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Predominant Lactic Acid Bacteria Involved in the Spontaneous Fermentation Step of Tchapalo Process, A Traditional Sorghum Beer of Cote D'ivoire

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Abstract: Fermented microflora involved in the spontaneous fermentation step of tchapalo processing was investigated. A total of 180 samples of the non fermented product (mash) and the fermented product (sour wort) were analysed by standard methods and Api 50 CH identification system. Difference in pH between mash (5.65) and sour wort (3.71) was significant ($p<0.05$). Lactic acid bacteria isolated in mash before fermentation belonged to the genus *Lactobacillus* (5.5×10^8 cfu mL^{-1}), *Enterococcus* (9.8×10^7 cfu mL^{-1}), *Pediococcus* (9.7×10^7 cfu mL^{-1}) and *Leuconostoc* (5.8×10^7 cfu mL^{-1}). During the spontaneous fermentation there was an increase of the load of *Lactobacillus*, which remained predominant in sour wort at the end of fermentation (89.6%). The load of other lactic acid bacteria and yeasts decreased and this evolution varied according to areas. Thus, this spontaneous fermentation was essentially governed by *Lactobacillus* strains. The physiological analysis of 114 *Lactobacilli* randomly selected revealed 25 different profiles indicating a diversity of *Lactobacillus* strains implicated in the spontaneous fermentation. Almost all profiles observed were specific to the studied area. Among the *Lactobacillus* isolates from physiological study, there were 82.5% heterofermentative strains including *Lactobacillus fermentum*, *Lactobacillus cellobiosus*, *Lactobacillus brevis*, *Lactobacillus coprophilus* and 17.5% homofermentative strains including *Lactobacillus plantarum*.

Key words: *Spontaneous fermentation*, lactic acid bacteria, mash, sour wort, traditional beer, tchapalo

INTRODUCTION

For centuries, sorghum has been an important staple food in the semi-arid tropical regions of Asia, Latino-America and Africa. It is therefore, one of the main source of carbohydrates, proteins, vitamins and mineral for millions of poor people in these regions (Michodjèhoun *et al*., 2005). Sorghum is involved in cooking of many foods such as porridges, pastes and traditional beverages in Africa. These beverages take different names according to regions, where they are produced; for example dolo in Burkina Faso (Sawadogo *et al*., 2007), pito in Ghana (Sefa-Dedeh *et al*., 1999), burukutu in Nigeria (Faparusisi *et al*., 1973), Amgba in Cameroun (Chevassus *et al*., 1976), bili-bili in Tchad (Nanadoum *et al*., 2006) and tchapalo in Cote d'Ivoire (N'Da and Coulibaly, 1996).

In Cote d'Ivoire, the sorghum is generally consumed as a beverage in both urban and rural areas. Indeed, in this country, production of sorghum is not in large quantities (an average of 30000 tons). Nevertheless, great part of this production is used to produce tchapalo. Tchapalo is an opaque, sour beer, which contains a large amount of insoluble material and is always fermenting (Yao *et al*., 1995). In addition, to therapeutic properties assigned, it has nutritional value contributing significantly to improve the diet of consumers. Tchapalo is generally consumed during rural work, popular festivities and funerals. It is also used to welcome and to cool down somebody. This beverage production is rooted profoundly in ethnics tradition of Northeast and North of the country and is essentially managed by women. For these women, tchapalo production is today a real economic activity productive of revenue throughout the whole country, particularly at Abidjan (N'Da and Coulibaly, 1996).

The tchapalo processing comes down in deplorable hygienic conditions with rudimentary equipments, expensive and laborious activities and the use of dried yeast harvested from previous tchapalo (Yao *et al*., 1995;

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N'Da and Coulibaly, 1996; Aka *et al*., 2008). It involves a spontaneous and an alcoholic fermentations. The spontaneous fermentation depends on environmental and climatic conditions. Consequently, the quality of finished product varies often from one production to another. This step is obligatory and very important because it determines the further to process, organoleptic properties and the preservation of the sweet wort.

In the objective to improve microbiology safety, organoleptic and nutritional quality, microbiological, biochemical and physical studies are widely carried out (Chevassus *et al*., 1976; Yao *et al*., 1995; Sefa-Dedeh *et al*., 1999) and also, molecular characterisation of yeasts (Van Der Aa Kuhle *et al*., 2001; Naumova *et al*., 2003; Nanadoum *et al*., 2005). Lactic acid bacteria involved in the spontaneous fermentation step of tchapalo production in Cote d'Ivoire has not been investigated.

The objectives of this study are to get available information on the dynamics growth of the predominant fermented microflora involved in tchapalo production and to develop starter culture. The present paper deals with isolation and identification of lactic acid bacteria involved in spontaneous step of tchapalo processing.

MATERIALS AND METHODS

Tchapalo processing: The tchapalo processing started with the malting about 33-50 kg of sorghum grain, sun-dried and milled to give malted sorghum flour. This flour was mixed out with around 200-300 L of water containing a sticky substance. This substance came from the bark of a shrub (Anogeissus leo carpus); it made decanting easier. The mixture obtained called mash was separated from supernatant and sediment. The sediment was cooked during 2-2 h 30 min, later mixed with the supernatant to give wort. The wort was left for a spontaneous fermentation during the night to give after percolation the sour wort. The sour wort was cooked during 5-6 h, cooled and inoculated with about 0.6-1.6% of dried yeast harvested from previous tchapalo for alcoholic fermentation during 9-12 h. The product obtained after alcoholic fermentation is tchapalo.

Sampling: Sampling was carried out on mash and on sour wort. They were collected from three areas (Abobo, Attecoube and Yopougon) randomly selected in the district of Abidjan. Within each area, one producer was also randomly selected. Sampling were collected in sterile small bottles, labelled and then transported to the laboratory in a box containing a freezing pack. A total of ninety mash samples and ninety sour wort samples were collected from these three areas.

Determination of pH: The pH was determined with a pH-meter (TOLEDO) and two independent measurements were made on each sample.

Enumeration and isolation of microorganisms: Ten millilitres of each sample were aseptically added into 90 mL of sterile 0.9% NaCl solution and mixed. Serial dilutions (10^{-1}-10^{-7}) are performed and 0.1 mL aliquot of the appropriate dilution is directly inoculated in duplicate on following media: Man Rogosa Sharpe agar (MRS, AFNOR, NF ISO 15214) incubated anaerobically at 30°C for 48 h for enumeration of *Lactobacillus* and *Pediococcus*; Bile Esculin Azide agar (BEA, ISO 7899/1) incubated at 37°C for 48 h for enumeration of *Enterococcus*; Mayeux agar (Mayeux *et al*., 1962) incubated at 25°C for 48 h for enumeration of *Leuconostoc*; Chalmer agar incubated at 25°C for 48 h for enumeration of *Lactococcus*; Sabouraud-chloramphenicol agar incubated at 25°C for 3-5 days (s) for enumeration of yeasts. Two hundred and twenty two colonies were picked randomly from plates of MRS (150), BEA (36), Mayeux (36) and purified by repeated plating. Isolates were cultivated in MRS broth and preserved in MRS broth using 15% (v v^{-1}) glycerol at -80°C.

Phenotypic characterizations: Presumptive lactic acid bacteria strains isolated from different media were assigned to a genus on the basis of key characteristics and tests (Kostinek *et al*., 2005; Ricciardi *et al*., 2005; Tamang *et al*., 2005; Yousif *et al*., 2005; Bahiru *et al*., 2006). Morphological and arrangement of cell were examined by microscopy. Growth at 15, 45 and 51°C in MRS broth was determined by visual turbidity after 1-5 days (s) of incubation. Gas production from glucose was assessed in MRS broth containing inverted Durham tubes. The ability to grow at different pH (3, 3.9 and 9) was tested. The salt tolerance was done using MRS broth containing 4 and 6.5% (w v^{-1}) NaCl.

Identification of the Lactobacillus at the species level: Twenty isolates of MRS were selected for identification to species level using the Api 50 CH galleries and Api 50 CHL medium (bio Merieux, l'Etoile, France). Tests were performed according to the manufacturer's instructions. The APILAB Plus database (bio Merieux, France) was used to interpret the result.

Statistical analyses: Significance in variation, in microbial count, in samples between and within areas was analysed using a one-way ANOVA method.

RESULTS

pH evolution: The average pH of mash was 5.65±0.36 and varied significantly ($p<0.05$) between productions but did

Table 1: pH and counts (cfu mL^{-1}) of microorganisms involved in the spontaneous fermentation in the tchapalo processing

	Mash				Sour wort			
Products microorganisms	X (cfu mL^{-1})	SD	Min. (cfu mL^{-1})	Max. (cfu mL^{-1})	X (cfu mL^{-1})	SD	Min. (cfu mL^{-1})	Max. (cfu mL^{-1})
Lactobacilli	5.5×10^{8}	7.4×10^{8}	1.0×10^{6}	2.89×10^{9}	1.2×10^{9}	1.2×10^{9}	7.0×10^{6}	4.9×10^{9}
Pediococci	9.7×10^{7}	1.8×10^{8}	3.0×10^{5}	6.6×10^{8}	9.8×10^{7}	2.6×10^{7}	0.9×10^{5}	7.8×10^{8}
Leuconostoc	4.3×10^{7}	5.8×10^{7}	9.0×10^{5}	3.17×10^{8}	3.4×10^{6}	9.3×10^{6}	0.6×10^{5}	3.7×10^{7}
Enterococci	9.8×10^{7}	1.7×10^{7}	2.0×10^{5}	1.08×10^{9}	1.5×10^{7}	2.3×10^{7}	0.3×10^{5}	1.22×10^{8}
Yeasts	1.2×10^{7}	1.8×10^{7}	2.5×10^{7}	1.06×10^{8}	2.5×10^{6}	2.9×10^{6}	$0,3\times10^{5}$	2.87×10^{7}
pH	5.65	0.36	4.94	6.29	3.71	0.23	2.99	4.25

X: Geometric mean, SD: Standard Deviation, Min: Minimum value, Max: Maximum value

Table 2: Characteristics of lactic acid bacteria isolated in the mash and the sour wort

Characteristics	*Lactobacillus*	*Pediococcus*	*Leuconostoc*	*Enterococcus*
Cell morphology	Rods/cocobacilli	Spherical	Spherical/lanticular	Spherical
Cellular arrangement	Single, pairs, chains	Tetrads	Pairs	Pairs/short chain
Catalase activity	-	-	-	-
Oxydase activity	-	-	-	-
Gas from glucose	±	-	+	-
Growth at temperature (°C)				
15	±	-	+	+
45	±	+	-	+
51	-	-	-	-
Growth in pH				
3	±	-	-	-
3.9	±	+	-	+
9	±	+	+	+
Growth in NaCl (%)				
4	±	+	±	+
6.5	±	-	-	±

+: Positive, -: Negative, ±: Response varied between species

not varied ($p>0.05$) between areas. After spontaneous fermentation, the pH of sour wort obtained fell down to 3.71±0.23 and it varied significantly ($p<0.05$) between productions and one area to another.

Enumeration of microorganisms responsible of the spontaneous fermentation: Lactic acid bacteria counts in mash before the spontaneous fermentation were higher than that of yeasts and constituted 98.4% of fermented microflora in mash. *Lactobacillus* ($5.5\times10^{8}\pm7.4\times10^{8}$ cfu mL^{-1}) was predominant; *Pediococcus* and *Enterococcus* had practically the same number in the mash ($9.7\times10^{7}\pm1.8\times10^{8}$ and $9.8\times10^{7}\pm1.7\times10^{7}$ cfu mL^{-1}, respectively). The lowest count of Lactic acid bacteria was that of *Leuconostoc* ($4.3\times10^{7}\pm5.8\times10^{7}$ cfu mL^{-1}) (Table 1). The same genus were found at the end of spontaneous fermentation and *Lactobacillus* ($1.2\times10^{9}\pm1.2\times10^{9}$cfu mL^{-1}) remained the predominant microflora (89.6%). Count of *Pediococcus* ($9.8\times10^{7}\pm2.6\times10^{7}$ cfu mL^{-1}) was practically the same than that observed in mash, while that count of *Enterococcus* ($1.5\times10^{7}\pm2.3\times10^{7}$ cfu mL^{-1}), *Leuconostoc* ($3.4\times10^{6}\pm9.3\times10^{6}$ cfu mL^{-1}) and yeast ($2.5\times10^{6}\pm2.9\times10^{6}$cfu mL^{-1}) have decreased a lot. *Lactococcus* was not isolated in all samples.

Physiological study of 222 strains randomly selected showed that *Pediococcus* and *Enterococcus* did not produce gas; thus, they were homofermentative strains. *Leuconostoc* were heterofermentative strains (Table 2). Among 114 *Lactobacilli*, 82.5% were heterofermentative strains and 17.5% were homofermentative strains. These *Lactobacilli* showed 25 different phenotypic profiles indicating a diversity of *Lactobacillus* strains involved in the spontaneous fermentation. Almost, all profiles observed were specific to the studied areas (result not shown).

Dynamics growth of microorganisms during the spontaneous fermentation: According to the evolution of fermented flora counts from mash to the end of the spontaneous fermentation, the samples were shared out among two groups (group A and B). In group A containing 162 samples, count of *Lactobacillus* increased, while the load of other lactic acid bacteria and yeasts decreased. This evolution varied according to the genus and the areas. In the samples of Abobo area, evolution count of *Lactobacillus* was higher than that of other areas. This count of *Lactobacillus* increased from 4.4 10^{8} cfu mL^{-1} in mash to 17.1 10^{8} cfu mL^{-1}at the fermentation end, that is 288.9% increasing (Fig. 1). *Lactobacillus* count varied from 4.6 10^{8}-11.8 10^{8} cfu mL^{-1} (156.7%) and from 4.7 10^{8}-11.6 10^{8} cfu mL^{-1} (145.8%) in the samples of Attecoube and Yopougon areas, respectively. The count of *Pediococcus* increased from 2.5 10^{7} cfu mL^{-1} in the mash to 8.5$10^{7}$ cfu mL^{-1}at the fermentation end (234.4%) in samples of Attecoube. This increase of *Pediococcus* is lower in samples of Yopougon (81%). In contrast, *Pediococcus* from Abobo samples was down

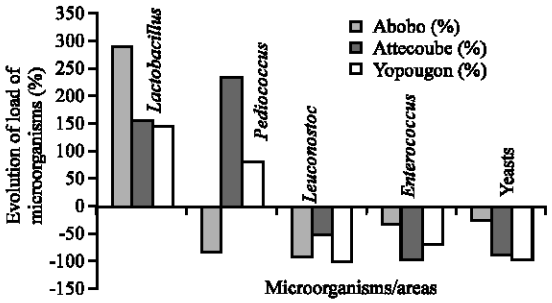

Fig. 1: Evolution of microorganisms from the mash to the sour wort of group A

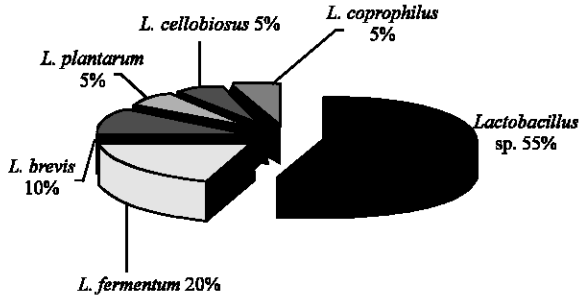

Fig. 2: Percentage of *Lactobacillus* species involved in the spontaneous fermentation of the tchapalo processing

82% in the sour wort. The counts of *Leuconostoc* and *Enterococcus* decreased a lot in all analysed samples. The highest decrease of *Leuconostoc* was observed in Yopougon area (99.6%). The highest count decrease of *Enterococcus* (94.2%) was observed in the samples of Attecoube whereas, the lowest count decrease (32%) was observed in samples of Abobo. The count of yeasts also decreased in all samples. The highest count decrease of yeasts (95.9%) was observed in the samples of Yopougon whereas, the lowest count decrease (24.6%) was observed in samples of Abobo.

In group B containing 18 samples distributed in areas, counts of all genus was smaller in the sour wort samples than in the mash ones, make believe that the microorganisms were not growth during the spontaneous fermentation (result not shown). Nevertheless, pH of these samples decreased from 5.60-3.81. It means that there was occurred a spontaneous fermentation.

Identification of *Lactobacillus* to species level: Twenty *Lactobacilli* strains randomly selected in the two products were characterized by Api 50 CH test (Fig. 2). Four belonged to *Lactobacillus fermentum*, one belonged to *Lactobacillus cellobiosus*, two belonged to *Lactobacillus brevis 3*, one belonged to *Lactobacillus coprophilus*, one belonged to *Lactobacillus plantarum* and eleven were identified from *Lactobacillus* sp.

DISCUSSION

The fermented microflora presents prior the spontaneous fermentation was made of yeasts and lactic acid bacteria. Michodjèhoun-Mestres *et al.* (2005) reported that microbial flora presents at the surface of the sorghum grain may have been developed during the malting process thus, explaining the higher initial count in the mash. Mohammed *et al.* (1991) and Tamminen *et al.* (2004) have also confirmed that in the natural fermentation, the microbial was associated with raw material. The main microorganisms found were similar in the three areas, but they proportions varied significantly ($p<0.05$). That is, the fermented microorganisms were the environment ones, but their load differed from one area to another. The number of yeasts (1.2×10^7 cfu mL^{-1}) in the mash was <1 of lactic acid bacteria. Thus, lactic acid bacteria constituted the dominant microflora (98.4%) in the mash before spontaneous fermentation (Nanadoum *et al.*, 2006).

The wort obtained after the mixed of supernatant and cooked sediment was left during the night, where occurred a spontaneous fermentation to give sour wort after percolation (Aka *et al.*, 2008). This step was very important and obligatory, because it determined the further process, organoleptic properties and the reproducibility of the finished product quality. During this spontaneous fermentation, the load of lactic acid bacteria increased.

The comparison of lactic acid bacteria counts in the sour wort and mash showed that *Lactobacillus* governed essentially this spontaneous fermentation. They constituted 92.8% in the sour wort. Counts of other lactic acid bacteria decreased a lot, therefore, their contribution was not obvious. This decrease also could be due to inhibitions or competition phenomenon between microorganisms. In the initial number of *Lactobacillus*, some strains may be bacteriocin producing bacteria. Indeed, these bacteria are isolated from food that normally contains lactic acid bacteria (Campanini *et al.*, 1993; Caplice and Fitzgerald, 1999; Bromberg *et al.*, 2004). Kostinek *et al.* (2005) also, observed that in the spontaneous fermentation of cassava for garis production, 63% of involved obligatory heterofermentative lactic acid were bacteriocin producing bacteria. On the other hand, in few samples, there was a decrease of all genus counts; their load was lower at the end of the fermentation than that observed in mash. This situation could be explained by the fact that the end of the

spontaneous fermentation was not appreciated at the same manner by producers, which had not the same time of fermentation (Yao *et al*., 1995). Indeed, the spontaneous fermentation could be ended when the mash gave sour or acidic taste. If not, the fermentation was extended. So, the end of the spontaneous fermentation is based on sensorial tests, which were not often mastered by producers. Probably, for these few samples where, all microorganisms decreased in the sour wort, the end of the spontaneous fermentation was estimated during the death phase of all microorganisms (Scriban, 1993). The decrease of all genus counts may be also due to the increasing of temperature of mash, which gave wort. Indeed, the wort was obtained after the mixed of supernatant and cooked sediment of mash. In these conditions, some bacteria were killed or inhibited (Sawadogo *et al*., 2007).

At the end of spontaneous fermentation, the pH dropped from 5.65 in the mash to 3.71 in the sour wort. Hydrolysed starch of sorghum was converted to organic acids that reduced pH (Iwuoha and Eke, 1996; Oyewole, 1997) dragging the inhibition of growth of pathogenic microorganisms (Caplice and Fitzgerald, 1999). Difference in the pH lowring was significant ($p<0.05$) in sour wort from areas. This could be due to the diversity of *Lactobacillus* strains, which showed 25 different phenotypic profiles. Recently, Sawadogo *et al*. (2007) have found a diversity strains of predominant lactic acid bacteria in dolo and pito wort for the production of sorghum beer in Burkina-Faso and Ghana.

Counts of yeasts also, decreased a lot in sour wort to the end of fermentation. This important disappearance of yeasts was probably due either to the raw material or count of lactic acid bacteria, which inhibited the growth of yeasts (Thomas *et al*., 2001). A similar observation was reported in literature on traditional fermented foods and beverages (Gotcheva *et al*., 2001; Blandino *et al*., 2003; Tamang *et al*., 2005). On the other hand, these results were contrary to those of Michodjèhoun-Mestres *et al*. (2005), which observed a concomitant increase of lactic acid bacteria and yeasts during fermentation of gowe.

Lactic acid bacteria found in the mash and the sour wort were identified as *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Enterococcus*. Kunene *et al*. (2000), Ben Omar and Ampe (2000) and Soomre *et al*. (2002) have also mentioned the presence of *Lactococcus*, *Streptococcus* and *Bifidobacterium* in spontaneous lactic fermentation of cereal products, but in this study these genus were not isolated.

Physiological study of 114 *Lactobacilli* stains randomly selected showed that 82.5% of strains including *Lactobacillus fermentum*, *Lactobacillus cellobiosus*, *Lactobacillus brevis* and *Lactobacillus coprophilus* were heterofermentative strains. Among 17.5% homofermentative strains, there was *Lactobacillus plantarum*. Similar species have been seen in fermentation of tej, cassava and cereal products (Hancioglu and Karapinar, 1997; Kunene *et al*., 2000; Gotcheva *et al*., 2001; Kostinek *et al*., 2005).

CONCLUSION

The spontaneous fermentation step of tchapalo processing is a natural lactic fermentation dominated essentially by *Lactobacillus* some of which were heterofermentatives. This fermentation was not often mastered by producers. This research is an essential preliminary research to improve quality and to produce standardized wort and tchapalo. Further research, will be focus on the molecular characterization of microorganisms species, organoleptic properties and trial fermentation with these microorganisms alone or co-culture. Microorganisms, which will be given characteristic wort, will be selected and used to produce starter for commercial product of wort and tchapalo.

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