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Diversity of lactic acid bacteria isolated from "kpètè-kpètè" a ferment of traditional beer "tchoukoutou" produced in Benin

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The counts and identification of lactic acid bacteria isolated from the ferment "kpètè-kpètè" of the traditional beer (*tchoukoutou*) collected from nine large producing cities of Benin were carried out. Out of 209 strains isolated, 135 strains were purified, identified and were member of four different types based on their morphological traits, biochemical tests, API 50CH gallery and genotypic characterization (PCR technique). They are of the genus *Lactobacillus* (72), *Enterococcus* (38), *Leuconostoc* (11), *Streptococcus* (7) and *Pediococcus* (7). But the study of the biochemical and physiological characteristics identified 128 strains of the following species: *Lactobacillus fermentum* (47), *Lactobacillus divergens* (17), *Lactobacillus bif fermentum* (2), *Lactobacillus fructuvoans* (2), *Lactobacillus casei* (2), *Lactobacillus acidophilus* (2), *Enterococcus faecium* (33), *Enterococcus faecalis* (5), *Streptococcus thermophilus* (7) and *Leuconostoc mesenteroides* (11). The genus *Lactobacillus* (56.25%) was dominant followed by the genus *Enterococcus* (25.78%).

Key words: Tchoukoutou ferment, lactic acid bacteria, *Lactobacillus* characterization, Benin.

INTRODUCTION

The lactic acid fermentation is a biological process in which a group of gram positive bacteria, growing under anaerobic conditions and using the carbon sources to produce lactic acid as a major organic acid is involved. In West Africa, the lactic acid fermentation has traditionally

been developed for a wide range of raw materials consisting essentially of starch ($\geq 80\%$ of dry matter) (Ketiku and Oyenuga, 1970; Mugula et al., 2003).

Therefore, cassava and cereals such as maize, sorghum and millet are crushed and fermented to obtain

non-alcoholic products (paste and porridge) and alcoholic beverages that are differently named according to the countries (Odunfa, 1985). For example, the sorghum beer is named “*pito*” or “*burukutu*” in Ghana and Nigeria, “*otika*” in Nigeria, “*dolo*” in Burkina Faso, “*tchapalo*” in Ivory Coast and “*tchoukoutou*” in Benin (Ekundayo, 1969; Chavan and Kadam, 1989; Demuyakor and Ohta, 1991; Iwuoha and Eke, 1996; Sawadogo-Lingani et al., 2007; Amane et al., 2005; Kayodé et al., 2007; Dje et al., 2008).

Generally, fermented foods are prepared exclusively using bacteria or bacteria-yeasts mixed culture. Yeasts and lactic acid bacteria plays an important role in the production of many traditionally fermented foods and beverages all over the world. Thus, many studies have examined the major role of yeast and lactic acid bacteria genera in different sorts of fermented food (meat, milk, cereals and alcoholic beverages) process (Brandt, 2007; Aidoo et al., 2006; Romano et al., 2006). Among those foods, alcoholic beverages such as “*tchoukoutou*” represent a vast diversity of products ranging from table wines, sake, cider, fruit wines, beer and distilled alcoholic products (Kayodé et al., 2007). Lactic acid bacteria, yeasts and molds may be responsible for the fermentation of “*tchoukoutou*” because, it ferment named “*kpètè-kpètè*” is dominated by lactic acid bacteria (7.8 ± 0.16 LogUCF/g) and yeast (8.2 ± 0.36 LogUCF/g) (Kayode et al., 2006).

However, the most studied alcoholic beverages of West Africa are the beers produced from three sorghum species (*Sorghum bicolor*, *Sorghum vulgare* and *Sorghum guineense*) (Steinkraus, 1983; Iwuoha and Eke, 1996). These popular fermented foods and drinks are produced at local scale by small production units (women's cooperatives). It has been reported that *Lactobacillus* are the major genus of lactic acid bacteria isolated from the micro-flora of these fermented beverages (Kayodé et al., 2007; Johansson et al., 1995; Hounhouigan, 1993). That is why the lactic acid bacteria are important for food industry, and are widely used in the production process of fermented foods (Ehrman et al., 1994).

The “*kpètè-kpètè*” is reported to be obtained from a wet deposit of previous “*tchoukoutou*” keeps for approximately one day for decantation. Overall, the producers use a perforated calabash or gourd (containing, “*kpètè-kpètè*” in dried form and stones) to ferment the traditional beer “*tchoukoutou*”. The fermentation is the most important step and has many advantages such as: the reduction of the risk of growth of pathogenic microorganisms by acidification of the medium, the degradation of some anti-nutritional factors (phytates, α -galactosides), the development of specific organoleptic properties by synthesis of organic acids and

aroma (Nout, 2009; Nout et al., 2003). The microbial flora of traditional beers has been widely studied by traditional microbiological tools (Faparusi et al., 1973; Sawadogo-Lingani et al., 2007; Kayodé et al., 2007). The molecular tools can also be used for the identification of several lactic acid bacteria (Atlan et al., 2000). *Lactobacillus* is reported to be the main genus isolated from the “*tchoukoutou*” samples (Kayodé et al., 2007) but the lactic acid bacteria contained in the ferment “*kpètè-kpètè*” of this beer are not yet identified.

Therefore, the present study aimed at documenting the Beninese food micro-flora and to determine the lactic acid bacteria of the ferment “*kpètè-kpètè*” and their genetic diversity in the present species. Through this, the study contributed to realize the first collection of lactic acid bacteria strains with possible application in the food industry. Therefore, once identified, these bacteria could be used as starter cultures to develop food ingredient with probiotic properties.

This study mainly focuses on six (*Lactobacillus*, *Enterococcus*, *Streptococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*) genera of lactic acid bacteria.

MATERIALS AND METHODS

Ferment samples collection

The study areas were divided by considering the large production of traditional beers in Benin. Traditional beer producers were chosen on the basis of a preliminary survey. A total of eighty-ten (90) samples (ten samples per Township) of “*kpètè-kpètè*” were collected in nine Townships (Natitingou, Tanguéta, Boukoumbé, Parakou, N'Dali, Tchaourou, Bantè, Glazoué and Dassa). The “*kpètè-kpètè*” samples were collected in Stomacher bags and kept in coolers with ice (-4°C) and transported within 24 h to the laboratory. Once in the laboratory, the samples were immediately analyzed for physico-chemical and microbiological parameters.

Counting and isolation of lactic acid bacteria

These species of lactic bacteria contained in the 90 samples of “*kpètè-kpètè*” (a wet deposit of former “*tchoukoutou*”) were counted, depending on the spices, on the MRSA, M17 or hyper-sucrose agar media according to previously described methods (Nout et al., 1989; Hounhouigan et al., 1993). The M17 agar medium (Tarzaghi agar, Difco) was used to isolate *Streptococcus* genus (37 to 45°C for 72 h), *Lactococcus* (30°C for 72 h) and *Enterococcus* (30°C for 48 to 72 h). The MRS medium agar (Man Rogosa and Sharpe, Difco) was used to isolate the genus *Lactobacillus* (30 to 45°C for 48 to 72 h) and *Pediococcus* (30°C for 48 to 72 h). The genus *Leuconostoc* was observed on the hyper-sucrose agar (Difco) at 30°C after 72 h of incubation. Thus, a volume of 0.1 mL of 4 dilutions (10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}) were sown in-depth on the agar preparation. After anaerobic incubation at various temperatures, the dishes containing between 15 and 300 colonies are retained for the calculation of the number of Unit Forming Colonies (UFC) of

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lactic acid bacteria per gram of sample. All the results are expressed in Log UFC/g of ferment. After the counting of microorganisms, representative colonies were isolated, inoculated on PCA medium and incubated at 37°C for 24 h. Once isolated, the pure strains aseptically stored brain-heart infusion supplemented with 20% glycerol, incubation for 24 h anaerobically at 37°C, and then stored at -40°C.

Phenotypic identification of lactic acid bacteria

The identification of isolates of lactic acid bacteria was carried out based on the morphology of the bacteria, the Gram test and biochemical characterization tests (catalase production, capability to ferment glucose). The isolates were then identified to species level based on the results obtained from API 50CH gallery tests, biochemical tests and identification criteria reported by several authors (Klein et al., 2001; Bissonnette et al., 2000; Samelis et al., 1994, Ben Amor et al., 2007). All the following described test were performed according to the methods previously described and used by Ben Amor et al. (2007).

Catalase test

A drop of bacterial suspension was transferred in a hydrogen peroxide droplet deposited on a slide. A positive reaction (presence of catalase) results in gas evolution of air bubbles.

Gas production from glucose

The test is to assess the type of metabolism by which the carbon source is transformed to CO₂ and is used to know if the bacteria are either homolactic or heterolactic. So the production of CO₂ incubation was performed at 30°C for 24 to 72 h on glucose MRS-BCP medium (Bacilli) or on glucose M17-BCP medium (*Enterococcus*).

Fermentation of carbohydrates

The fermentation of carbohydrates by lactic acid bacteria isolates was determined using the API 50CH gallery (Bio Merieux, France) and by following the instructions of the manufacturer. The strains were tested twice to determine the reproducibility of the tests and the fermentation profiles were performed.

Sherman test and the thermo-resistance

Some *Enterococcus* was able to grow on milk medium + 0.1% methylene blue after 48 h incubation at 30°C (Samelis et al., 1994). The thermo-resistance bacteria are able to grow at 60°C. Thus, the selection of thermo-resistance species was performed into tubes containing MRS and M17 media at 60°C for 30 min. From the tubes exposed to high temperature culture a fresh culture was performed, and then incubated at 30°C for 24 h to select the strains that resist the temperature (Stiles and Holzapfe, 1997).

Growth tests

This test was carried out for cocci and bacilli, and use to classify strains as thermophilic or mesophilic. Growth was performed in the appropriate medium (MRS or M17 broth) and incubated at 10°C (*Leuconostoc*, *Streptococcus* and *Lactococcus*) for 5 to 7 days,

37°C (*Leuconostoc*), 40°C (*Pediococcus*, *Streptococcus* and *Lactococcus*) and 45°C (*Streptococcus*, *Lactococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* at 45°C) 24 to 48 h.

Production of arginine dihydrolase (ADH) and esculin hydrolysis

This property was detected by using M16-BCP agar (lactose, arginine and purple bromocresol) following the method describe by Thomas (1973). After 24 to 48 h of incubation at 30°C, the lactose-utilizing bacteria acidify the medium (yellow), while those using arginine re-alkalize the medium (purple color).

Growth in the presence of NaCl

The ability to grow on M17 and MRS media + different concentrations of NaCl (2% and 4%) and at various pH values (4.5 and 6.5) was observed after 2 to 3 days of incubation. The isolates were tested according to their genera as follows:

1. For *Streptococcus*, *Lactococcus* and *Lactobacillus* at 2%, 4% of NaCl and pH 4.5 and 6.5
2. For *Leuconostoc* at 6.5% of NaCl and pH 4.5, 4.8 and 6.5 and
3. For *Pediococcus* and *Enterococcus* at 4% and 6.5% of NaCl and pH 4.5, 6.5, 9.6.

It should be noted for Cocci group on MRS and M17 the adequate pH is 9.6 and *Enterococcus* grow in hyper-saline medium (6.5% NaCl).

Production of dextran from sucrose

The MSE agar medium composed by Mayeux et al. (1962) was used only for *Leuconostoc* isolates whereas the citrate utilization was evaluated on Kempler and McKay (1980) medium for isolation of *Streptococcus* and *Lactococcus*.

Production of acetoin

Clark and Lub medium was used to carry out this test (Leveau and Bouix, 1993). This medium was used for the study of two reactions: the test of methyl red (MR) and the Voges-Proskauer test (VPI and VP II).

Genotypic identification of strains to genus level

Extraction and purification of genomic DNA from Gram-positive bacteria

The genomic DNA extraction was performed using QIAamp DNA Mini kit (Qiagen, France) on a 16 h culture of strains (incubated at 30°C) in 5 ml of MRS broth following the manufacturer's instructions. The extracted DNA was stored at -20°C until used.

Amplification of 16S rDNA genes of lactic acid bacteria by PCR (Polymerase Chain Reaction)

The genes (1500 bp) encoding for the 16S rDNA of the lactic acid bacteria were determined by the polymerase chain reaction using specific primers (F and R) of 5 different species (Table 1).

Table 1. List of primer sequences used for PCR.

Genus/ species	Primers	Anneling temperature (°C)	Sequences (5'-3')	References
<i>Lactobacillus</i> sp.	LbF LbR	60	GGA ATC TTC CAC AAT GGA CG CGC TTT ACG CCC AAT AAA TCC GG	Bakar et al. (2010)
<i>Leuconostoc</i> sp.	LnF LnR	57	GAT CCA TCT CTA GGT GAC GCC G CAC CGC TAC ACA TGG AG	Ampe et al. (1999), Savadogo et al. (2004)
<i>Lactococcus</i> sp.	LcF LcR	53	CTT TGA GTG ATG CAA TTG CAT C CAC CGC TAC ACA TGG AG	Ampe et al. (1999), Savadogo et al. (2004)
<i>Pediococcus</i> spp.	PdF PdR	58	GTA AAG TGG CGT GTG TAC CTC AAGCAC CGC TAC ACA TGG AG	Heilig et al. (2002), Savadogo et al. (2004)
<i>Streptococcus</i> spp.	StF StR	57	AGAGTTTGATCCTGGCTCAG GTACCGTCACAGTATGAACTTTCC	Karsidani et al. (2010)
<i>Enterococcus</i> sp.	EnF EnR	55	TAC TGA CAA ACC ATT CAT GAT G AAC TTC GTC ACC AAC GCG AAC	Ammor et al. (2005), Jamaly et al. (2010)
Bacteriocins	BcF BcR	56	AG AGT TTG ATC CTG GCT CAG CTA CGG CTA CCT TGT TAC GA	Diop et al. (2008)

Preparation of PCR mixture and amplification

The PCR reactions were performed in a total volume of 25 µl containing 5 µl of 5x buffer, 3 µl of MgCl₂ (25 mM) 2 µl dNTP (10 mM), 1 µl of each primer (F and R), 0.15 µl of *Taq* polymerase (5 U /µl) and 10 µl of the DNA sample. In the negative control, 10 µl of DNA were replaced by 10 µl of sterile distilled water. The mix were then placed in a thermo-cycler (3 PRIME BASE/02/ Serial :30150, UKA) for amplifications. The initial denaturation was performed at 95°C for 2 min. This denaturation was followed by 30 cycles (denaturation at 95°C/ 45 s, variable hybridization temperature according to the primers (Table 1) for 45 s and elongation at 72°C / 1 min), and a final elongation at 72°C for 5 min.

Electrophoresis of the PCR reaction products

At the end of amplification, an aliquot of 10 µl of each amplification product was mixed with 2 µl of a loading dye. The various mixtures were migrated on 1.2% agarose gel (ethidium bromide) ¾ h at 5 V/cm. The hyper-ladder marker 100 bp (Promega, USA) was used as a molecular weight marker of DNA fragments. The gel was then photographed under ultraviolet rays (Ultraviolet radiation type T. 05X20-2A; 254 nm) with numeric camera.

Identification of lactic acid bacteria

The different results of microscopic observation, catalase

and oxidase tests, the growth test and carbon sources assimilation test (API gallery) were subject to bacteria identification process using the IBIS (Intelligent Bacteria Identification system, the Netherland) software following the description of Wijtzes et al. (1997).

Factorial correspondence analysis (FCA) of genera and species vary by township

The analysis was performed with the MINITAB 16 software to show the grouping of genera and species, and to classify them. This analysis was carried out based on the two groups (G1 and G2) reported on the study previous results on physico-chemical and microbiological characteristics of "kpètè-kpètè" (N'Tcha et al., 2015). The

Table 2. Overall morphological and physiological of presumed genera of traits lactic acid bacteria.

Macromorphology	Micro morphology	Type of fermentation	T°C	Presumed genera	Number of isolates
White, round or lenticular colonies	Cocci, Diplococciand in small chains	Homo-fermentative	37-45	<i>Streptococci</i>	7
Transparent, verysmall and round colonies	Cocci, oval, in small chains	Hetero-fermentative	30	<i>Leuconostoc</i>	11
White, round colonies	Cocci, in small chains	Hetero-fermentative	30	<i>Enterococcus</i>	38
Smooth, rounded greyish colonies	Cocci in tetrads	Homo-fermentative	30	<i>Pediococcus</i>	7
Small white colonies with brown center and curved	Coiled or filamentous long rods, singly or in chains	Homo-fermentative	45	<i>Lactobacilli</i>	22
Small white colonies, round or lenticular	Small rods in chains	Homo-fermentative and Hetero-fermentative	30	<i>Lactobacilli</i>	50

T°C: Growth temperature.

first group G1 was divided into two subgroups G1A (samples of Boukoumbé, N'dali, Parakou and Tchaourou) and G1B (samples of Tanguiéta, Bantè, Glazoué and Dassa). Whereas the G2 group is characterized by the ferments having a low dry matter rate and a very high load in lactic bacteria. The ferment of this group has a low concentration of sugar.

RESULTS

Selection of isolates

A total of 209 isolates was identified from the 90 ferment samples collected. Then the study selected the bacteria that showed specific characteristics of lactic acid bacteria by taking into consideration previous work of several authors (Bekhouche and Boulahrouf, 2005; Badis et al., 2004; Axelsson, 2004; Bizzarro et al., 2000). Thus, 135 isolates were selected, all Gram-positive, catalase negative, immobile and non-sporulating. Microscopic observation of these isolates revealed two types of cells (coccus and rod). The cocci (diplococci in small chains) were 46.67% (63/135) of total number and rod-like cells represent 53.33% (72/135). These 135 isolates, representative of the dominant flora of the ferment, have been identified by phenotypic and

genotypic methods.

Phenotypic identification of isolates

The 135 isolates were divided into 5 genera and classified by dominance order as follows: *Lactobacillus* (72 isolates, 53.33%), *Enterococcus* (38 isolates, 28.15%) *Leuconostoc* (11 isolates, 8.15%), *Streptococci* (7 isolates, 5.19 %) and *Pediococcus* (7 isolates, 5.19%). Overall morphological and physiological of presumed genera are presented in Table 2.

Carbohydrates fermentation of API 50 CH gallery by lactic acid bacteria

In this study, the fermentation of carbohydrates of 128 LAB isolates was examined. The pre-identification was completed by the study of the fermentative properties of strains using API 50CH gallery. The gallery comprises 49 sugars that were tested to identify the strains of lactic bacteria during growth. The results of this Characterization were used as criteria for selecting strains of lactic acid bacteria. Out of 135 isolates of bacteria, 128 isolates were characterized because the isolates belonging to the genus *Pediococcus* (7) were not

identified. The results obtained with the API system allowed us to group species into 10 groups. All isolates fermented glucose, fructose, maltose and mannose. In addition, some strains of the genus *lactobacillus* such as *Lb. Acidophilus* (2); *Lb. plantarum* (2) used esculin and fermented melibiose while others did not utilized esculin for instance *Lb. fermentum* (47). Others did not ferment lactose nor maltose and nor utilized esculin: *Lb. divergens* (17). As regards the cocci, the group 7 was the most dominant and was represented by the species *Enterococcus faecium* (33). This group of bacteria fermented galactose, glucose, fructose, mannose, maltose, sucrose, mannitol and sorbitol but did not ferment lactose and raffinose but utilized esculin. Groups 9 and 10 were represented by the species *Streptococcus thermophilus* (7) and *Leuconostoc mesenteroides* (11) respectively. These groups fermented the same sugars as in group 7 except that it did not use esculin.

Biochemical characteristics of identified genera and species

Lactobacillus

They are rod shaped bacteria. The results of the

Table 3. Physiological and biochemical characteristics of the genus *Lactobacillus* strains.

Tests	Site 1 (3)	Site 2 (12)	Site 3 (13)	Site 4 (18)	Site 5 (8)	Site 6 (5)	Site7 (4)	Site 8 (8)	Site 9 (1)
CO ₂ on glucose	-	-	-	-	-	-	-	-	-
Growth at 10°C	+	+	+	-	+	+	+	+	+
Growth at 30°C	+	+	+	+	+	+	+	+	+
Growth at 45°C	+	+	+	-	+	-	+	+	+
Growth at PH 4.5	+	+	+	+	+	+	+	+	+
Growth at PH 6.5	+	+	+	V	+	+	+	+	+
2%NaCl	+	+	-	+	+	+	+	-	+
4%NaCl	-	-	+	V	-	-	-	-	+
Resistance at 60°C	+	-	+	-	+	+	-	+	+
ADH	+	+	+	-	+	+	+	+	+
Esculin	+	+	+	+	+	-	-	-	-
Lactose	+	+	+	-	+	+	+	+	+
Melibiose	+	+	-	+	+	+	+	+	+
Raffinose	+	+	+	-	+	+	-	+	+
Sucrose	+	-	+	+	+	+	+	-	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: Production of Dihydrolase Arginin; (ND) No Determined.

77 isolates of lactobacilli have led us to classify them in 6 species. The different species were differentiated by their type of carbohydrates fermentation (Table 3). This classification was also performed based on the works of lactic acid bacteria characterization of several authors (Carr et al., 2002; Gunter et al., 1998). The results of identification of 72 isolates of *Lactobacillus* at species level are shown in Table 4. Also, on the basis of different growth temperatures, hydrolysis of arginine and the type fermentation, the genus *Lactobacillus* was subdivided into three groups:

Group of thermophile and strict homo-fermentative lactobacilli: The isolates of this group fermented exoses by producing exclusively lactate, but did not ferment pentose. The associated species were:

- Lb. Acidophilus* (02 isolates) possessed a typical fermentation of carbohydrates (glucose, fructose, mannitol, sucrose).
- Lb. fructivorans*

Group of mesophile and facultative homo-fermentative lactobacilli: The isolates of this group fermented hexoses by producing exclusively lactate, and did not produce gas from glucose. They can ferment pentose. Three species belonging to this group were isolated: *Lb. casei* (2 isolates) typical fermentation

Group of mesophile or thermophile and strict hetero-fermentative Lactobacilli: The isolates of this group fermented hexoses into lactate, acetate and / or ethanol and CO₂. The species isolated were *Lb. fermentum*

(47 isolates and 40% fermented arabinose) and *Lb. fermentum* (2 isolates).

Enterococcus

Thirty-eight (38) isolates belonging to the genus *Enterococcus* were identified. Generally, these strains grew at 10°C in the presence of 6.5% NaCl, and showed a thermo-resistance at 60°C for 30 min. Among the selected strains, thirty-three (33) were citrate negative which bring them closer to the species *Enterococcus faecium*. Additionally, the remaining five (5) strains produced citrate and showed a positive growth in the presence of potassium tellurite. These characteristics are similar to *Enterococcus faecalis* (Table 4).

Leuconostoc

All strains of the genus *Leuconostoc* grew at 37°C, and were resistant to heat treatment of 60°C/30 min (Table 5). These strains also produced dextran. The 11 isolates of *Leuconostoc* species were associated with *Ln. mesenteroides*. The heterogeneity in the fermentation of sugars was observed for the isolates of this species to a very high degree.

Streptococcus

The 7 isolates identified from the genus *Streptococcus* was *Streptococcus thermophilus*. The results obtained with these strains are highly variable (Table 6). These 7 strains were able to grow rapidly at pH 6.5, and slowly at

Table 4. Physiological and biochemical characteristics of strains of the genus *Enterococcus*.

Tests	Site 1 (6)		Site 2 (4)		Site 3 (3)		Site 4 (2)		Site 5 (6)		Site 6 (6)		Site 7 (5)		Site 8 (4)		Site 9 (3)	
10°C	-	+	+	+	+	+	+	+	V	+	V	+	+	+	+	+	+	+
45°C	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-
pH 4.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	+
6.5% NaCl	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-
9.6% NaCl	+	+	+	+	V	+	+	+	+	+	+	+	+	+	+	+	+	+
Sherman bleu	+	-	-	-	-	+	-	-	-	-	-	-	V	+	V	+	+	+
Resistance at 60°C	+	+	+	+	V	V	+	+	+	+	+	+	+	V	+	+	+	V
Acetoin	+	+	+	+	+	-	+	+	-	+	-	-	+	-	+	+	+	-
Citrate	+	+	+	+	+	-	+	+	V	+	+	+	V	-	V	+	+	-
ADH	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CO ₂ on citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: Production of Dihydrolase Arginine.

Table 5. Physiological and biochemical characteristics of strains of the genus *Leuconostoc*.

Tests	Site 1 (1)	Site 2 (1)	Site 3 (1)	Site 4 (1)	Site 5 (1)	Site 6 (1)	Site 7 (3)	Site 8 (1)	Site 9 (1)
Growth at 10°C	-	-	+	-	-	+	-	+	-
Growth at 37°C	+	+	+	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+	+	+	+
Growth at pH 9.6	+	+	+	-	-	-	+	+	+
6.5% NaCl	+	-	-	+	+	+	+	+	+
Resistance at 60°C	+	+	+	+	+	+	+	+	+
Acetoin	+	+	+	+	+	+	+	+	+
Dextran	+	+	+	+	+	+	+	+	+
Esculin	-	-	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-	-
sucrose	-	+	+	+	+	+	-	+	+
fructose	+	+	+	+	+	+	+	+	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: production of dihydrolase arginine.

pH 4.5 and 4.8, they were thermo-resistant, did not produce acetoin and could not hydrolyze esculin, fermented differently some sugars (43% fermented lactose and galactose, 20.6% fermented melibiose, 18% fermented raffinose and 14% fermented melezitose).

Pediococcus

The 7 isolates presumed to belong to this genus showed very variable results. Among the strains belonging to this genus, 4 isolates demonstrated a positive growth at pH 5, 37°C and 45°C and did not ferment maltose. On the other

hand, 3 isolates exhibit the same characteristics as the other strains, but they fermented maltose and showed negative growth at 15% NaCl.

Genotypic characterization of lactic acid bacteria

The PCR amplification using specific primers revealed the presence of the specific gene in various proportion according to the species (Figure 1). The amplification products obtained after amplification of *Lactobacillus* DNA showed bands ranging in size from 200 to 290 bp (Figure 1a).

Table 6. Physiological and biochemical characteristics of strains of the genus *Streptococcus*.

Tests	Site 1 (1)	Site 7 (2)	Site 8 (2)	Site 9 (2)
Growth at 10°C	+	-	+	-
Growth at 45°C	+	+	+	+
Growth at PH 6.5	+	+	+	+
Growth at PH 9.6	+	-	+	-
Resistance at 60°C/ 30 min	+	+	+	+
6.5% NaCl	+	+	+	+
CO ₂ on Citrate	-	-	-	-
Growth on milk " Sherman blue"	-	-	-	-
Acetoin	-	+	-	+
Citrate	-	-	-	-
ADH	+	+	-	-
Esculin	+	+	+	+
Lactose	+	+	+	+
Glucose	-	-	+	+
Mannitol	+	+	+	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: Production of Dihydrolase Arginine.

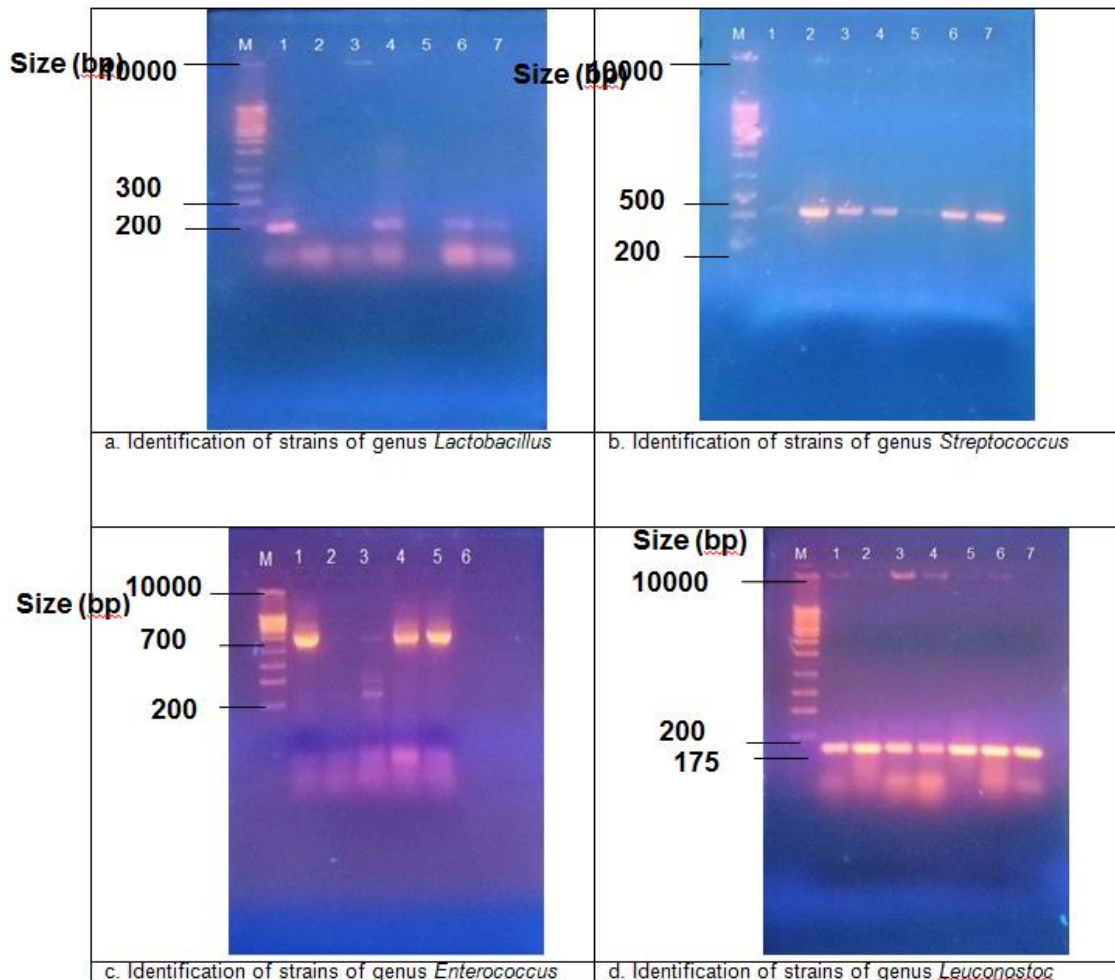


Figure 1. Identification of different strains of Lactic Bacteria using PCR at genus level.

Table 7. Overall of lactic acid bacteria identified.

Site I (Natitingou)	Site II (Boukoubé)	Site III (Tanguiéta)
<i>Lacobacillus fermentum</i> (3)	<i>Lacobacillus fermentum</i> (5)	<i>Lacobacillus fermentum</i> (11)
<i>Enterococcus faecium</i> (5)	<i>Lacobacillus divergens</i> (5)	<i>Lacobacillus divergens</i> (2)
<i>Enterococcus faecalis</i> (1)	<i>Lacobacillus acidophilus</i> (2)	<i>Enterococcus faecium</i> (2)
<i>Leuconostoc mesenteroides</i> (1)	<i>Enterococcus faecium</i> (4)	<i>Enterococcus faecalis</i> (1)
<i>Streptococcus thermophilus</i> (1)	<i>Leuconostoc mesenteroides</i> (1)	<i>Leuconostoc mesenteroides</i> (1)
Site IV (Parakou)	Site V (N'Dali)	Site VI (Tchaourou)
<i>Lacobacillus fermentum</i> (8)	<i>Lacobacillus fermentum</i> (7)	<i>Lacobacillus fermentum</i> (1)
<i>Lacobacillus plantarum</i> (2)	<i>Lacobacillus casei</i> (1)	<i>Lacobacillus casei</i> (1)
<i>Lacobacillus divergens</i> (6)	<i>Enterococcus faecium</i> (5)	<i>Lacobacillus divergens</i> (3)
<i>Lacobacillus fructivorans</i> (2)	<i>Leuconostoc mesenteroides</i> (1)	<i>Enterococcus faecium</i> (5)
<i>Enterococcus faecium</i> (2)		<i>Enterococcus faecalis</i> (1)
<i>Leuconostoc mesenteroides</i> (1)		<i>Leuconostoc mesenteroides</i> (1)
Site VII (Bantè)	Site VIII (Glazoué)	Site XV (Dassa)
<i>Lacobacillus fermentum</i> (4)	<i>Lacobacillus fermentum</i> (8)	<i>Lacobacillus fermentum</i> (1)
<i>Enterococcus faecium</i> (4)	<i>Enterococcus faecium</i> (4)	<i>Enterococcus faecium</i> (4)
<i>Enterococcus faecalis</i> (1)	<i>Leuconostoc mesenteroides</i> (1)	<i>Enterococcus faecalis</i> (4)
<i>Leuconostoc mesenteroides</i> (3)	<i>Streptococcus thermophilus</i> (2)	<i>Leuconostoc mesenteroides</i> (1)
<i>Streptococcus thermophilus</i> (2)	-	<i>Streptococcus thermophilus</i> (2)

Table 8. Value and proportion of information concentrated on the axes.

Informations sur les axes	Value	Proportion	Cumulative
Axis1	0.2269	0.9301	0.9301
Axis 2	0.0170	0.0699	1.0000

The 733 bp bands correspond to the genus *Enterococcus* (Figure 1b), the ones of 175 to 200 bp correspond to *Leuconostoc* (Figure 1c), those of 500 to 600 bp correspond to the genus *Streptococcus* (Figure 1d), and those of 500 to 1000 bp correspond to a toxin (bacteriocin) produced by lactic acid bacteria. None gene encoding for the *Pediococcus* were identified.

Distribution of lactic acid bacteria species isolated from ferment “kpètè-kpètè”

The results of the distribution of lactic acid bacteria according to the species are presented in Table 7. The number of species identified varied from one collection site to another. The species, *Lb. fermentum* (47) were found in all areas of the study, while *Lb. acidophilus* (2) and *Lb. fructivorans* (2) were found only in Boukoubé and Parakou respectively.

Factorial correspondence analysis (FCA)

Table 8 shows the values and the proportions of information concentrated on the axis. Two major

components accounted for 100% of the total variation of information. Axis 1 stands for 93.01% of the information and axis 2 account for 6.99% of the initial information. The study can therefore consider the two axes for the interpretation of results.

Correlation between the principal components and the original variables

Table 9 shows the correlations between the principal components and the original variables. The analysis of the results show that the parameters, *Lb. divergens*, *Lb. plantarum*, *Lb. fructivorans*, *Lb. casei*, *Lb. acidophilus*, *En. faecalis*, *Ln. mesenteroides* and *St. thermophilus* were positively correlated to the first axis (PC1). *En. faecium* parameter was correlated to the axis (PC2) while *Lb. fermentum* was positively correlated to the two axes (PC1 and PC2).

Grouping of genera and species according to their similarities

Figure 2 shows the FCA with the different group of ferments according to their similarities in relation to the

Table 9. Correlation between the principal components and the initial variables.

Variable	PC1	PC2
<i>Lactobacillus fermentum</i>	0.417	0.583
<i>Lactobacillus divergens</i>	0.991	0.009
<i>Lactobacillus plantarum</i>	1.000	0.000
<i>Lactobacillus fructivorans</i>	1.000	0.000
<i>Lactobacillus casei</i>	1.000	0.000
<i>Lactobacillus acidophilus</i>	1.000	0.000
<i>Enterococcus faecium</i>	0.049	0.951
<i>Enterococcus faecalis</i>	0.994	0.006
<i>Leuconostoc mesenteroides</i>	0.971	0.029
<i>Streptococcus thermophilus</i>	0.963	0.004

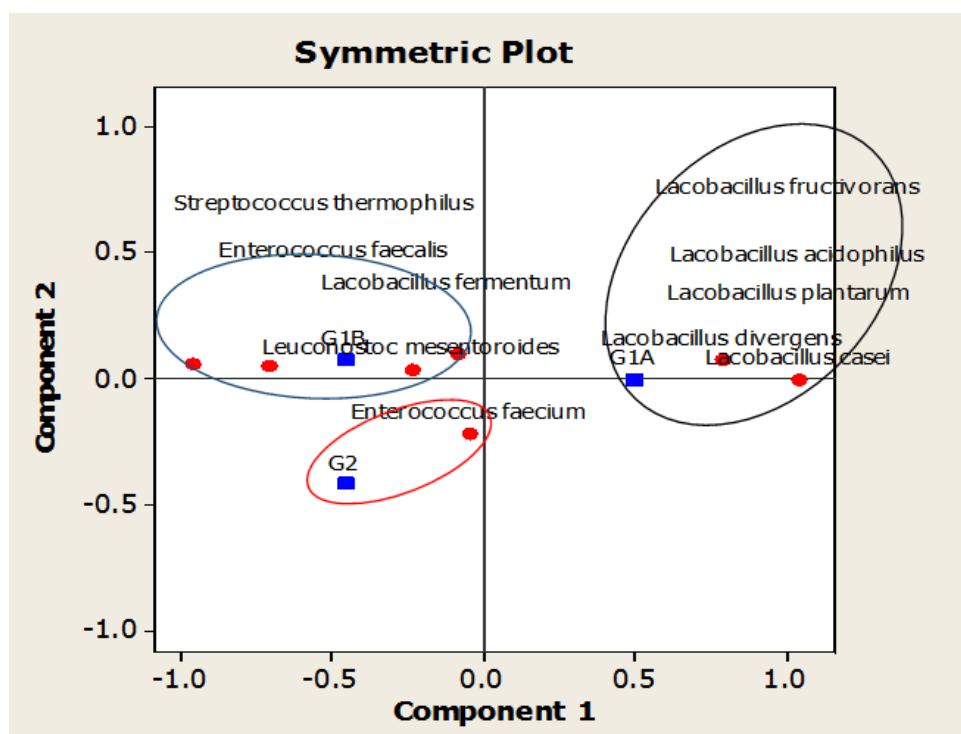


Figure 2. Grouping of lactic acid bacteria identified according to their similarity.

genera and species. The axis 1 shows a correlation between the subgroup G1A and 5 lactic acid bacteria (*Lb. divergens*, *Lb. plantarum*, *Lb. fructivorans*, *Lb. casei* and *Lb. acidophilus*). The subgroup G1B and 3 lactic acid bacteria (*En faecalis*, *Ln. mesenteroides*, *St. thermophilus*) were negative correlated to the Axis 1. The axis 2 connects ferment of group G2 and 2 species of bacteria (*Enterococcus faecium* and *Lb. fermentum*). Thus, Figure 2 shows that the ferments of the subgroup G1A is characterized by lactic acid bacteria such as *Lb. divergens*, *Lb. plantarum*, *Lb. fructivorans*, *Lb. casei*, *Lb. acidophilus*. The second subgroup (G1B) is composed of ferments from Tanguiéta, Bantè and Glazoué. These

ferments were essentially characterized by lactic acid bacteria such as *Lb. fermentum*, *En faecalis* and *Ln. mesenteroides* *St. thermophilus*. The second group (G2) includes only the ferments from Natitingou. These ferments were characterized by lactic bacteria such as *En. faecium* and *Lb. fermentum*. This results shows that the strains are unequally distributed in the ferment coming from various cities.

DISCUSSION

The study of morphological, biochemical, physiological and genotypic characters of 128 isolates isolated from

the ferment “*kpètè-kpètè*” of traditional beer produced in Benin revealed 4 genera (*Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Streptococcus*) and 10 different species. Their number varies from a collecting site to another. The most frequently isolated genus were *Lactobacillus* (56.25%). Five (5) species of *Lactobacillus* (*Lb. fermentum*, *Lb. divergens*, *Lb. bif fermentum*, *Lb. fructivorans* and *Lb. casei*, *Lb. acidophilus*) were rod shaped and found in all the ferment samples from the nine collecting sites. The physiological and biochemical properties of species identified in “*kpètè-kpètè*” were similar to those reported by Hounhouingan et al. (1993) and kayodé et al. (2007). The predominance of the genus *Lactobacillus* was reported by kayodé et al. (2007) in their study on the characterization of lactic acid bacteria in traditional beer “*tchoukoutou*”. The authors reported the predominance of the genus *Lactobacillus* in the traditional beer *tchoukoutou* represented by 8 species (*Lb. Divergens*, *Lb. fermentum*, *Lb. bif fermentum*, *Lb. fructivorans*, *Lb. viridescens*, *Lb. hilgardii*, *Lb. kandleri*, *Lb. casei*). The composition of lactic acid bacteria is relative and depends on different criteria used in each study as reported by Bissonnette et al. (2000). In this study, *Lb. fermentum* (36.72%) was largely dominant and isolated from 47 samples of strains collected in the 9 sites.

These results are also similar to those of Hounhouigan (1993) in their study on composition of microbiological and physical attributes of “*mawè*,” a fermented maize dough from Benin. This study results are also similar with those obtained by Vieira-Dalodé et al. (2007) during the process of “*gowé*” fermentation when they found that *Lb. fermentum* was the most predominant species of *Lactobacillus*. Muyanja et al. (2003) also enumerated the predominance of *Lb. fermentum* in “*bushera*” a Ugandan traditional fermented beverage. In Tanzania, Mugula et al. (2003) reported the same findings in the production of “*togwa*” (fermented drink). In Saudi Arabia, Gassem (2002) observed on the “*sobia*” (fermented beverage) that *Lb. fermentum* was the most dominant species throughout the fermentation process of the fermented beverage.

Leuconostoc mesenteroides isolated from “*kpètè-kpètè*” has also been isolated from *mawè* (Hounhouigan et al., 1993) and from *gowé* (Vieira-Dalodé et al., 2007). Moreover, *Lactobacillus* sp. and *Leuconostoc* were the main microorganisms identified, respectively, during the fermentation of sorghum for the production of “*pito*” and “*burukutu*” in Nigeria (Ekundayo, 1969; Faparusi et al., 1973). Lactic acid bacteria belonging to the genera *Lactobacillus* and *Leuconostoc* were also identified during the fermentation of “*pito*” in Ghana (van der AaKühle et al., 2001). Recently, the dominant micro-flora during the transformation of “*dolo*” and “*pito*” (fermented beverage) from four production sites in Burkina Faso and Ghana were studied (Sawadogo-Lingani et al., 2007). Moreover, several other authors (Kunene et al., 2000;

Steinkraus, 1996) have shown the dominance of *Lb. plantarum* in the fermented food produced from cereal (sorghum, maize). The differences in quantity and quality noticed in our study are probably related to the difference of the physico-chemical composition of these ferments. In general, specific species of lactic acid bacteria become dominant in any particular ecological niche and based on the physicochemical niche diversity, exhibit metabolic diversity (Ali et al., 2012)

Among the identified lactobacilli, most belongs to the group of thermo-bacterium because they grew at 45°C (*Lb. fermentum*, *Lb. acidophilus*, *Lb. casei*) (Robinson, 2002; Bedi et al., 2005). Other lactic acid bacteria were also identified in “*kpètè-kpètè*”: *St. thermophiles*, *En. faecium* and *En. faecalis*. These species have been reported in several cereal fermentation (Muyanja et al., 2003; Mugula et al., 2003). The role of *En. faecium* in “*kpètè-kpètè*” may warrant further investigation despite the question of its potential pathogenetic. The occurrence of *St. thermophilus* in “*kpètè-kpètè*” samples; may indicate different fermentation conditions. The growth of these species has been shown to be favoured by high temperatures and they have been isolated from food materials fermented at higher temperatures (Muyanja et al., 2003). *St. thermophilus* was also found to be effective in acidifying wet maize slurry, but it is usually not acid tolerant and is quickly inhibited by low pH values.

The microscopic observations and the phenotypic characterization were associated with the API 50CH system to better identify the strains. However, these identification methods do not always provide a sufficient basis for reliable identification of *Lactobacillus* as reported by other authors (Nigatu, 2000; De Angelis et al., 2001; Muyana et al., 2003). Therefore, these identification methods have been supplemented by genotypic characterization through the PCR technique, which is more reliable instrument of identification.

The factorial correspondence analysis (FCA) allowed us to group the genera and species identified in the ferments of different townships in our study. Diversity in the distribution of genera and species in ferments was observed. The analysis of the results showed that there was a highly significant difference between dry matters and highly significant between charges in lactic acid bacteria. This significant difference between these parameters could explain the distribution of genera and species of ferments from different townships. As a matter of fact, in metabolic reactions, there is use of the dry matter and water production by lactic acid bacteria (Hounhouigan et al., 1993). . On the other hand, the ferments characterized by high dry matter values promote the growth of genera such as *Lactobacillus* and *Streptococcus*. These results are in agreement with the studies of several authors (Greppi et al. 2013; Sefa Dedeh et al. 1999) who reported that the distribution frequency of microbial species isolated from African

beers vary according to locality and ingredients for brewing.

Fermentation is the only way to preserve the traditional beer "tchoukoutou" for three days. To improve this traditional fermentation, controlled fermentation using starter culture is very important in the production of this beer well consumed in Benin. Starter cultures have important industrial features. Our study contributes to find new strains of lactic acid bacteria in traditional products. It is essential to continue research and study the probiotic properties of lactic acid bacteria for their use in the manufacture of foods for nutrition of domestic animals and fish and humans. The differences in quantity and quality of species noticed in this study are probably related to the difference of the physico-chemical composition and the process use to product these ferments.

Furthermore, these results have indicated that several different species of lactic acid bacteria can be contained in the ferment "kpètè-kpètè".

Conclusion

In general, the morphological, physiological, biochemical and genotypic analysis showed a variety of lactic acid bacteria's genera, and species isolated from the ferment of Beninese traditional beer "tchoukoutou". The results of the identification tests showed 128 strains which demonstrated various distributions from the "Kpètè-kpètè". Therefore, there is a need for investigation into the selection of the most suitable strains that induce the best control of "tchoukoutou" fermentation. The starch-fermenting strains might be important in the development of the starter cultures and for its use in the development of small-scale commercial production of "kpètè-kpètè". The potential use of strains as starter cultures will depend on various parameters such as probiotics properties.

Conflict of interests

The authors declare that they have no conflict of interests

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