rDNA sequence analysis.

4. Discussion

The physicochemical analyses of the traditional beverages show that, for "Tchoukoutou", pH ranges from 3.65 to 3.92, while the Brix degree varies from 1° to 12° Brix across the three studied zones. For the "Atan" beverage, the pH ranges from 3.71 to 4.04, and the Brix degree from 1° to 14° Brix. These values highlight a proportional relationship between pH and Brix degree, which gradually decreases as fermentation progresses (Figures 2 and 3). This observation aligns with findings by [31], who reported that dry matter content progressively decreases during fermentation. As fermentation advances, sugar levels drop and acidity increases, creating an environment that enhances the metabolic activity of microorganisms, particularly yeasts, leading to the conversion of fermentable sugars into ethanol.

The study of the morphological characteristics of the isolated yeasts revealed that all yeast strains share the same shape, cellular arrangement, reproductive mode, and

motility: they are all immobile, ovoid in shape, composed of single cells, and reproduce by budding (asexual reproduction). Microscopic examination of yeast isolates from both "Tchoukoutou" and "Atan" showed that all cultures originated from pure strains. This result is consistent with the findings of [32], who identified the cellular morphology of vineyard yeasts under the microscope and observed cells with similar shapes.

After amplifying the D1/D2 region by PCR for all isolated yeast strains, bands ranging from 520 to 580 bp were obtained. The similarity in sequence length suggests that they could belong to the same genus or species, with slight variations among the strains. These results differ from those obtained by [31], who studied a traditional sorghum beer (tchapalo) in Côte d'Ivoire and reported sequence sizes ranging from 473 to 586 bp. These results also differ from those [33] reported, where the D1/D2 amplicon size for yeasts was 600 bp. These discrepancies could be attributed to several factors, including: The limited resolution of agarose gel, which may introduce bias due to its difficulty in distinguishing fragments differing by only a few nucleotide pairs; The PCR conditions, such as the primers used or cycle parameters, which can influence the sizes of the amplicons obtained; Differences between yeast species or genera: the microorganisms studied may belong to distinct populations influenced by the substrate or local environment.

Molecular identification by sequencing of the 26S rRNA gene revealed yeast diversity among the isolates at the species level. A total of five (05) yeast species were identified in the "Tchoukoutou" beverage and six (06) in the "Atan" beverage.

The observed rich biodiversity could influence the characteristics of fermentation and the quality of the final product. Indeed, each yeast strain has different abilities to convert sugars into alcohol, gas (CO_2) and aromatic compounds. Greater biodiversity means a richer metabolic palette, producing a variety of organic acids, esters, higher alcohols and phenols. As a result, the beverage contains more complex aromas with a unique taste profile (fruity, floral, spicy notes, etc.). Furthermore, not all yeasts have the same ability to produce ethanol or tolerate high alcohol concentrations. Some also produce glycerol or other compounds that soften the drink. Biodiversity, therefore, implies a balance between alcohol, sweetness and acidity, influencing the drinkability of the product. The species Schizosaccharomyces pombe is predominant in the "Tchoukoutou" beverage. This finding aligns with that of [34], who worked on kombucha and found *Schizosaccharomyces* spp., Brettanomyces spp., and Zygosaccharomyces spp. as dominant species in that drink. However, it differs from the results obtained by [35], who reported that the yeast species predominant in the Benin opaque sorghum beer tchoukoutou was Saccharomyces cerevisiae. Our results also differ from those obtained by [9], who studied a traditional sorghum beer produced in sub-Saharan Africa and found *Saccharomyces cerevisiae* as the dominant species. Nevertheless, non-saccharomyces species such as *Schizosaccharomyces pombe*, C. tropicalis, *P. kudriavzevii, Kloeckera apiculata, Pichia anomala, Torulaspora delbrueckii,* and *Kluyveromyces africanus* have been reported as non-dominant by other researchers outside Benin [9, 36, 37] in traditional sorghum beers. These variations in results could be attributed to several factors, such as the raw materials, production technology, and fermentation used during production. *Schizosaccharomyces pombe* is an uncommon yeast in traditional African beverages. Its identification in the tchoukoutou produced in Benin is original and potentially new for this geographical and technological context. This suggests that S. pombe may play a specific role in the fermentation of tchoukoutou (alcohol production, aromas, acidity...). In fact, S. pombe favors the metabolic level of the mevalonate pathway (high expression of ACCAT1, HMGCS1 and HMGCR1 genes) to degrade a high concentration of acetic acid, which improves the beverage's organoleptic quality. It also improves the concentration of a precursor of terpenes, to enhance the taste and quality [38].

This result reflects the natural microbial diversity of traditional fermentations, which is often underestimated. Regarding the "Atan" beverage, *Saccharomyces cerevisiae* is the predominant species involved in its fermentation. This result is comparable to that obtained by [39], who studied "Atan" palm wine produced in Nigeria and found *Saccharomyces cerevisiae* as the predominant species. It is also comparable to the findings of [40], who worked on raffia palm wine (Raphia hookeri) produced in Côte d'Ivoire and reported *Saccharomyces cerevisiae* as the dominant species. The species Schizosaccharomyces pombe (44.44%) and *Saccharomyces cerevisiae* (66.66%) are thus the predominant species involved in the alcoholic fermentation of "Tchoukoutou» and "Atan", respectively.

The phylogenetic tree derived from the drink "Tchoukoutou" strains shows that they belong to an ancient common ancestor, divided into two groups (I and II). Group I consists of two subgroups, with the first subgroup being the most represented. This subgroup includes the strains a1; j3; k3; a3, identified as belonging to the species *Schizosaccharomyces pombe*, and the strain j1, identified as belonging to the species *Candida parapsilosis*, which is divergent from the other strains. This divergence of strain j1 compared to the other strains (a1; j3, k3; a3) could be explained by the fact that they shared a recent common ancestor and might be genetically linked. Meanwhile, the second subgroup includes strain k1, identified as belonging to Pichia sp. Feni and strain Tf3, identified as belonging to the species *Saccharomyces cerevisiae*. This divergence of strain k1 compared to strain Tf3 might be explained by the fact that they shared a recent common ancestor and could therefore be genetically linked. Group II consists solely of strain h1, identified as belonging to the species Saccharomyces cerevisiae, clearly separated from the first group, which marks an evolutionary divergence.

Regarding the strains derived from the drink "Atan," the phylogenetic tree shows that they belong to an ancient common ancestor, divided into three groups (I, II, and III). Group I comprises strains 1a and IIIa1, identified as belonging respectively to the species *Pichia kudriavzevii and Pichia ethanolica*, which are slightly divergent. This divergence of strain IIIa1 compared to strain IIIa1 could be explained by the fact that they shared a recent common ancestor and might therefore be genetically linked. Group II consists of two subgroups, with the first subgroup being the most represented. It contains strains 1c; F3; IIb1; C3; F1; B1; D3; B3; IIIb1, identified as belonging to the species *Saccharomyces cerevisiae*. This first subgroup presents a complex internal structure, composed of multiple subgroups, which demonstrates significant genetic diver-

sity within the species Saccharomyces cerevisiae, likely linked to specific ecological or industrial adaptations. Meanwhile, the second subgroup, which includes strains 5a and 5b1, identified as belonging to the species *Candida parapsilosis*, is separated from the first subgroup, marking an ancient evolutionary divergence and indicating that the two species share an ancient common ancestor. Group III consists solely of strain 28a, identified as belonging to the species *Schizosaccharomyces osmophilus*, which is separated from the first group, marking an ancient evolutionary divergence and indicating that the species of all three groups (I, II, and III) share an ancient common ancestor as reported by [41].

Knowledge of the biodiversity of yeast species involved in the fermentation of Atan and Tchoukoutou produced in Benin would enable the genetic improvement of the most common species in these fermented beverages with a view to improving industrial or artisanal fermentation processes. This could be achieved, firstly, by creating mutant yeasts capable of regulating the alcoholic fermentation of these beverages with a view to their sustainable preservation and industrial-scale production. Secondly, through the production of molecules of interest, certain strains could be used to produce antioxidants, vitamins, probiotics, flavours or other functional compounds, adding nutritional or health value to these beverages.

This study reveals that five (05) yeast species are involved in the fermentation of the traditional beverage "Tchoukoutou" and six (06) yeast species in the traditional beverage "Atan". The species *Schizosaccharomyces pombe*, a yeast not commonly found in traditional African beverages, is the predominant yeast species in "Tchoukoutou," followed by *Saccharomyces cerevisiae*, while *Saccharomyces cerevisiae* is the predominant yeast species in "Atan," followed by *Saccharomyces boulardii* and *Candida parapsilosis*. This data provides valuable information for developing genetic engineering strategies to control their alcoholic fermentation, facilitate packaging, and increase their shelf life for industrial-scale commercialization.

Conflicts of interest

The authors declare that they have no conflicts of interest

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