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Biochemical properties of three lactic acid bacteria strains isolated from traditional cassava starters used for attieke preparation

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Variable sensorial quality of attieké (a fermented cassava product like couscous) is mainly due to different types of artisanal starters used for fermentation of cassava dough. Biochemical properties of eleven lactic acid bacteria identified as *Lactobacillus plantarum* strains isolated from these traditional cassava starters were evaluated *in vitro*. Three of these isolates (Lp 210, Lp 140 and Lp 19) presented suitable properties (fermentation at 45°C, important acidification rate, enzymatic activities, osmotolerance, thermotolerance) necessary for their potential use in drastic environment. These rapid acid producers' strains also induced a rapid drop of pH of MRS broth under pH 4 which is a major food safety factor. These potentialities confer to these strains ability to be selected as microbial starters for the reliable and reproducible lactic fermentation of cassava dough into attieké in order to optimize and standardize the quality and organoleptic characteristics of this staple food.

Key words: Attieké, cassava, lactic acid bacteria, biochemical properties, lactic fermentation.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the third agricultural resource after rice and maize as a source of calories in tropical countries (FAO, 2008; De Oliveira et al., 2015; Ngobisa et al., 2015). In Africa, tendency for cassava's use is almost 40%, and represents nearly

twice of that of the world (Tetchi et al., 2012). For 200 million people (more than a quarter of the continent's total population), cassava represents staple food necessities of which each consumes more than 100 kg per year (Pierre, 2012). Cassava is traditionally processed into

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a wide variety of fermented products such as attiéké, gari, lafun, fufu, baton de manioc or chips, particularly suited to transportation, trade and rapid preparation of meals (Pierre, 2012; Kouamé et al., 2012). Through thousands of years, demand for the production and consumption of fermented foods has extremely increased and accordingly, those foods occupied a substantial part of the diet worldwide (Elyas et al., 2015; Ngobisa et al., 2015).

In Côte d'Ivoire, the most popular food derived from fermented cassava is attiéké (Djeni et al., 2008; Kouamé et al., 2012). Quality of cassava derived-food is largely dependent on technologies processing which consist of a combination of steps such as peeling, boiling, steaming, punding, slicing, grating, roasting, soaking, pressing and fermentation (Pierre, 2012). Fermentation of cassava dough by bacteria and yeasts does not only enhance detoxification; it also improves food quality and safety by product preservation, flavor development, cyanide reduction and changes in functional properties (Obilie et al., 2003; Padonou et al., 2010).

In attiéké production, producers use a traditional cassava starter, which constitutes the main source of microorganisms which are predominant in the later fermentation of cassava dough. Djeni et al. (2011) established characteristics of each of the three "attieke" type and the differences between them, probably due to the differences in their traditional starter used to conduct the fermentation. Microflora of these traditional starters has not been studied so far, but similar studies on fermented cassava derived-food such as agbelima, lafun and chikwangue which shows that the active microorganisms in cassava fermentation process are generally lactic acid bacteria (LAB) because the cassava dough fermentation results mainly from a lactic fermentation (Coulin et al., 2006).

LAB generally regarded as safe (GRAS), play an essential role in the majority of food fermentations and preservation, and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable, and bakery products (Elyas et al., 2015; Gemechu, 2015). They contribute to the enhancement of sensory, quality and safety features of these fermented foods (Holzapfel and Wood, 2014). Their antimicrobial activity has been attributed to produced metabolites such as organic acids, carbon dioxide, hydrogen peroxide, diacetyl and bacteriocins which can inhibit pathogenic and spoilage microorganisms, extending the shelf life and enhancing the safety of food products (Piard and Desmazeaud, 1992).

Lactobacilli represent one of the major microbial groups involved in these desirable fermentations. Among them, *Lactobacillus plantarum* is regularly noted among cassava dough fermentative germs (Kostinek et al., 2007; Edward et al., 2012). Therefore, this study was carried out to select LAB strains as potential microbial starters for

cassava dough fermentation into attiéké.

MATERIALS AND METHODS

Samples collection and isolation of fermentative microorganisms

The biological material used was constituted of "Adjoukrou" traditional cassava inocula ready-to-use, collected in small-scales production of attiéké from thirteen high production locations in Abidjan, Côte d'Ivoire. The collected samples wrapped in sterile poly ethylene sacs (Stomacher, Laboratoire Humeau, Rennes, France), were immediately transported in ice box to the laboratory in less than 1 h, where they were mixed and then subdivided into five aliquots. Preparation of stock solutions, inoculation of agar plates and cultivation of the various microorganisms were carried out (Coulin et al., 2006). Total viable (LAB) count in each sample was analyzed by spread plating the tenfold diluted samples into de Man, Rogosa and Sharpe (MRS), Bile Esculin Azide (BEA), Mayeux, Sandine and Elliker (MSE), and M17 agar plates (all from OXOID, Basingstoke, Hampshire, UK), respectively to obtain the widest possible variety of LAB associated with fermenting traditional cassava starters. The media were supplemented with nystatin (1%) to inhibit fungal growth. Petri dishes were then incubated in an anaerobic jar at 30°C for 48 h.

Morphological and biochemical characterization of isolates

Phenotypic characteristics

Isolates were performed by Gram staining method and catalase test. For mobility test, each colony was sub-cultured in MRS broth, and the medium was then incubated at 30°C for 24 h. After incubation, the mobility was determined by using of an optical microscope (Primo Star, Zeiss, Marly-Le-Roi, France). The isolates characterized as Gram-positive rods and catalase negative, were tested for carbohydrate fermentation ability using the API 50 CH strips with API CHL medium (API system LaBalme le Grottes, Montalieu Vercieu, France), incubated at 30°C for 48 h following manufacturers instruction.

Acid producing strains were identified as earlier described (Dicks and Van Vuren, 1987) with slight modification. Each strain was inoculated at 30°C in 5 mL MRS agar medium without beef extract, and with 0.004 g/L of bromocresol purple in tubes. Acid production was monitored during three days by formation of yellow area in the tube and acidification capacity was analyzed by a visual evaluation of the yellow area's spread.

Then tests of aerobic and anaerobic growth were assessed using two inoculated tubes containing 5 mL of MRS broth supplemented with 0.004 g/L of bromocresol purple of which one was incubated aerobically at 30°C, and the other anaerobically in an anaerobic jar. If growth was possible, the purple would turn yellow. Homo- or heterofermentative assimilation of glucose was assessed using 5 mL MRS agar broth in tubes. The heterofermentative character was analyzed by a visual evaluation of the breakdown of the agar broth due to CO₂ gas production at the bottom of the tube. An isolate was deemed to be a homofermentative lactic acid producer if no gas was produced.

Eleven strains were selected from all these experiments, and tested for their ability to lactic acid fermentation at 45°C and 50°C to select the possible thermotolerant of them in MRS broth without beef extract, and with 0.004 g/L of bromocresol purple in tubes. An 18 h culture of each isolate was used as the inoculums whereby the cells were spun down, re-suspended in 0.85% saline, and 100 µL of

the suspension was inoculated into each test bottle. Three of them were then selected and also tested at 15, 30 and 37°C and subjected to lactic acid concentrations of 0.5 and 1% and to NaCl concentrations of 2, 5, 7 and 9 % (w/v). Four pH were tested, that is, 3, 5, 7 and 9. The basal medium was adjusted with 1M phosphoric acid and 1M NaOH, and the tubes were placed at the specific temperatures or at 37°C concerning tests dealing with pH, concentrations of lactic acid and NaCl, respectively. After 42 h, growth level was evaluated by visual inspection by the color change and turbidity of each bottle was noted as a simple indication of growth or no growth.

All experiments were done in triplicate.

Assay of acidification capacity of isolates

The three thermotolerant strains were selected and tested during 42 h for their capacities to reduce pH to less than 4.2 (Kostinek et al., 2007). For this purpose, 5 mL of MRS broth (into tubes) adjusted to pH 6.5 before autoclaving (pH 6.2 after autoclaving) was inoculated with 0.2% of an overnight preculture ($OD_{600} = 1$) and cultures were grown aerobically at 37°C. Acid production was determined by measuring the culture pH after 6, 12, 18, 24, 30, 36 and 42 h by a pH-meter (pH₇₀₀, Eutech Instruments, Adelaide, Australia). During microbial growth, the pH of MRS broth media was automatically measured by a pH-meter (pH₇₀₀, Eutech Instruments, Adelaide, Australia). Biomass evolution was followed by measuring absorbance at 600 nm. Acidity was titrated against 0.1N NaOH using phenolphthalein as indicator and the total titratable acidity was calculated as a percentage of lactic acid. Strains able to induce drop of MRS broth pH from 6.2 to less than 4.2 at the end of the fermentation were selected for further screening studies (Kostinek et al., 2007).

Screening of enzymatic activities of isolates

For alpha-amylase and cellulase production, LAB isolates were grown on modified MRS agar plates containing 20 g/L cassava starch or carboxymethylcellulose (CMC) as the sole carbon source on Petri dishes. The plates were incubated at 37°C for 24 h, and flooded with iodine. Production of amylase or cellulase was evident by a clear area surrounding the colonies (Ouattara et al., 2008).

Identification of isolates

Strains were identified by MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (ABSciex, Framingham, MA, USA)) according to Doan et al. (2012). Bacterial cells were grown on MRS-agar under aerobic conditions for 48 h at 28°C, and sub-cultured twice. Cell extractions were prepared according to the formic acid and acetonitrile extraction protocol as described by Freiwald and Sauer (2009). One μ L of freshly prepared cell extract was spotted on a 384 Opti-TOF stainless steel MALDI target plate (ABSciex, Framingham, MA, USA) and dried at room temperature. Next 1 μ L of 0.5% (w/v) α -cyano-4-hydroxycinnamic acid (α -CHCA) in 50: 48: 2 acetonitrile: water: trifluoroacetic acid solution was added and allowed to dry.

Bacterial fingerprints were acquired using the 4800 Plus MALDI TOF/TOF™ Analyzer (ABSciex, Framingham, MA, USA) in linear positive ion mode. Ions were generated by a 200-Hz-tripled UV Nd: YAG laser, accelerated in a 20-kV electric field through a grid at 19.2 kV and separated according to their m/z ratio in a 1.5-m long linear field-free drift region. For each spotted extract, 40 laser shots at 50 random positions within the spot were collected automatically

in the mass range from 2000 to 20 000 Da. A maximum laser intensity of 5200 pdu (power distribution unit) resulted in base peak signal intensities varying between 5.0×10^2 and 1.0×10^4 cps (continuous periodic signal).

Prior to the analyses, calibration was performed with a protein calibration standard that includes the adrenocorticotrophic hormone (ACTH) fragment 18–39 MALDI-MS standard (m/z 2465.7), insulin (m/z 5734.6), ubiquitin I (m/z 8565.9), cytochrome C (m/z 12361.5) and myoglobin (m/z 16952.3). The raw data were extracted as t2d files from the 4800 plus MALDI TOF/TOF™-analyzer software, imported into the Data Explorer 4.0 software (Applied Biosystems) and transformed to text files.

Next, these text files were imported into the BioNumerics 5.0 software package (Applied- Maths, Sint-Martens-Latem, Belgium) and converted to fingerprints for further analysis. Pearson's product moment correlation coefficient was used to determine similarity between the spectra by which the spectra were subsequently clustered using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm. To validate the tentative identifications of the isolates, their profiles were clustered against all previously characterized lactic acid producing bacteria (Doan et al., 2012).

RESULTS AND DISCUSSION

Isolation and screening of microorganisms from natural sources has always proved to be a successful way for obtaining industrially interest strains (Adejumo, 2014). It is well understood that LAB, which grow as the adventitious microflora of foods or that are added to foods as starter cultures, are generally considered to be harmless (Elyas et al., 2015).

In this study, eleven presumptive LAB strains (regular rod shape (bacilli), positive for Gram staining, negative for catalase and oxydase and non-endospore forming) were isolated from traditional cassava starters used for attieké fermentation and identified as *L. plantarum* via MALDI-TOF MS. Figure 1 overviews the protein profiles of these isolates, including the reference spectrum of the type strain of *L. plantarum* (LMG 6907^T). The strains Lp 210, Lp 140 and Lp 19 shows more than 80% spectral similarity with the *L. plantarum* type strain. These homofermentative strains among the eleven with greater lactic acid fermentative ability on glucose (produced titratable acidity more than 1% after 12 h), presented growth and fermentative ability at 45°C, and were confirmed as *L. plantarum* according to their sugar fermentation profile by API 50 CHL kit (Percentage ID more than 99%).

Surprising, strains LP 210, LP 140 and Lp 19 were isolated from BEA agar plates. *L. plantarum* strains, by their biochemical properties, presented suitable characters for their use as pure microbial starters for cassava dough fermentation (Edward et al., 2012). Ability for microbial growth and fermentation at high temperature such as 45°C is a suitable feature for their industrial exploitation, especially for the thermotolerance in tropical regions where ambient

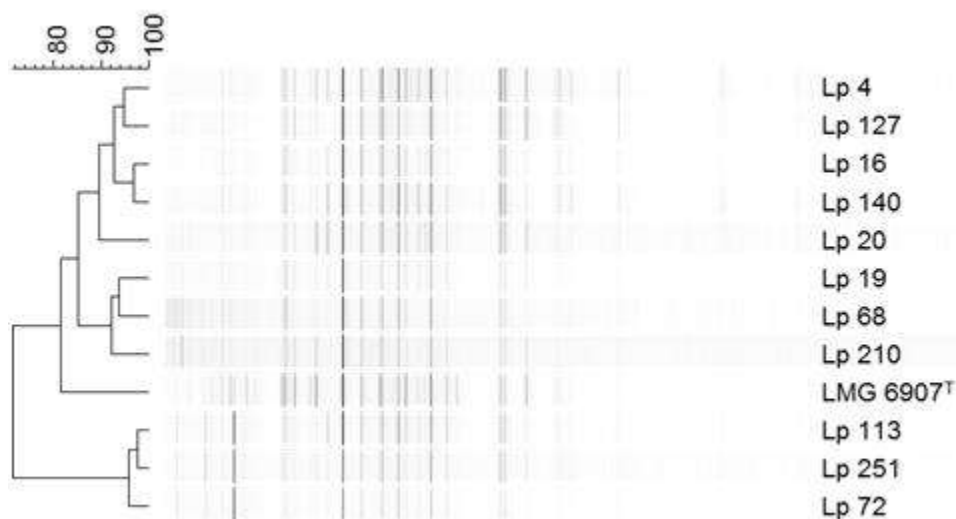


Figure 1. UPGMA dendrogram based on the Pearson correlations between the mass spectra of 11 isolates and the *L. plantarum* type strain. Protein profiles are displayed as gel views ranging from 2000 m/z to 20000 m/z.

Table 1. Microbial growth according to different environmental conditions.

Environmental conditions	Isolates		
	Lp 210	Lp140	Lp 19
Temperature (°C)			
15	+	+	+
30	+	+	+
37	+	+	+
45	+	+	+
50	-	-	-
Lactic acid (%W/v)			
0.5	+	+	+
1	-	-	-
NaCl (%W/v)			
2	+	+	+
5	+	+	+
7	+	+	+
9	-	-	-
pH			
3	+	-	-
5	+	+	+
7	+	+	+
9	-	-	-

temperature is generally higher than 30°C (Ndoye et al., 2006).

Indeed, this property makes it possible to reduce in a

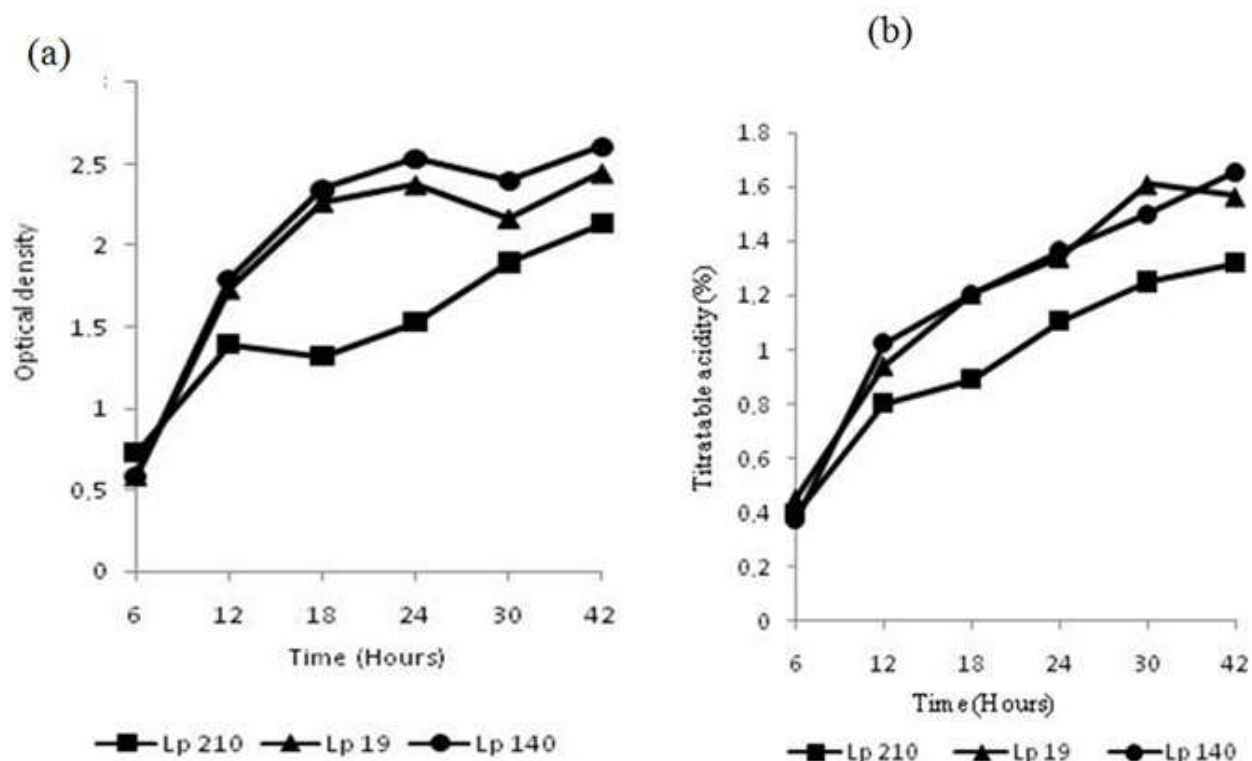
considerable way the cooling water expenses during production in bioreactor and to prevent great mortality during storage after processes such as atomization, freeze-drying (Ndoye et al., 2006). Moreover, a high fermentation temperature also reduces contamination by other microorganisms. In addition, these three strains presented resistance to drastic environment (ability to growth and to ferment at 7% NaCl which requires an osmotolerance property, and ability to resist at low pH for Lp 210 (that is, pH = 3) indicating their acidophilic character (Table 1).

High osmotolerance ability and acidophilic character are suitable features for an industrial LAB. They all grew as well-in the absence and presence of oxygen, possessed trace of amylase and cellulase for Lp 210 but did not degrade starch into fermentable sugars able to be directly fermented. These strains showed similar fermentation profile excepted for raffinose and Potassium-gluconate of which the fermentation was strain-dependant. Strain Lp 140 fermented Potassium-gluconate and Strains Lp 210 and Lp 19 did not. Raffinose fermentation is met for strains Lp 140 and Lp 19 (Table 2). Cassava roots contain raffinose (Dossevi et al., 1980). Ability for fermentation of indigestible sugars like raffinose or stachyose is an interesting property. Indeed, in humans, these sugars are metabolized by microorganisms in the large intestine, liberating huge amounts of gas, which can then cause gastrointestinal disorders (LeBlanc et al., 2004).

Cellulase is a cell wall degrading enzyme required to break down cassava tissue and to induce a desired textural modification by softening cassava derived-foods

Table 2. Enzyme activities and technological properties of the three isolates.

Strains	Cellulase	α -amylase	Raffinose fermentation	pH < 5.3 at 6 h	pH < 4.2 at 12h	Fermentation at 45°C
Lp 210	+	+	-	+	+	+
Lp 140	-	+	+	+	+	+
Lp 19	-	+	+	+	+	+


Figure 2. Changes in growth curve (a) and titratable acidity (b) during the isolates growth.

(Obilie et al., 2003). In addition to this enzyme, amylase is required in starchy food to improve their digestibility. However, in many studies, only a few amylolytic LAB have been isolated from starchy fermented foods in Africa such as gari (a cassava fermented partially gelatinized granular-product) production in Benin (Kostinek et al., 2007).

An important feature for a potential LAB starter strain is its ability to rapidly acidify its environment, as the acid production and the accompanying pH decrease is well-known to extend the lag phase of sensitive organisms including food borne pathogens. Suitability for lactic acid fermentation of isolates was studied on MRS broth over 42 h. Microbial growth and produced titratable acidity were presented in Figure 2. Strain Lp 140 presented better growth followed by Lp 19 and then by Lp 210. Peaks of growth ($OD_{600} = 2.1-2.6$) and the produced amount of

lactic acid (1.3-1.7 %) were obtained after 42 h.

In cassava dough for attiéké preparation, titratable acidity increased until 0.7 ± 0.05 % after 24 h (Coulin et al., 2006). Also, in traditional cassava starters used for attiéké preparation, titratable acidity ranged from 0.02 to 0.09% (Kakou et al., 2010; Tetchi et al., 2012). These low rates of titratable acidity met during the process of attiéké preparation compared to the obtained levels in this study could be explained by the MRS broth composition, which is very rich comparatively to cassava dough, mainly consists of starch (89%), protein (2.5%), fat (1%) and other minerals (more than 1 %), respectively (Pierre, 2012).

The high content of produced titratable acidity (more than 1% after 12 h on MRS broth) shows that these isolates may be suitable for industrial production of lactic acid and, also as pure starters for lactic acid fermentation

of cassava dough. Generally, *L. plantarum* has the best ability for lactic acid fermentation than others strains such as bacteria, yeasts or moulds (Kostinek et al., 2007). In addition, LAB, especially *L. plantarum* strains show antimicrobial properties (production of organics acid, bacteriocin and hydrogen peroxide) contributing to inhibition or reduction of pathogens (Kostinek et al., 2007; Yasmeen et al., 2015).

Vieira-Dalodé et al. (1994) reported that lactic acid fermentation for the production of máwe (a fermented cereal in Bénin), reduced the *Enterobacteriaceae* population below the detection level ($< \log_{10} 1.7$ cfu/g) after 24 h of fermentation. The pH of MRS broth (6.2 after autoclaving) decreased faster during the first hours for these strains. These isolates induced a rapid pH decrease to below 5.3 after 6 h and 4.2 after 12 h (that is, pH = 4.08, 3.92 and 3.93 for isolates Lp 210, Lp 19 and Lp 140, respectively) and then, the pH remains constant to a value of 3.50 to 3.70 after 24 to 42 h.

Comparatively to Kostinek et al. (2007) and Edward et al. (2012), these isolates are rapid acid producer's strains. In fact, a pH less than 4.2 constitutes a major food safety factor. The required pH to inhibit undesirable bacteria should be at least pH 4.2. This is because spoilage bacteria, as well as pathogens, notably those including members of *Enterobacteriaceae* family, do not grow below this pH level (Kostinek et al., 2007; Edward et al., 2012). Coliforms have been mentioned in traditional cassava starters and in cassava dough during attiéké preparation. Their presence is evidence of possible faecal contamination, through water or materials used or from the environment (Tetchi et al., 2012).

Thus, from both a quality and safety perspective, the use of starter cultures is recommended, as it would lead to a rapid acidification, inhibition of spoilage and pathogenic bacteria (Holzapfel, 2002), and to a safe and constant quality product. Antimicrobial activities against bacteria causing diarrhoea have been related to LAB involved in fermentation of uji, a Kenyan indigenous fermented cereal gruel (Mbugua and Njenga, 1991).

Several studies have shown that at pH < 4.0 diarrhoea-causing pathogens will be inhibited in traditional ready-to-eat fermented food products (Steinkraus, 1996). Thus, in regarding to such technological properties, these three isolates would be confirmed in further steps by pilot plant fermentations. These results also are in agreement with literature considering *L. plantarum* as suitable starters culture for cassava dough fermentation into cassava derived-foods like gari and kivunde (Giraud et al., 1983; Kimaryo et al., 2000; Kostinek et al., 2008).

Conclusion

Three *L. plantarum* strains isolated from traditional cassava starters with important lactic acid fermentative

ability induced a rapid decline of the initial pH of MRS broth (main food safety factor). These strains were thermotolerant and osmotolerant, presented cellulase for Lp 210 and alpha-amylase activities necessary for cassava dough softening, and fermented raffinose (an indigestible sugar) for Lp 140 and Lp 19. In regards to these features, they may be suitable for their industrial exploitation and, particularly as microbial starters for reliable and reproducible fermentation of cassava dough to optimize and standardize the quality of attiéké.

Conflict of Interests

The authors have not declared any conflict of interests.

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