Lactic Acid Bacteria and Yeasts Associated with Cassava Fermentation to attiébé in Burkina Faso and Their Technological Properties

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Abstract Attiébé is a cassava fermented food introduced in Burkina Faso some decade ago. It has been spread out in all the country. The production is still traditional using a spontaneous way of fermentation. The present study aims to evaluate the main microorganisms groups associated with attiébé production and to identify yeast and Lactic Acid Bacteria (LAB) species according to biochemical (API 20 C AUX and API 50 CHL) and molecular (PCR) methods. Ninety-six (96) samples were collected from three (3) producers during four successive productions. LAB (1.34 up to 6.94 log CFU/g) are the main microorganisms associated to cassava fermentation to attiébé followed by yeasts (1.31 up to 6.25 log CFU/g). Thermo-tolerant Coliforms (1.56 log CFU/g) have been counted in traditional starter only while total coliforms were numbered in both traditional starter and attiébé (1.94 and 0.98 log CFU/g). Candida kruzei/incospicua, Candida norvegensis, Candida parapsilosis, Candida rugose, Candida boidini, Candida tropicalis, Saccharomyces cerevisiae, Trichosporon ashiii were identified as the main yeast species. Enterococcus spp, Lactobacillus brevis, Leuconostoc mesenroides spp mesenteroides/dextranicum, Lactococcus lactis spp lactis, Leuconostoc mesenteroides spp mesenteroides/dextranicum, Lactobacillus fermentum, Lactobacillus plantarum and Lactobacillus buchneri were identified as LAB species associated. A difference between the biochemical and the molecular identification has been observed. The technological properties of these microorganisms need to be highlight for a selection of starter culture. The pH varies from 6.05 to 4.21 and the acidity from 0.5 to 4.8. The final cyanogen content is very low. The cyanogenic potential decrease from 8.6 to 0.07, free cyanogenic acid from 1.7 to 0.064 and the no cyanogenic glucogenic from 3.84 to 0.046. For an efficient fermentation of cassava to attiébé, there is then a need to select appropriate bacterial strains with starch fermenting properties to reduce the production time with high biochemical properties.

Keywords: Attiébé, yeasts, lactic acid bacteria, technological properties

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1. Introduction

Attiébé is a cassava (Manihot esculenta, Crantz) semolina with an agglomerated appearence obtained from peeled fresh cassava tubers which are cut, cleaned, grounded, fermented, wrung, granulled, sifted, pre-dried, zonked, firing steam. It’s then a cassava fermented food [1]. Attiébé is originante from Ivory Coast and was previously produced by Avikams, Alladjans, Adjoukrous and Ebriés people groups [2,3,4,5]. Its production and consumption are now spread in many countries in West Africa and beyond. Firstly imported from Ivory Coast, attiébé is now produced in several areas in Burkina Faso [6]. There is sometimes a difference between attiébé produced in Burkina Faso and the Ivory Coast one. The spontaneous fermentation of cassava roots into attiébé follows a traditional way of process [7]. During the grating operation unit, the cassava dough is mixed with a traditional inoculum as starter culture called magnan. The inoculum is prepared by storing boiled or roasted cassava
roots for three days in a jute bag [7]. The microorganisms associated in cassava fermentation include a wide lactic acid bacteria and Bacillus species. Yeasts and moulds are also part of this fermentation process [8]. Some of these microorganisms are highly desired for their biochemical compounds, which preserve the food, enhance aroma and flavour and reduce anti-nutrients as well as cyanogen. The main microorganisms involved in cassava-fermented food have been described in others countries. However, the associated microorganisms with attiéké production in Burkina Faso is still undescribed. The climatic and environmental conditions as well as the cassava varieties used may have an impact on this microflora loads and diversity. There is also a need for interesting strains from spontaneous fermentation to be select as starter culture in order to control the quality of attiéké. The present study aims then to evaluate the microbial charge of the main products of the process and to identify the dominant microorganisms species associated with cassava fermentation into attiéké in Burkina Faso.

2. Material and Methods

2.1. Sampling

The inoculum or traditional starter culture called magnan, the fermented cassava dough and the final product attiéké of four successive production were sampled. Ninety six (96) samples were then collected from three producing units of different localities in Burkina Faso: Gaoua (Poni province, Sud-Ouest region), Banfora (Comoé province, Cascades region) and Orodara (Kénédougou province, Houet region). Samples were collected using sterile stomacher bags placed in icebox and transported in the microbiology laboratory of Département Technologie Alimentaire (DTA/IRSAT/CNRST), where the main analyses were realized.

2.2. Enumeration of Lactic Acid Bacteria (LAB), Yeasts and Molds and Enterobacteria

The preparation of samples and tenfold dilutions for inoculation were carried out on peptone buffer water according to [9]. For all enumerations, 10 g of each sample were homogenized with 90 mL of sterile peptone buffer water in a stomacher bag. The tenfold serials dilutions were prepared and spread-plated for microorganisms counts. Yeasts and molds were cultivated on Sabouraud-Chloramphenicol Agar (Oxoid LTD, Basingstore, Hampshire, England) after incubation at 25°C for 4 - 5 days and counted according to ISO 7954 [10]. Lactic acid bacteria were cultivated on De Man, Rogosa and Sharpe Agar (MRS, Merck 10660, Merck, Darmstadt, Germany) incubated anaerobically (anaerobic jar) at 37°C for 2-3 days and counted according to [11,12]. Enterobacteria were cultivated on violet red bile glucose agar (VRBG) and after incubation for 24h ± 2h at 37°C and 44°C respectively for total and thermotolerant coliforms, counted according to ISO 4832 [13].

2.3. Isolation and Initial Characterization of LAB

Microorganisms colonies were randomly picked and purified by successive streaking onto the corresponding agar as described by [14]. Morphological characteristics such as colony and cell aspects, forms, grouping mode, mobility were registered. The phenotypical characterization was based upon Gram test, catalase reaction and salt tolerance. Gram test was carried out using KOH (3%) as described by [15]; catalase production was determined by adding to a colony on a glass slide a drop of H₂O₂ solution (30%). For long-term storage, purified isolates were stored at −80°C. The aerobic mesophilic bacteria were stored in Nutrient broth (Oxoid, Basingstoke, UK) containing 20% glycerol (w/w) and LAB were stored in MRS broth (Merck, Darmstadt, Germany) containing 20% (w/w) glycerol. Working cultures were kept at 4°C on MRS agar or broth and were then conserved at -20°C.

2.4. Initial Characterization of Yeasts in Liquid Medium

Each isolated yeast colony was introduced in tubes containing 10 mL of nutrient broth and incubated at 25°C for 3 days up to a week. The grown of yeasts along tubes, the development of a ring in the surface as well as the presence of a deposit on the bottom of the tubes and the turbidity are then noted and used for a primary grouping of associated yeasts in the fermentation.

2.5. Biochemical Identification of LAB and Yeasts Isolates

All the isolates were firstly grouped based on theirs colonies aspects, their forms, mobility, Gram test, catalase. Isolates from each group were randomly picked for the biochemical identification. API 50 CH gallery and API 20 C AUX gallery were used for the biochemical identification of LAB and yeasts respectively according to BioMérieux [16]. For yeasts identification, a cell culture of 18 up to 24 hours is homogenized in the physiological water. The optical density (OD) of the culture was adjusted to have an equivalent of 1 McFarland. Cupules were filled avoiding bubble formation and the gallery are incubated at 30°C for 48 up to 72 hours. The interpretation was based on the turbidity of the cupule and then compare to a witness. For LAB identification, a cell culture of 18 up to 24 hours was homogenized in an API medium. The optical density (OD) of the culture was adjusted to have an equivalent of 1 McFarland. Cupules were filled avoiding bubble formation. Cupules were covered with parafin oil to create anaerobic conditions and then incubated at 37 °C for 24 to 48 hours. Positive cupules were those that coloration was different to the witness.

2.6. Molecular Characterization of Yeasts and LAB

The protocol of [17] was used for the molecular identification of the isolates. The total DNA was extracted
from bacterial culture of 18 up to 24 h, according to the thermal shock method [18]. A loopful of isolated colony of each bacterial was added to 100 µL of sterilized demineralized water and then firstly frozen at -20°C for about twenty minutes. Then, they were introduced in a water bath at 100 °C for ten minutes. The separation of the DNA from the other cell components was obtained by a centrifugation at 13 000 rpm for one minute. The supernatant containing the DNA was collected in PCR tubes. A volume of 2.5 µL of each extracted DNA was homogenized with 4 µL of the Master Mix PCR (5x FIREPol® Master Mix Solis BioDyne, Estonia), 0.5 µL of each primers (forward and reverse) and 12.5 µL of demineralized water. The primers used are showed in table I. DNA fragments were separated by applying 10 µL of each PCR product in 2% (v/p) of agarose gel (SIGMA, Germany) for electrophoresis using electrophorus curve (APPELEX, France). The gel was prepared in 0.5X Tris Borate EDTA (TBE) buffer containing 100 mM of Trizma base, 100 mM of boric acid, 2 mM EDTA and 0.5 µg µL-1 of ethidium bromide (BET). A DNA molecular marker (Solis BioDyne, Estonia) of 100 pb was included as standard for the determination of the size of the fragments. The gel was runned in 0.5 XTBE buffer for 90 minutes at 100 V and 100 mA. The visualization of the strips was done with an ultraviolet light illumination (SYNGENE 1295, UK) and photographed. Primers used are in following Table 1.

2.7. pH and Acidity Variation

Ten gram (10 g) of each sample were dissolved in 50 ml of sterile peptoned buffered water and mixed. The pH was directly measured with a numeric pH-meter (WTW multil line P4). For Total acidity, 10 g of each sample are mixed with 50 ml of distilled water in an erlenmeyer. 10 mL of the dilution were then titrated against 0.1 N KOH using phenolphthalein as indicator; the total acidity was then calculated as a percentage of lactic acid.

2.8. Reduction of Cyanogen Content Base on Enzymatic Method

Both cyanogenic potential content (CNp), free cyanogen content and non-glycosidic cyanogen content was concerned with the cyanogen determination. The enzymatic method is used. After extraction followed an addition of 0.1 ml of linamarase (SIGMA Aldrich, UK) solution with 5 EUml-1 as activity. Free cyanide content was quantified using 0.1ml of each extract. Then 3.9 ml of phosphate buffer pH 4 and 0.1 ml Choramine T (SIGMA Aldrich, UK) reagent was added. The absorbance at 605 nm was measured after 10 min of incubation. Non-glycosidic cyanogens was calculated as the difference between the total cyanogenic potential and the free cyanide content.

2.9. Biochemical and Technological Properties of the Associated Microorganisms

The biochemical and technological properties concerned bile tolerance, evaluated using Bile Esculin Agar, acid production based on Klingberg et al. (2005), their capacity to degrade starch using modified MRS agar which contain starch as only source of carbon. The evaluation of the acidification power of some strains made by the dosage of the acidity of the medium each 6h during 24h. Exopolysaccharides (EPS) production of LAB strains has been putted in evidence using the method of sowing in dials on MRS agar containing 5% of sucrose according to Sawadogo-Lingani et al., (2010). Pathogen reduction in traditional starter, fermented dough and attiéké is evaluation by enumerating total coliforms and fecal coliforms using standard methods [11].

2.10. Statistical Analysis

The data collection as well as the diagrams were performed with Excel version 2010. Analysis of variance and average were done using XLSTAT 2014.5.03.

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### Table 1. List of the select primers

| Primer | Sequence (5' – 3') | References |
|--------|-------------------|------------|
| Candida krusei | CkFKSfor359 | CATTGGCCGTTCATTTGCTTC | [19] |
| Saccharomyces cerevisiae | SC-5fw | ACGAGTCGCTTCTTCTAAG | [20] |
| Lactobacillus sp. | LbF | GGA ATC TTC CAC AAT GGA CG | [21] |
| Lactococcus sp | LcF | CTT TGA GTG ATG CAA TTG CAT C | [22,23] |
| Pediococcus | PdF | GTA AAG TGG CGT GTG TAC TCT AAG CAC CGC | [22,23] |
| Streptococcus spp. | StF | AGA GTT GTA TCC TGG CTC AG | [24] |
| Enterococcus sp. | EnF | TAC TGA CAA ACC ATT CAT GAT G | [25,26] |
| Bactéries lactiques | fDl | (AGAGTTTGATCCTGGCTCAG) | [22, 23] |

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3. Results

3.1. Average Loads of Associated Microorganisms

Lactic acid bacteria are the main bacteria associated with cassava fermentation. The fermented cassava dough showed the high level of microbial charge followed by attiééké and magnan, the traditional starter. The count of yeast and molds varied from 1.31 log CFU/g (traditional starter) to 6.25 CFU/g for fermented cassava dough with an average of 4.33 log CFU/g. The counts of LAB ranged between 1.34 and 6.94 log CFU/g with an average of 4.66. Total coliforms count ranged between 0.98 and 1.94 log CFU/g with an average of 0.97 and the charge of thermotolerant coliforms varied from < 1 to 1.56 log CFU/g with an average of 0.52 log CFU/g. The count of yeast and molds in the traditional starter magnan, is low compare to those obtained in the fermented cassava dough and attiééké. Thermo-tolerant coliforms were found only in the traditional starter. The high level of fermenting bacteria in fermented cassava dough is correlated with an absence of coliforms. The microbiological counts varied according to the producer but also according to productions in the same producing unit. The count of microorganisms in traditional starter, fermented cassava dough and attiééké were shown in Figure 1.

3.2. Basically Characteristics of Yeasts Involve in Cassava Fermentation

About hundred and two (102) isolates of yeasts were picked out from traditional starter (25 isolates), fermented cassava dough (39 isolates) and attiééké (38 isolates) of the three producers. These isolates were grouped based on their aspects on plate count agar, their cell form in microscopy, and their properties in nutrient broth. The properties in nutrient broth include the growth on the top or in the bottom of the tube (oxygen affinity), the density of turbidity and deposit in the bottom. Based on these properties, twenty-five yeast isolates were selected for the biochemical characterization.

3.3. Yeasts Identities Based on API 20 C AUX System

From the twenty one (21) isolates used for API 20 C AUX test and identification, height yeast species were found. They were seven Candida krusei/incospicua (33.33% of isolates), seven Candida norvegensis (33.33%), two Candida parapsilosis (9.52%), one Candida rugosa (4.76%), one Candida boidinii (4.76%), one Candida tropicalis (4.76%), one Saccharomyces Cerevisiae (4.76%), one Trichosporon ashi (4.76%). The percentage of similarity ranged between 62.7 and 99.8%. Candida krusei and Candida norvegensis were the main yeasts species associated with cassava fermentation into attiééké. The characterized isolates are summarized in Table 2 and Table 3. Sugars are differently appreciate by yeasts. D-Glucose is the most used while pectin sugar, D-cellulobiose and D-Melezitose are less used.

The percentage of similarity of the isolates is ranged between 62.7 and 99.8 as it showed in table III. C. krusei/incospicua (n=7) and C. norvegensis (n=7) are the most represented of yeasts species associated in cassava fermentation to attiééké.

There is a difference in the diversity of yeasts associated to attiééké production according to the nature of the samples. Attiééké was found to have the most diversity in yeasts compared to cassava fermented dough and the traditional starter. Some species was specific to the traditional starter (T. ashi), some was specific to the fermented dough (C. tropicalis) and other to attiééké (C. parapsilosis, C. rugose, C. boidinii). This difference is highlight in the following Table 4.

![Figure 1. Microbial loads of cassava products](image-url)
Table 2. Sugar assimilation profile of yeasts isolates based on API 20 C AUX

| Isolates code | L2366, L2130, L3307, L1357, L2314, L2199, L2318, L2194, L3301, L3308, L2215, L3306, L3312, L3309, L3300, L0014, L0011, L0013, L1355 |
|---------------|----------------------------------------------------------------------------------------------------------------------------------|
| D-Glucose     | +                                                                                                                                |
| Pectin sugar  | -                                                                                                                                |
| Xylose        | +                                                                                                                                |
| Galactose     | +                                                                                                                                |
| D-Cellulobiose| -                                                                                                                                |
| D-Lactose     | +                                                                                                                                |
| D-Maltose     | -                                                                                                                                |
| D-Sucrose     | -                                                                                                                                |
| D-Trehalose   | -                                                                                                                                |
| D-Melezitose  | -                                                                                                                                |
| D-Rafinose    | -                                                                                                                                |

Table 3. Yeasts identities according to API 20 C AUX test

| Isolates code | Corresponding species                | Similarity (%) | Percentage of isolates |
|---------------|--------------------------------------|----------------|------------------------|
| L1370, L2366, L2130, L3307, L1314, L2199 (n = 7) | Candida kruzei/incospicua            | >98.60         | 33.33                  |
| L3243, L2318, L2194, L3301, L3308, L2215, L3306 (n=7) | Candida norvegensis                  | 62.7 - >98     | 33.33                  |
| L3312, L3309 (n=2) | Candida parapsilosis                | 99.80          | 9.52                   |
| L3300 (n=1) | Candida rugosa                       | 96.20          | 4.76                   |
| L3014 (n=1) | Candida boidinii                     | 98.20          | 4.76                   |
| L2011 (n=1) | Candida tropicalis                   | 84.80          | 4.76                   |
| L3013 (n=1) | Saccharomyces cerevisiae             | 98             | 4.76                   |
| L1355 (n=1) | Trichosporon ashiii                  | 63             | 4.76                   |

Table 4. Yeast diversity according to the nature of the products

| Species                                | Repartition (%) |        |        |        |
|----------------------------------------|-----------------|--------|--------|--------|
|                                        | Traditional     | Cassava fermented dough | Attiéqué |
|                                        | starter         |                   |         |
| Candida kruzei/incospicua             | 28.57           | 57.14             | 28.57   |
| Candida norvegensis                   | 0               | 50                | 50      |
| Candida parapsilosis                  | 0               | 0                 | 100     |
| Candida rugosa                        | 0               | 0                 | 100     |
| Candida boidinii                      | 0               | 0                 | 100     |
| Candida tropicalis                    | 0               | 100               | 0       |
| Saccharomyces cerevisiae              | 0               | 0                 | 100     |
| Trichosporon ashiii                   | 100             | 0                 | 0       |

Figure 2. Electrophoresis gel analysis of PCR-amplified 16S rDNA fragments obtained with Candida krusei Group Specific Primer, Lane M: 100-bp DNA molecular mass marker (hyperLadder IV), Lane T: negative control, Lxxxx: strains code
3.4. Molecular Characteristics of the Yeasts Isolates Associated to the Fermentation of Cassava

The molecular characteristics aims to certificate previous characterization of isolates. The primers of *Candida krusei* and *Saccharomyces cerevisiae* used revealed that isolates L1370, L2366, L2130, L3307, L1355, L2314 and L2199 belong to *Candida krusei* species and isolate L0013 belongs to *Saccharomyces cerevisiae* specie. The hybridization of the primers and presumed *Candida krusei* DNA strain (359bp) are showed in the Figure 2.

3.5. Basically Characteristics of LAB Isolates

The morphological and physiological characterization of the LAB isolates collected from the traditional starter *magnan*, the fermented cassava dough and the *attiéké* samples showed that all the cells were Gram positive, catalase negative, no motile, spherical or rod (short or long) and grow at different temperatures (10°C, 25°C, 37°C and 45°C). Three hundred twenty (320) LAB isolates were collected.

3.6. LAB Identity According to API 50CHL

The biochemical characterization of the selected LAB using API 50 CHL revealed height (8) different species: *Lactobacillus brevis 1* (16.67%), *Lactobacillus fermentum 2* (5.55%), *Leuconostoc mesenroides spp mesenteroides/dextranicum2* (5.55%), *Lactococcus lactis spp lactis1* (27.77%), *Leuconostoc mesenteroides spp mesenteroides/dextranicum1* (16.67%), *Lactobacillus fermentum* (15.55%), *Lactobacillus plantarum1* (11.11%), *Lactobacillus buchneri* (11.11%). The more representative LAB species according to the biochemical properties are *Lactococcus lactis spp lactis1* followed by *Leuconostoc mesenteroides spp mesenteroides/dextranicum1* and *Lactobacillus brevis 1*. The percentage of similarity of these strains ranged between 75% and 99.90%. The biochemical profile of each strain according to their carbohydrates metabolism is showed in Table 5. L-Fucose, D-Fucose, L-Sorbose, L-Rhamnose, L-Xylose and D-Arabinose are not use as carbon source by any bacterial strain. L-Arabinose, D-Ribose, D-Galactose, D-Glucose, D-Maltose, D-Melibiose are used by all bacterial strain while other sugars are used differently (Table 5).

| Table 5. Lactic acid bacteria profile according to their carbohydrates metabolism |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                   | Bb24, Bc17, Bg07, B119, B219, Bg01 | B02b, B03b, B279 | B255             | B292             | Bb25, Bb31, Bb28 | B166-1, B102     | Bb18, Bb12       |
| D-Arabinose       | -                | -                | +                | +                | +                | +                | +                |
| L-Arabinose       | +                | +                | +                | +                | +                | +                | +                |
| D-Ribose          | +                | +                | +                | +                | +                | +                | +                |
| D-Xylose          | +                | -                | +                | +                | +                | +                | +                |
| L-Xylose          | -                | -                | -                | -                | -                | -                | -                |
| D-Galactose       | +                | +                | +                | +                | +                | +                | +                |
| D-Glucose         | +                | +                | +                | +                | +                | +                | +                |
| D-Fructose        | ?                | +                | +                | +                | +                | +                | +                |
| D-Mannose         | -                | +                | +                | +                | +                | +                | +                |
| L-Sorbose         | -                | -                | -                | -                | -                | -                | -                |
| L-Rhamnose        | -                | -                | -                | -                | -                | -                | -                |
| D-Cellobiose      | -                | +                | +                | -                | +                | -                | -                |
| D-Maltose         | +                | +                | +                | +                | +                | +                | +                |
| D-Lactose         | ?                | +                | +                | +                | +                | +                | +                |
| D-Melibiose       | +                | +                | +                | +                | +                | +                | +                |
| D-Saccharose      | +                | +                | +                | +                | +                | +                | +                |
| D-Trehalose       | -                | +                | +                | +                | +                | +                | +                |
| D-Mélezitose      | -                | -                | -                | -                | -                | -                | -                |
| D-Raffinose       | +                | -                | +                | +                | +                | +                | +                |
| Amidon            | -                | -                | -                | -                | -                | -                | -                |
| D-Turanose        | -                | -                | +                | +                | +                | +                | +                |
| D-Lyxose          | -                | -                | -                | -                | -                | -                | -                |
| D-Tagatose        | -                | +                | -                | -                | -                | -                | -                |
| D-Fucose          | -                | -                | -                | -                | -                | -                | -                |

![Lactic acid bacteria profile according to their carbohydrates metabolism](image-url)
Table 6. LAB identity according to API 50 CHL

| Isolates       | corresponding species                                      | Similarity (%) | Isolate (%) |
|----------------|------------------------------------------------------------|----------------|-------------|
| Bb24,          | Lactobacillus fermentum 1                                  | >81.20         | 5.55        |
| Bc17, Bg07, B119, B219, Bg01 | Lactococcus lactis spp lactis 1                          | >79            | 27.77       |
| B02b, B03b, B279 | Leuconostoc mesenteroides spp mesenteroides/ Dextranicum1 | >79            | 16.67       |
| B255           | Leuconostoc mesenteroides spp mesenteroides/ dextranicum2  | >76            | 5.55        |
| B292           | Lactobacillus fermentum 2                                  | 75%            | 5.55        |
| Bb25, Bb31, Bb28 | Lactobacillus brevis 1                                    | 99.70%         | 16.67       |
| B166-1, B102   | Lactobacillus plantarum 1                                 | 99.90          | 11.11       |
| Bb18, Bb12     | Lactobacillus buchneri                                     | 92.90          | 11.11       |

The identity of the selected LAB isolates according to API 50 CHL is as presented in Table 6. The percentage of similarity is ranged between 75 and 99.90 percent.

3.7. Molecular Characteristics and Identity of LAB Associated in Cassava Fermentation into Attiéké

The molecular identification was carried on to certificate the identity of the associated microorganisms previously performed according to API, based on biochemical properties. The LAB strains is firstly used to confirm that all isolates LAB are really LAB. Then, specific species strains are used for the identification. Figure 3 shows the molecular identification of LAB strains. LAB strains previously characterize reveal themselves to really LAB as it showed in Figure 3.

For Lactobacillus spp. Identification, Lactobacillus spp group specific primer was used. The isolates Bb25, Bb31, B102, Bb28, Bb12, Bb24 that are been formally identified as LAB were found to belong the genus Lactobacillus spp. They was previously identify as Lactobacillus in biochemical level (API 50 CHL). Figure 4 shows the electrophoresis gel analysis of PCR-amplified 16S rDNA fragments obtained with Lactobacillus spp.

Enterococcus spp. group specific primer is used for Enterococcus spp identification. Two isolates (B258 and B214) were identified as Enterococcus spp. They was previously identify as Enterococcus spp according to API 50 CHL results. The electrophoresis gel analysis of PCR-amplified 16S rDNA of Enterococcus spp. Is showed in Figure 5.

Figure 3. Electrophoresis gel analysis of PCR-amplified 16S rDNA fragments obtained with LAB Group Specific Primer, Lane M: 100-bp DNA molecular mass marker (hyperLadder IV), Bxxx: strains code

Figure 4. Electrophoresis gel analysis of PCR-amplified 16S rDNA fragments obtained with Lactobacillus spp Group Specific Primer, Lane M: 100-bp DNA molecular mass marker (hyperLadder IV), Lane T: negative control, Bxxx: strains code
3.8. The Technological Properties of the Associated Microorganisms

Some physiological properties such as Gram, catalase, form, mobility, grouping mode, growth at 25°C, 37°C and 45°C was firstly use for a basic grouping of associated microorganisms in cassava transformation to attiéké. It from those groups that forty (40) lactic acid bacterial isolates was picked up for the technological properties evaluation.

### Table 7. Technological properties of some bacterial strains

| Strains code | EPS Production | Starch Fermentation | Sucrose Fermentation | Bile tolerance | Gas Production | NaCl 6.5% | NaCl 18% | pH<5.3 at 6h | pH<4.8 at 12h | pH<4.2 at 18h | pH<3.85 at 24h |
|--------------|----------------|---------------------|----------------------|----------------|----------------|----------|---------|-------------|---------------|---------------|---------------|
| B413         | -              | -                   | +                    | +              | +              | -        | -       | +           | -             | -             | -             |
| B415         | -              | -                   | +                    | -              | -              | +        | -       | -           | -             | -             | -             |
| B424         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
| B119         | -              | -                   | +                    | -              | +              | +        | -       | -           | -             | -             | -             |
| B272         | -              | -                   | +                    | -              | +              | -        | -       | -           | -             | -             | -             |
| B102         | -              | -                   | +                    | -              | -              | +        | -       | -           | -             | -             | -             |
| B192         | -              | -                   | +                    | -              | -              | +        | -       | -           | -             | -             | -             |
| B222         | -              | -                   | +                    | -              | -              | +        | -       | -           | -             | -             | -             |
| B384         | -              | -                   | +                    | -              | -              | +        | -       | -           | -             | -             | -             |
| B351         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
| B219         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
| B220         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
| B513         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
| B515         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
| B516         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
| B504         | -              | -                   | +                    | -              | -              | -        | -       | -           | -             | -             | -             |
The technological properties concerned are their capacity to use the main carbohydrates in cassava as carbon source, their capacity to reduce cyanogen content level in the final product, to growth in particular conditions, to produce EPS and to preserve food spoilage throughout antimicrobial activity. All the selected microorganisms are EPS negative, use sucrose as carbon source but none use starch. The others properties depend on each isolate as presented in following Table 7.

3.9. Acid Production during Fermentation

During cassava fresh root fermentation to attiéké, pH decrease significantly when acidity increase. The pH vary from 6.05 to 4.21 and the acidity from 0.5 to 4.8 as showed in the following Figure 6.

3.10. Cyanogen Reduction

The cyanogen content is also significantly reduce during the fermentation. The final cyanogen content is very low. The cyanogenic potential (CNP) decrease from 8.6 to 0.07 ppm, free cyanogenic acid (HCN) from 1.7 to 0.064 ppm and the no cyanogenic glycogenic (NGC), from 3.84 to 0.046 ppm. The decrease curve is as showed in the following Figure 7.

![Figure 6. Variation on pH and acidity during attiéké fermentation](image1)

![Figure 7. Cyanogen content reduction during cassava fermentation to attiéké](image2)
4. Discussion

The microorganisms counts, varied according to the type of products (Traditional starter, cassava fermented dough and attiéké). It may be tribute to the hygienic conditions of the process, their nutrient content, their water activity and their physicochemical parameters. The traditional way of the process may be the main factor of the variation of microorganisms counts according to both product and producer. These factors may also explain the difference in the diversity according to the products. The absence of thermo-tolerant coliforms in the final products attiéké could be the result of the fermentation effect that increase the acidity of the product, avoiding undesirable bacterial growth. The results are similar to those found previously by [7]. The content of microorganisms in the inoculum (the traditional starter) is less than those obtained in Ivory Coast by [2] which was 7.75 to 9.23 log CFU for LAB and 7.74 to 8.30 log CFU for yeasts. There is a diversity of the microorganisms associated to attiéké production. Spontaneous fermentation is the main way of cassava fermentation to produce attiéké even in Ivory Cost [2,27,28,29]. Yeasts species identified in this study are diversified (Candida krusei/incospicua, Candida norvegensis, Candida parapsilosis, Candida rugose, Candida boidinii, Candida tropicalis, Saccharomyces cerevisiae, Trichosporon ashi). There is also a difference in the identities of the species between the biochemical and the molecular methods used. The difference between molecular and biochemical identification could be also found in previous study [30]. However, the few number of specific yeast primers of molecular identification could also explain this difference. The spontaneous nature of the fermentation influences on the diversity of yeasts involved in cassava fermentation into attiéké. Yeasts contribute to synthetize volatile organic compounds and unicellular protein in fermented food [31,32,33,34]. But, yeasts also contribute to food spoilation [35]. Some yeast species such as Candida validatied, Candida holmii, Candida krusei, Kloeckera japonica, Saccharomyces cerevisiae were previously identified in traditional starter of attieke made in three villages in Ivory Coast. However Candida tropicalis was the only yeast species identified to be associated to attiéké fermentation according to Coulin et al., (2006). This difference in the diversity of yeast strains in attiéké production may be attribute to many factors. The main factors are the environmental and climatic conditions of production zones [36,37], the mastery of the fermentation process by producers as well as the spontaneous nature of fermentation [7,38,39] the variety of cassava used. On average, yeasts were more representative in fermented doughs and in attiéké than in the traditional starter because of the water activity.

There is also a diversity in LAB among the microorganisms associated with cassava fermentation into attiéké. However, the primers used in the molecular identification were not so specific to species. There is a need of DNA sequencing for a formal identification. Previous studies on the main microorganisms associated with cassava fermentation into attiéké in Ivory Coast found the following species: L. plantarum, L. fermentum, L. celtobiusus, L. brevis, L. mesenteroides, P. acidilactici, W. cibaria; Lactobacillus sp; E. faecium [2,40,41]. A spontaneous fermentation of cassava in Nigeria revealed L. plantarum, L. rhamnosus, L. hilgardii, L. paracasei, W. paramesenteroides, L. mesenteroides, E. faecium, E. casseliflavus, P. acidilactici, as main lactic acid bacteria [42]. There is then a difference in the microbial diversity during cassava fermentation according to areas. The climatic and environmental conditions have an impact on the microbial diversity in cassava fermentation. Djeni et al. (2015) have also confirmed this hypothesis during their investigation among attiéké producers in three localities in Ivory Coast. The concerned communities have some specificities in the production process of attiéké. The same cassava variety was used for a spontaneous fermentation. The mastery of the production process especially the fermentation step has an impact on the microbial diversity, biochemical, nutritional and sanitary parameters as it has been already observed (Guira et al., 2016a). A high level of attiéké production then requires the quality management in units, the mastery of the all production process and specifically the fermentation one. A selection of specific microorganisms with pectinolytic, polygalacturonase enzyme activity for cassava fermentation into attiéké is necessary for more valorization of this fermented food [31,43,44,45,46,47].

The fermentation lead to a great reduction of cyanogen content in the final product (attiéké). The acidity of the environment and the linamarase activity contribute to the reduction of the cyanogen content. The impact of the fermentation on the cyanogen reduction have been already proved in previous studies [8,31,48,49,50,51]. The residual content of cyanogen in attiéké is neglected. The variation in the acidity between cassava root and attiéké is important. But the decrease on the acidity is low compare to the Ivorian and the Beninese one [29,52]. The technological properties of the associate microorganisms explain the kinetic profile. In MRS nutrient broth the pH decrease in to lower than in cassava dough. Not all the isolated LAB strains are fermenting starch and more of them don’t ferment sucrose. On the other hand, starch in the main carbohydrate in cassava. The no fermentation of cassava lead to a long time of the fermentation. Ivorian producer spend few time in the fermentation process than burkinabé because some of the isolated strains in Ivorian attiéké production process can use starch and sucrose as carbohydrate source [2,40]. For an efficient fermentation of cassava to attiéké, there is then a need to select appropriate bacterial strains with starch fermenting properties to reduce the production time with a high biochemical properties [8,53,54].

5. Conclusion

A diversity of microorganisms including yeasts and LAB are associated to the fermentation of cassava into attiéké. Candida spp. is the most dominant yeast whereas Lactobacillus spp, Lactococcus spp, Enterococcus spp, Leuconostoe spp are the main LAB. The environmental conditions and the physicochemical parameters may have been influenced the microbial diversity. A formal identification throughout DNA sequencing is needed as well as the technical and nourishing profile of each strain. The fermentation process contribute to reduce
significantly ce cyanogen content of the cassava fresh root. The spontaneous nature of the process leads to uncontrolled results of the final product. The fermentation of starch by associate spontaneous microorganisms require the use of starter with specific bacterial strains which have high technological properties for quality attiéké production.

**Conflict of Interest**

The authors declare any conflict of interest.

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**Ethical Statements**

This study does not involve any human or animal testing.

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