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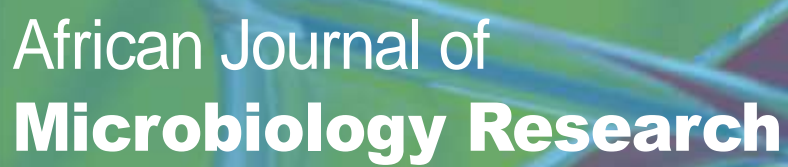

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African Journal of Microbiology Research

Full Length Research Paper

Molecular characterization of *Listeria monocytogenes* isolated from a ready-to-eat fermented milk and cereal product, *Fura-de-Nunu*

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This study was conducted to determine the occurrence of *Listeria (L.) monocytogenes* in *Fura-de-Nunu,* a ready-to-eat (RTE) fermented milk (*Nunu*) and cereal (*Fura*) blend, the serogroups as well as the virulence of the isolates. A total of 75 *Fura* and 75 *Nunu* samples were examined. *Listeria* species were isolated on PALCAM medium and *Listeria* chromogenic agar, and identified phenotypically according to International Standardization Organization (ISO) procedures. Identification of *L. monocytogenes,* serogrouping and detection of virulence genes were carried out by polymerase chain reaction (PCR). *Listeria* spp. were recovered from 23 (30.67%) and 41 (54.67%) samples of *Fura* and *Nunu*, respectively. The bioloads of *Listeria* ranged from 10^3 to 10^5 CFU/ml. Six presumptive species of *Listeria* were identified from the samples, with *L. monocytogenes* accounting for 21.00 and 20.64% of isolates from *Fura* and *Nunu,* respectively. Out of the three major serogroups (1/2a, 1/2b and 4b) associated with human disease, only 1/2a and 4b were identified among the isolates. Some of the isolates tested positive for the presence of virulence genes, *hlyA* and *iap*. Results from this study show that *Fura-de-Nunu*, may represent a risk for transmission of listeriosis to consumers.

Key words: *Listeria monocytogenes*, Fura-de-Nunu, fermented milk, ready-to-eat, Listeriosis.

INTRODUCTION

The genus *Listeria* currently comprises seventeen species of Gram-positive, non-spore forming, and catalase-positive bacteria: *Listeria (L.) aquatica, L. booriae, L. cornellensis, L. fleischmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri, L. weihenstephanensis,* and *L. welshimeri* (Weller et al., 2015). The haemolytic species (*L. monocytogenes, L. ivanovii* and *L. seeligeri*) are associated with human disease, although *L. ivanovii* and *L. seeligeri* are more commonly involved in diseases of animals and are rare causes of human disease (Guillet et al., 2010; Jamali et al., 2013; Abdellrazeq et al., 2014).

L. monocytogenes has emerged as a very important food-borne pathogen, causing the human disease, listeriosis (Marnissi et al., 2013; Mansouri-Najand et al.,

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2015). It has been detected in various foods, including raw milk and other dairy products, such as cheese and ice cream (Jamali et al., 2013). Infectious doses are reported to range from 10^1 to 10^5 CFU/g of food (Maijala et al., 2001; Ooi and Lorber, 2005; Yang et al., 2007; Marnissi et al., 2013; Jamali et al., 2013; Mansouri-Najand et al., 2015; Oyinloye, 2016). However, according to the European food safety regulations, generally, food samples containing more than 100 CFU/g of *L. monocytogenes* are considered unsatisfactory. In foods intended for infants or foods where the shelf life is determined without consideration of the organism, there should be complete absence of *L. monocytogenes* /25 g of the food before the food leaves the immediate control of the producer (Food Standards Agency, 2006; Public Health England, 2014). Listeriosis is a disease of serious public health concern because of the high mortality rate associated with the disease; especially in pregnant women and infants, the elderly and immunocompromised individuals, who are at the greatest risk for the disease (Gillespie et al., 2010; Barton et al., 2011; Abdellrazeq et al., 2014; Oyinloye, 2016). A survey in 2010, by the Europe PMC Funders Group, estimated that listeriosis resulted in 23,150 illnesses, 5,463 deaths and 172, 823 disability adjusted life years (DALYs) globally. The proportion of perinatal cases was 20·7%. This led to a call by the World Health Organization (WHO) for more studies, particularly in developing countries (de Noordhout et al., 2014).

The presence of *L. monocytogenes* in ready-to-eat (RTE) foods and the low infectious dose of the organism are a cause for concern as such foods could constitute health hazards to consumers. *L. monocytogenes* has been reported from humans, environment and various foods in Nigeria, although most of these reports have come from studies based on phenotypic and biochemical characterization of the organism (Nwaiwu, 2015). *Fura-de-Nunu*, a fermented milk-cereal mix, is a street-vended, RTE food, consumed in various parts of Nigeria, especially in Northern Nigeria. In Nigeria, about 90% of the dairy cattle belong to the Fulani agro-pasturalists and their women strictly control the processing and marketing of their milk products (Chukwuma, 2009; Okeke et al., 2014). Most of these women are illiterate and have no knowledge of critical control points of food production. *Fura* is a semi-solid dumpling millet-based meal. The millet is blended with spices and water, molded into dough balls and then cooked. To make the complete meal, *Fura-de-Nunu,* the cooked dough balls are broken up and mixed into the fermented milk (*Nunu)*, to form porridge (Kordylasi, 1990; Jideani et al., 2001).

Fura-de-Nunu, like many other indigenous fermented foods, depends on spontaneous fermentation initiated by natural microorganisms on raw materials and equipment, the hands of producers and the local environment. In addition, some aspects of the production of this widely consumed product, such as use of leftover products as starter for fresh fermentation of the *Nunu* and molding of the *Fura* by hand, all increase the chances of contamination of the product. This study therefore aimed to determine the prevalence of *L. monocytogenes* in RTE *Fura-de-Nunu* samples, the PCR serogroups as well as virulence genes of the isolates.

MATERIALS AND METHODS

Samples collection

A total of 150 samples, comprising 75 *Fura* and 75 *Nunu,* were purchased from vendors in different communities within Nsukka metropolis. *Nunu* samples were collected in a sterile large screw capped bottles while *Fura* balls were placed in sterile polyethylene bags and transported in ice-packed containers to the Microbiology Laboratory, University of Nigeria, Nsukka for analysis.

Isolation and enumeration of organisms

The procedure recommended by the International Organization for Standardization (ISO) 11290 (2004) was used as follows: A 25 g representative portion from each *Fura* sample, mashed into a semi-powdery paste or 25 mL representative portion of each *Nunu* sample was introduced aseptically into a sterile conical flask containing 225 mL of Half Fraser Broth to obtain a 1:10 sample dilution. The samples were homogenized for 1 min and incubated for 24 h at 37°C. After incubation, a 0.1 mL aliquot from each Half Fraser Broth culture was added to 9.9 mL of Fraser Broth and incubated for 24 to 48 h at 37°C. Following incubation, the culture was serially diluted ten-fold and 0.1 mL from 10^{-3}, $10^{-4}$and 10^{-5} dilutions was spread on *Listeria* selective agar (PALCAM; Oxoid, UK). Samples were also inoculated onto plates of Brilliance *Listeria* chromogenic agar (Oxoid, UK), selective for *Listeria* spp. The plates were incubated for 24 to 48 h at 37°C and examined for grayish colonies, typical of *Listeria.* Total viable counts were taken from plates containing countable colonies.

Biochemical confirmation of the isolates

Colonies showing typical morphology of *Listeria* were streaked onto Tryptone Soya Agar supplemented with 0.6% yeast extract powder (TSYEA; Oxoid, UK) and incubated at 37°C for 24 h to further obtain pure cultures for identification and characterization (Alsheikh et al., 2013). The isolates were identified and characterized using the following tests: Gram staining, oxidase, catalase, motility test, sugar fermentation (Glucose, Rhamnose, Mannitol, methyl-D-mannose, xylose), and haemolysis test. Results from suspected *L. monocytogenes* isolates were compared to those of a reference *L. monocytogenes* strain ATCC13932

Haemolysis test

The isolates were inoculated onto 7% Sheep Blood Agar (SBA) by piercing into the medium and incubated at 37°C for 24 h. The haemolytic zones around the colonies were examined after incubation (Alsheikh et al., 2013; Momtaz and Yadollahi, 2013).

Christie, Atkins, Munch-Petersen (CAMP) test

This was performed according to the procedure described by Yadav et al. (2010). A β-haemolytic strain of *Staphylococcus aureus* was

grown overnight on 7% SBA at 37°C and a colony was streaked again on freshly prepared 7% SBA in a manner that the streak allows for streaking of *Listeria* colonies. *Listeria* isolates was streaked at 90° angle and 3 mm apart before incubating them at 37°C for 24 h. A positive reaction was indicated by an enhanced zone of haemolysis at the intersection of the test and indicator strain.

Determination of pH of samples

For the *Fura* samples, 10 g of each sample was homogenized with 20 mL of distilled water in a beaker, for 1 min and the pH of the homogenate determined using digital pH meter calibrated with standard buffer solutions. For the *Nunu* samples, the samples were mixed properly and 25 mL of each sample was poured into a beaker and the pH determined using a digital pH meter.

Determination of titratable acidity of the samples (using phenolphthalein indicator)

Ten millilitres (*Nunu*) or 10 g (*Fura*) of each sample was added to 50 mL of distilled water in a 250 mL beaker and homogenized properly. Then, 3 drops of phenolphthalein were added to the mixture. A 0.1 M solution of NaOH was slowly titrated into the mixture, while carefully swirling the beaker to ensure thorough mixing. The point of neutrality was reached when the indicator changed colour from colourless to pink, which must remain stable for at least 30 s. The amount of NaOH used on the burette was recorded and result expressed as percentage acid, using the following formula:

$$\text{Percent acid} = \frac{\text{Titre} \times \text{acid factor} \times 100}{10 \text{ mL of sample}}$$

Acid factor for Lactic acid = 0.0090

Determination of NaCl (salt content) with standard silver nitrate solution

To each sample neutralized as described above, about 1 mL of 5% aqueous potassium chromate solution was added and titrated with 0.1 N $AgNO_3$ solution to produce red-brown end point.

$$\text{NaCl \%} = \frac{\text{Titre value} \times \text{Normality of } AgNO_3 \times 58.4 \times 100}{\text{Weight of the sample} \times 1000}$$

Determination of moisture content

About 5 mL and 5 g of well mixed samples (*Nunu* and *Fura*) were weighed in a well dried oven sterilized glass tray. The tray was placed in a hot air oven maintained at 105 ± 2°C and dried for at least 2 h. It was then cooled in a dessicator and weighed. The process of heating, cooling and weighing was repeated until the difference between two successive weighing was less than 1 mg and the lowest weight recorded.

$$\text{\% Moisture content} = \frac{100\,(M_1 - M_2)}{M_1 - M}$$

Where; M_1 = Weight in gram of dish with material before drying; M_2 = Weight in gram of dish with the dried material, and M = Weight in gram of empty dish

Determination of water activity (a_w)

This was done using water activity meter (Buchi, Switzerland). About 10 g of each *Fura* and 10 mL of each *Nunu* sample was placed on the sample scale pan and readings recorded.

DNA extraction

Genomic DNA was extracted from the isolates using an extraction kit (Zymo Research, U.S.A.) following the procedure of the manufacturer. A 72- h tryptoy soya broth culture of the isolates was transferred in an Eppendorf tube and centrifuged for 5 minutes to pellet the cells. A 200 µL volume of nuclease free water was added to the cell pellet and vortexed for 1 min in a vortex mixer to wash the cells, it was then centrifuged for 1 min and the supernatant was decanted out. A 50 to 100 mg weight of the bacterial cells was resuspended in 200 µL of nuclease free water and transferred to a ZR Bashing Bead Lysis tube. Then, 750 µL of lysis solution was added to the tube. The tube was then vortexed in a vortex mixer vigorously for 5 to 10 min to lyse the cells. The ZR Bashing Bead Lysis tube was then centrifuged for 1 min at 10,000 × *g.* About 400 µL of supernatant was transferred to a Zymo-Spin IV Spin Filter in a collection tube and centrifuged at 7,000 × *g* for 1 min. To the filtrate in the collection tube, 1,200 µL of bacterial DNA Binding Buffer was added. About 800 µL of the mixture was transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 × *g* for 1 min. The flow through the collection tube was discarded and the process repeated. Then, 200 µL of DNA Pre-wash buffer was added to the Zymo-Spin IIC column in a new collection tube and centrifuged at 10,000 × g for 1 min. Next, 500 µL of bacterial DNA Wash Buffer was added to the Zymo-Spin IIC column and centrifuged at 10,000 × *g* for 1 min. The Zymo-Spin IIC column was finally transferred to a clean Eppendorf tube and 50 µL of DNA Elution Buffer was added directly into the column matrix and centrifuged at 10,000 × *g* for 30 s to elute the DNA.

PCR identification of *L. monocytogenes*

A 25 µL PCR supermix comprising 12.5 µL One Taq Quick-Load 2× Master Mix with Standard Buffer (New England Biolabs, U.S.A.), 0.5 µL of each primer (10 µM), 4.5 µL of nuclease free water and 5 µL of bacterial genomic DNA solution was subjected to thermocycling conditions, in a TECHNE thermocycler (Bibby Scientific, UK), according to the following schedule: Initial denaturation, 95°C for 5 min; 35 thermal cycles of 95°C, 30 s; 58°C, 30 s; 72°C, 45 s); final extension, 72°C for 5 min; and hold, 4°C. The PCR products were analysed on a 1.5% agarose gel containing ethidium bromide (10 mg/mL) and specific DNA bands were visualized using a bench top UV transilluminator (Upland, U.S.A.). Primers used for PCR are shown in Table 1. Identification of *L. monocytogenes* was by detection of the 370 and 456 bp amplicon of the *prs* and *hlyA* genes, respectively.

Serogrouping of *L. monocytogenes* and dectection of virulence genes

Serogroups of the isolates were determined by PCR using specific primers (Table 1) for the three major serogroups (1/2a, 1/2b and 4b) associated with human listeriosis. The PCR conditions were: Initial denaturation, 94°C for 5 min; 35 thermal cycles of (94°C, 40 s; 53°C, 75 s; 72°C, 75 s); final extension, 72°C for 7 min; and hold,

Table 1. Nucleotide sequences of primers used in this study.

Primer name	Primer sequence (5'-3')	Product size (bp)	Primer target	References
prs	F: GCTGAAGAGATTGCGAAAGAAG R: CAAAGAAACCTTGGATTTGCGG	370	Genus *Listeria*	Doumith et al., 2004
lmo0737	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691	**Lm 1/2a*	Doumith et al., 2004
ORF 2819	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471	*Lm 1/2b*	Doumith et al., 2004
ORF 2110	F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597	*Lm 4b*	Doumith et al., 2004
plcA	F:CTGCTTGAGCGTTCATGTCTCCATCCCCC R: CATGGGTTTCACTCTCCTTCTAC	1484	*plcA* gene	Momtaz & Yadollahi, 2013
hlyA	F: GCAGTTGCAAGCGCTTGGAGTGAA R: GCAACGTATCCTCCAGAGTGATCG	456	*hlyA* gene	Swetha et al., 2012
iap	F: ACAAGCTGCACCTGTTGCAG R: TGACAGCGTGTGTAGTAGCA	131	*iap* gene	Swetha et al., 2012

**Lm* = *L. monocytogenes.*

4°C. The isolates were also screened for the presence of virulence genes, *hylA* and *iap* by PCR, under the same conditions described under DNA amplification above.

RESULTS AND DISCUSSION

Out of a total of 75 *Fura* and 75 *Nunu* samples examined in this study, 23 (30.7%) and 41 (54.7%) samples, respectively, yielded *Listeria* spp, based on growth on PALCAM *Listeria* agar, *Listeria* chromogenic agar and biochemical characterization. These values indicate that both the cereal and milk used in preparation of the RTE *Fura-de-Nunu,* were contaminated with *Listeria* spp. The occurrence recorded in this study are very much higher than the values of about 5 to 7% reported by some authors for raw milk and dairy products (Jamali et al., 2013; Marnissi et al., 2013; Mansouri-Najand et al., 2015; Shamloo et al., 2016), but lower than 78% reported from a study in south western Nigeria on an RTE soft cheese product (Oyinloye, 2016). Six presumptive *Listeria* species were identified from the samples. The distribution of the presumptive *Listeria* spp. in the *Fura* was as follows: *L. ivanovii* (36.7%) > *L. monocytogenes* (30.0%) > *L. seeligeri* (20.0%) > *L. grayi* (10.0%) and *L. welshimeri* (3.3%), while for *Nunu*, it was *L. ivanovii* (46.4%) > *L. monocytogenes* (26.8%) > *L. seeligeri* (16.1%) > *L. innocua* (5.4%) > *L. grayi* (3.6%) and *L. welshimeri* (1.8%). Another study in Nigeria had also reported these species to be the most predominant *Listeria* species in raw milk and attributed their presence in milk to unhygienic milking practices (Yakubu et al., 2012). Among the *Listeria* species, *L. monocytogenes* is the most commonly associated with human listeriosis. Although the illness is relatively rare, it can have mortality rates as high as 30%, especially among vulnerable groups such as infants, pregnant women and the elderly (Mansouri-Najand et al., 2015). For this reason, most western countries have developed set-down policies and guidelines to monitor and control *L. monocytogenes* in foods. Unfortunately, the same cannot be said for Nigeria and some other African countries. The mean *Listeria* counts for the samples in this study were 7.7×10^4 CFU/g and 8.6×10^4 CFU/mL, for *Fura* and *Nunu*, respectively. Although these values represent total *Listeria* counts and not *L. monocytogenes* counts, they are still a cause for concern, considering that *L. monocytogenes* was the second most predominant species isolated from the food samples. According to international standards for acceptable levels of *L. monocytogenes* in foods, foods with a shelf life of less than five days or foods with the potential to support the growth of *L. monocytogenes* should not contain more than 100 CFU/g of food; and when the food is intended for infants, there should be complete absence of *L. monocytogenes* per 25 g of the food (EC 2073/2005; FSA UK, 2010; Health Canada, 2011; FOOD Standards Australia, 2014). The high frequency of *L. monocytogenes* in this study and the high total *Listeria* counts suggest a possibility that the *L. monocytogenes* counts in these products could well exceed the acceptable limit of 100 cfu/g of food. Moreover, as *Fura-de-Nunu* is a street-vended food,

Table 2. Intrinsic parameters of *Fura* and *Nunu* samples.

Sample	a_w	%LA	%NaCl	%Wc	pH
Fura	0.76±0.02	0.31±0.04	2.6±0.17	58.11±8.59	4.5±0.1
Nunu	0.94±0.01	1.17±0.02	3.3±0.12	91.13±1.87	3.6±0.1

Values represent means ± standard deviation of triplicate experiments; a_w = Water activity; LA = lactic acid; NaCl = Sodium chloride; Wc = Water content.

there is no specification for the groups of individuals that can consume the product. It is available to all groups of consumers including infants, pregnant women and the elderly; it is in fact considered a complete food, suitable for weaning babies and should therefore meet the standard of 'complete absence of *L. monocytogenes* / 25 g of food'. Other studies conducted in Nigeria have also reported high loads of *L. monocytogenes* from different foods, including RTE foods (Chukwu et al., 2006; Ikeh et al., 2010; Nwaiwu, 2015).

According to Food Safety guidelines, methods that have been recommended to control the growth of *Listeria* in foods including the use of antibacterial treatments, pasteurization, reduction of pH, refrigeration and consumption within safe periods (FSA UK, 2010; Health Canada, 2011; FOOD Standards Australia, 2014). However, for the RTE food studied here, none of these measures are usually applied. The products are made crudely by local Fulani women and then hawked in pans and containers on the streets, under conditions which would surely support growth of the *Listeria* organisms.

Evaluation of the physico-chemical properties of the samples showed that the pH and water activity (a_w) values of the *Fura* and *Nunu* samples were 4.5± 0.1, 0.76±0.0 and 3.6 ±0.1, 0.94±0.0, respectively (Table 2). Although these values appear to be within the safe ranges (pH < 4.4 and a_w <0.92), the lack of specified shelf life and the conditions under which the foods are vended still leave reasons for concern. According to the Canadian Bureau of Microbial Hazards policy on *L. monocytogenes* in RTE foods (2011), growth of *L. monocytogenes* is assumed to occur in RTE foods if the pH and a_w values fall outside the safe range, unless the RTE food processor is able to present data, to be reviewed by regulatory authorities which demonstrates that the growth of *L. monocytogenes* will not occur within the specified shelf life (Health Canada, 2011). Sadly, for the RTE *Fura de Nunu*, these criteria are neither considered nor met, before the street-hawking of the products.

Representative *L. monocytogenes* isolates (n=23) identified by phenotypic and biochemical methods were subjected to PCR, which has proved to be a very useful and rapid method for detection of *Listeria* (Mansouri-Najand et al., 2015). Screening of the presumptive *L. monocytogenes* isolates by PCR showed that only about 77% of the isolates could be confirmed as *L. monocytogenes*, by detection of the 370 bp amplicon and 456 bp amplicon of the *prs* and *hlyA* genes, for identification of genus *Listeria* and *L. monocytogenes*, respectively (Figures 1 and 2). This result suggests that non-molecular methods of detection of these organisms may not be very specific and may lead to artificially high prevalence values. Thus, more specific molecular methods such as PCR must be used when evaluating *L. monocytogenes* contamination of food samples. The value of molecular methods of identification and typing has also been emphasized by other authors. For instance, it has been suggested that molecular methods are useful not just for accurate identification, epidemiological and trace-back investigations, but also for understanding the diversity and evolution of the organism (Doumith et al., 2004; Laksanalamai et al., 2014). It has also been suggested that although the classical biochemical methods of identification are standard, they can sometimes give false positives (Nwaiwu, 2015).

L. monocytogenes has 13 serotypes (1/2a,1/2b,1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7), but serogroups 1/2a, 1/2b and 4b are believed to be responsible for about 95% of human listeriosis (Kathariou, 2002; Jamali et al., 2013). Out of 24 *L. monocytogenes* (23 isolates and reference strain) screened by PCR-serogrouping in this study, only seven (including the reference strain) could be placed in any of the three serogroups tested for; and of these, only 1/2a and 4b were identified (Table 3). The 1/2a isolates were positive for *lmo0737* gene alone while the 4b isolates showed the presence of *ORF 2110.* Therefore, even though it has been suggested that variant strains of one serotype can sometimes acquire the gene clusters of another as in the reports of Laksanalamai et al., (2014), no such variants were detected in this study. Due to the epidemiological and clinical relevance of serotypes of the organism, serotyping is usually recommended for suspected *L. monocytogenes* isolates, particularly since the development of a PCR-based grouping scheme by Doumith et al. (2004). This molecular serogroup-related PCR typing has been endorsed by other researchers (Huang et al., 2011; Laksanalamai et al., 2014).

It has been suggested that pathogenic *L. monocytogenes* may be identified based on detection of some virulence markers, including internalins (*inlA, inlC, inlJ*), listeriolysin O (*hlya*), actin polymerization protein

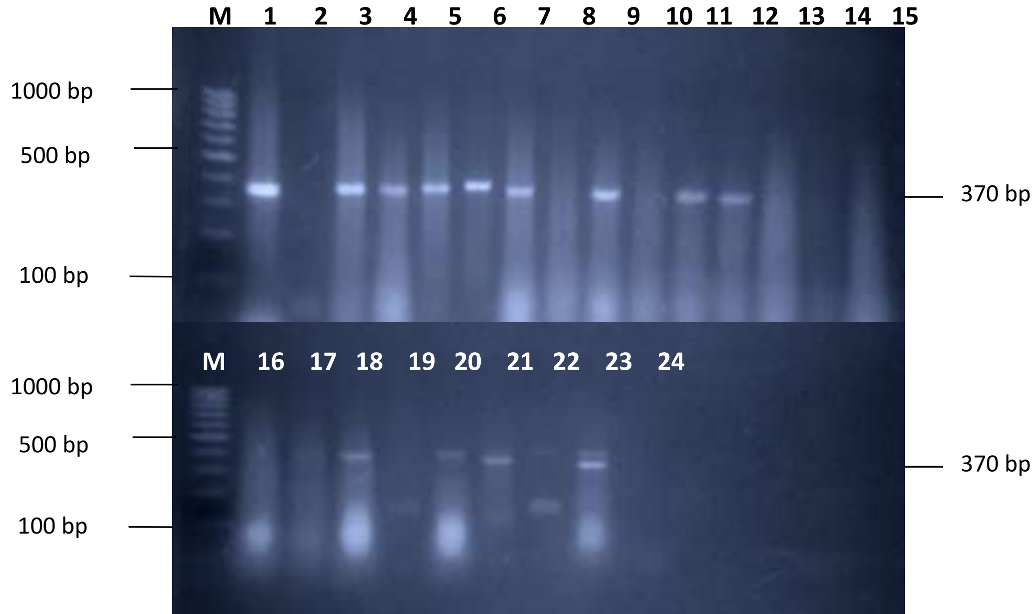

Figure 1. PCR detection of 370 bp amplicon of *prs* gene for *identification* of *Listeria* species. Lane M, 100 bp DNA ladder; lane 1, positive control (*L. monocytogenes* ATCC13932); lanes 2-23, presumptive *L. monocytogenes* isolates; lane 24 negative control. Lanes 3, 4, 5, 6, 7, 9, 11, 12, 18, 20, 21 and 23 are positive.

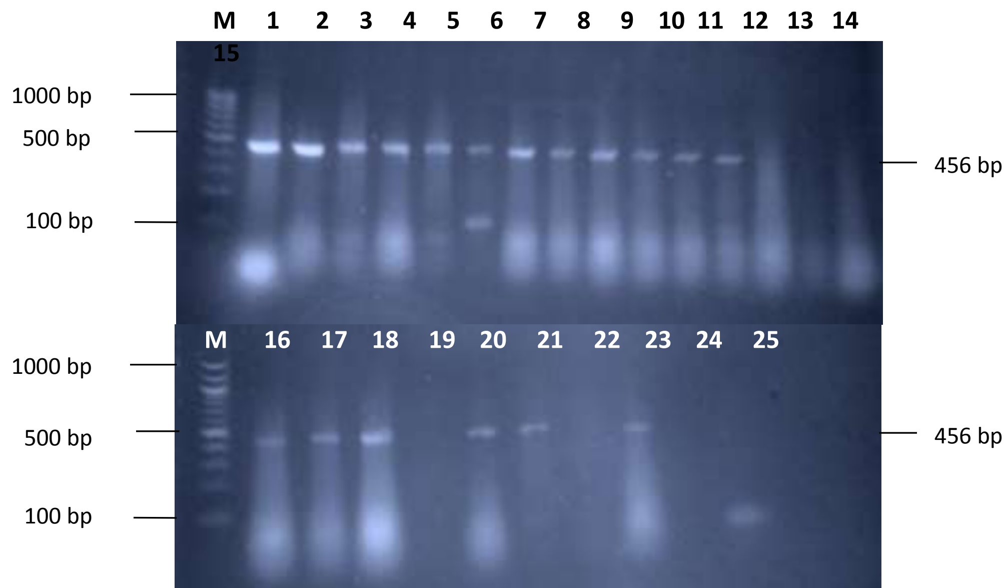

Figure 2. PCR detection of 456 bp amplicon of *hlyA* gene for identification of *L. monocytogenes*. Lane M, 100 bp DNA ladder; lane 1, positive control (*L. monocytogenes* ATCC13932); lanes 2-23, presumptive *L. monocytogenes* isolates; lane 25 negative control. Lanes 2-12, 16-18, 20, 21 and 23 are positive.

(*actA*), phosphatidyl-inositol-phospholipase C (*plcA*), invasive associated protein (*iap*) and virulence regulator (*prfA*), which are believed to be important in *L. monocytogenes* infection and pathogenesis (Liu et al., 2007; Di Ciccio et al., 2012). PCR-based methods have also been used to identify pathogenic *L. monocytogenes* strains possessing these genes (Huang et al., 2011; Laksanalamai et al., 2014; Swetha et al., 2015). The

Table 3. Genetic characteristics of some *L. monocytogenes* isolates from *Fura* and *Nunu.*

Isolate ID	Source	PCR amplification reaction						PCR serogroup
		prs	*hlyA*	*iap*	*Lmo 0737*	*ORF 2819*	*ORF 2110*	
N21	Nunu	+	+	+	-	-	+	4b
N54	Nunu	+	+	+	-	-	+	4b
N69	Nunu	+	+	+	-	-	+	4b
N71	Nunu	+	+	+	+	-	-	1/2a
N72	Nunu	+	+	+	+	-	-	1/2a
F3	Fura	+	+	+	+	-	-	1/2a
TC	ATCC 13932	+	+	+	-	-	+	4b

isolates in this study were screened for the presence of two virulence genes: *hlyA* and *iap* by PCR and both genes were detected in some of the isolates (Table 3). Detection of these virulence genes suggests that the *L. monocytogenes* isolates from this study are pathogenic. Further studies will aim to carry out full genome sequencing on these isolates, in order to further characterize and document the strains. The isolation of virulent *L. monocytogenes* from the RTE product, *Fura-de-Nunu,* in this study is a significant cause for concern. *Fura de Nunu* is considered to be a complete food and is relatively cheap. Therefore, in a country with a large population of low-income earners, it comes highly recommended as a means of reducing malnutrition and is widely consumed across demographic groups, including pregnant women, infants and the elderly, who are among the groups most at risk for listeriosis. The results from this study highlight a need for the development and implementation of food safety policies and standards to guide the production and distribution of RTE foods in Nigeria. Producers and consumers of RTE products should also be made aware of the need to observe the highest possible standards of hygiene during production of RTE and street-vended foods to reduce the risk of listeriosis and other food-borne diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Emphasis on functional properties of cocoa-specific acidifying lactic acid bacteria for cocoa beans fermentation improvement

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Lactic acid bacteria (LAB) strains isolated from six main Ivorian cocoa producer regions were investigated based on their biochemical properties in order to select the best one as potential starter. Three main technological and useful properties for good cocoa beans fermentation were monitored among the 568 isolated LAB strains. Thus, between the 408 cocoa-specific acidifying LAB strains identified, 05.88% (24 isolates) of them were able to maintain this activity in pH and temperature conditions as same as fermentation heap with an acidifying rate ranged within 0.03±0.00 to 1.19±0.070%. All these 24 cocoa-specific acidifying LAB strains displayed the ability to degrade the citrate while six (25%) of them were able to produce acetoin. These six LAB strains namely T_1Gb_8, T_6C_5, T_6C_{12}, T_7C_8, T_9C_9 and $T_{11}C_5$ were identified as *Lactobacillus plantarum.* They were the best able to continuously produce lactic acid in the required standards. Firstly under varying condition of temperature (30 à 45°C) and pH (3 à 6) with sharp acid (acetic, lactic and citric acids) influence. Secondly beyond 8% of ethanol content. The highlighted properties in this study and the performance of these six strains may make them best candidate as starters for cocoa fermentation control.

Key words: Lactic acid bacteria**,** cocoa-specific acidifying, technological properties, starter, Ivorian cocoa, fermentation.

INTRODUCTION

Cocoa beans constitute a basic export product for many tropical countries in America (Central and South), Asia (South and Southeast) and mainly West Africa. Export of raw cocoa beans is of great economic importance for West African countries which supply two thirds of the world's cocoa crop. Côte d'Ivoire is the world's leading producer of cocoa beans since 1978 (Deheuvels, 2003). Today, this country is highly dependent on this crop

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which accounts for 50% of national export income and 22% of the Gross Domestic Product (ICCO, 2016).Cocoa beans are an important source of cocoa powder and the principal raw material of chocolate. The sensory and physical properties of West African cocoa make it a much sought-after product, although overall production quality seems to be declining (Afoakwa et al., 2013). Unfortunately, ensuring the marketable quality of cocoa beans remains difficult due to variability in the organoleptic quality of this product. This inconsistency in cocoa quality is mainly linked to post-harvest processing including fermentation, drying and roasting of the crude and fresh cocoa beans. Fermentation is considered as the most important processing step influencing the quality of cocoa beans. Indeed, during this process, there is initiation of flavor precursors, color development and a significant reduction in bitterness and astringency of the beans (Afoakwa et al., 2013). These changes involve the action of diverse microrganisms naturally found in the cocoa pulp. The succession of these microbial groups has been clearly established and lactic acid bacteria (LAB) population is considered as the second microbial group growing in mucilaginous pulp of cocoa beans after yeast population (Crafack et al., 2013). This micro-biota group is mainly dominating until 24 to 48 h of fermentation (Kouame et al., 2015). The most important roles of LAB are the degradation of the citric acid in the pulp, leading to increase in pH, the production of ethanol and both volatile and non-volatile organic acids such as acetic and lactic acids (Penia et al. 2013). Organic acid will diffuse into the beans cotelydon and induce the enzymatic reactions to form precursor of flavor, aroma and color (Afoakwa et al., 2013). The diffusion of certain amount of acid into the bean during fermentation is essential for the development of flavor precursors. Indeed, the presence of excessive acidity usually correlates with poor development of chocolate flavor (Serra et al., 1997). Thus, acidity is a critical factor in defining the quality of cocoa used in chocolate manufacturing. The control of cocoa fermentation process is very challenging for farmers in cocoa producing countries because cocoa fermentation has always been a natural process which sometimes results in poorly fermented cocoa. Therefore, studies suggest the use of starter microbial culture as the best approach to improve the fermentation process. In this perspective, LAB strains as monoculture or co-culture would be essential component of starter culture aimed at the control of cocoa beans fermentation process. Studies conducted by differents authors, such as Papalexendratou et al. (2013), Penia et al. (2013) etc., on cocoa fermentation showed that the maximum rate of lactic acid produced ranged between 0.5 and 1.2%. As excessive acidity, mainly lactic acid, produces unsuitable raw cocoa for chocolate making, it appears more judicious to isolate, screen and characterize LAB strains with cocoa-specific acidifying potential included in this limit of 1.2%. These strains may positively and durably influence the quality of a controlled cocoa fermentation especially if they provide useful additional properties such as citrate degradation and acetoin production (Rodriguez-Campos et al., 2012). Information on cocoa-specific acidifying LAB strains with adequate lactic acidifying potential, citrate degradation and acetoin production may be a starting point for the development of starter culture. Thus, the aim of the research was to select strains of LAB that best meet these different criteria in order to optimize and standardize the cocoa fermentation process.

MATERIALS AND METHODS

Fermentation condition

Cocoa pods were harvested on farms from six cocoa producing regions of Ivory Coast: Cavally (6° 25' 0 North -7° 30' 0 West), Gkôklè (4° 57′ 04″ north 6° 05′ 19″ West), Gôh (6° 15’ 0’’ North 5° 55’ 0’’ West), Haut-Sassandra (7° 0' 0 North -6° 34' 59 West), San-Pedro (4° 44' 54 North -6° 38' 10 West) and Tonkpi (7° 24’ 45’’ North 7° 33’ 14’’ West). Spontaneous cocoa bean fermentation was performed using traditional conditions, with temperature and humidity ranging respectively, from 28 to 30°C, and 60 to 65% for 6 days. The fermenting mass (50 kg) set on banana leaves and covered with banana leaves were constituted of mixed genotypes (Forastero, Trinitario and Criollo cultivars).

Sampling

The fermenting heap was mixed and 100 g beans were collected, at the beginning and each 12 h of fermentation, in sterile Stomacher bag. A total, 13 samples of each fermenting cocoa according to the locality were collected for physico-chemical and microbial analysis.

Isolation of bacterial strains

Isolation of LAB was performed according to the standard method described by Pereira et al. (2012). An amount of 25 g of fermenting cocoa beans was homogenized in 225 mL sterile peptone water in a Stomacher bag (Seward, Worthington, United Kingdom) for 5 min at room temperature. After appropriate dilution of samples in sterile saline solution, 0.1 mL from each dilution was inoculated onto duplicate plates of MRS agar (Oxoid) supplemented with 50 µg/mL of nystatin to inhibit the fungal growth. Plates were incubated at 30°C for 48 to 72 h, under anaerobic conditions.

Preliminary biochemical test of lactic acid bacteria

LAB isolates, identified using conventional colonial morphology, gram staining and biochemical reactions (oxidase and catalase tests), were stored in cryotubes at -20°C in MRS broth supplemented with 20% glycerol for further investigations.

Screening of cocoa-specific acidifying LAB strains

Cocoa-specific acidifying LAB strains were screening based on their acidification capacity at 30°C according to the protocol of Guha et al. (2013). 100 µL of a preculture recording an optical

density (OD) of 1 at 600 nm against a control (MRS broth) were used to inoculate 5 mL of MRS broth. The seeded broths were incubated at 30°C in a water bath (Julabo TWB 12) for 48 h. Then, fermented broths were centrifuged at 4500 trs/min for 10 min to pellet out the bacterial growth. Supernatant was used to estimate lactic acid content by titrimetric method with 0.1 NaOH. The percentage of lactic acid was calculated using the formula in equation 1. Each treatment was tested in triplicate.

$$\%\ Lactic\ acid = V(NaOH) \times N \times 0{,}09 \times 100/V(test) \quad (1)$$

V(NaOH) : Volume of NaOH solution used for titration
N : Normality of the NaOH solution (0.1)
V (test): Sample Volume

Analysis of additional properties of cocoa-specific acidifying lactic acid bacteria isolates

Screening of LAB strains for citrate degradation

The ability of lactic acid bacteria to degrade citrate was investigated using the method described by Kempler and McKay (1980). A basal medium containing 1% milk powder, 0.25% casein peptone, 0.5% glucose and 1.5% agar was first prepared and then 1L of the medium, was supplemented with 10 mL of potassium ferricyanide (10%) solution and 10mL of mixed iron citrate and sodium citrate (2.5%) solution. The medium was inoculated with pure 24 h preculture of LAB strains and incubated in an anaerobic jar at 30 °C for 48 to 72 h. The citrate metabolism was assessed by the formation of a blue complex surrounding the colonies.

Screening of LAB strains for acetoin production

The production of acetoin was investigated using the method described by King (1948). Acetoin production from citrate was checked in the basal liquid medium. Clark and Lubs medium was seeded with pure 24 h preculture of LAB strains and incubated at 30°C for 48 h. After incubation time, acetoin production was revealed in 1mL of cell free supernatant by adding two drops of an alcoholic solution of alpha-naphthol (6%) and two drops of sodium hydroxide (16%) solution. The presence of acetoin was assessed by appearance of red color.

Effect of environmental stress on lactic acid production by cocoa-specific acidifying LAB strains

Influence of temperature and pH

The influence of temperature and pH on the acidification capacity of cocoa-specific acidifying lactic acid bacteria strains was evaluated by Guha et al. (2013) method. Seeded broths were incubated at 30, 35, 40, 45 and 50 °C to assess the influence of temperature and at pH 3; 4; 5 and 6 for pH influence, in a water bath (Julabo TWB 12) for 48 h.

Influence of alcohol and organic acids on lactic acid production by cocoa-specific acidifying LAB strains

To analyze the acidification potential of cocoa-specific LAB strains in alcoholic and acidic conditions, a MRS liquid medium was prepared. After autoclaving, this medium was cooled and maintained in liquid state at 45 °C and supplemented with alcohol (2, 4, 6 and 8%), acetic and lactic acid (0.2; 0.4; 0.6; 0.8; 1; 1.2%), citric acid (0.5; 1; 1.5; 2; 2.5 and 3%). The standard inoculum was prepared as follow: a 18 h preculture was suspended in a saline solution to obtain an absorbance OD 1 at 600 nm. 100 µL were used to inoculate 5 mL of MRS broth. The seeded broths were incubated at 30°C in a water bath for 48 h. After incubation time, the amount of total acid produced by each strain was determined by titration as previously described.

Molecular characterization of lactic acid bacteria starters

16S rRNA gene PCR and sequence analysis

To perform PCR reactions, The isolates of LAB strains were grown for 24 h at 30°C on agar plates. A loopful of pure culture was suspended in 100 µL of sterile distilled water for colony PCR.

PCR amplification of ribosomal 16S gene

The hypervariable regions (V1, V2 and V6) of the different LAB strains were amplified by using universal primers F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R520 (5'-ACCGCGGCTGCTGGC-3') (Ouattara et al., 2011) for colony PCR. Each mixture (final volume 50 µL) contained about 1 µL of sample 1.25 U of Taq DNA polymerase (Biolabs, Lyon France), 5 µL of 10x Taq Buffer (10 mM TrisHCl, 50 mM KCl, 1,5 mM MgCl2), 1 µL of 10mM dNTP, 2 µL of each primer (10 µM) (Eurofins, Genomics, Germany) supplemented by sterile water Milli-Q. PCR amplification was carried out in a thermocycler (Sensoquest Labcycler) as described previously (Ouattara et al., 2011). After an initial denaturation at 95 °C for 4 min, reactions were run for 35 cycles. Each cycle comprising denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1min; and final extension at 72 °C for 10 min.

Gel electrophoresis

After migration of the products realised at 70 V for 2 h in a tank, amplicons were revealed by incubating on 0.8% agarose gel electrophoresis in a 1x phosphate TAE buffer containing bromide of ethidium. The molecular weight of the products was estimated in reference to a molecular weight marker (Biolabs, France) of 500 bp.

Sequencing and analysis of hypervariable sequence of 16S RNA gene amplified

The PCR products were purified using the nucleospinR Gel and PCR Clean-up kit (MachereyeNagel, Germany). Then, they were sequenced by MWG Eurofins using the primer F27. The basic local alignment search tool (BLAST, blast N) from the NCBI database site (blast.ncbi.nlm.nih.gov/ BLAST/) was used to find the closest sequences relative to the amplified 16S RNA genes in order to identify our LAB strains.

RESULTS AND DISCUSSION

Acidification capacity of LAB strains isolates

A total of 568 strains characterized as gram positive, oxydase negative and catalase negative identified as

Table 1. Distribution of LAB strains isolates based on their acidifying capacity.

Regions	S.I.	A.C. of total LAB strains isolates			C.S.A.LabStrains (A.R.≤1.2%)
		Low acidity LAB strainsA.R.< 0.45%	Medium acidity LAB strains0.45<A.R.≤1.35%	High acidity LAB strainsA.R.≥ 1.35%	
Cavally	104 (18.31)	00 (00)	40 (12.23)	64 (46.71)	036 (08.82)
Gboklè	087 (15.33)	05 (04.81)	78 (23.85)	04 (02.92)	078 (19.12)
Goh	048 (08.45)	15 (14.42)	15 (04.59)	18 (13.14)	028 (06.86)
H. S.	109 (19.19)	25 (24.04)	84 (25.69)	00 (0.00)	107 (26.23)
S. P	122 (21.47)	59 (56.73)	63 (19.27)	00 (0.00)	121 (29.66)
Tonkpi	098 (17.25)	00 (00)	47 (14.37)	51 (37.23)	038 (09.31)
Total Strains	568 (100)	104(8.31)	327 (57.57)	137 (24.12)	408 (71.83)

Note: With regard to the acidity property, C.S.A.LAB strains (A.R.≤1.2%): correspond to the Low and Medium acidity LAB strains with acidifying potential ≤1.2% ; H. S.: Haut sassandra; S. P.: San pédro; S.I.: Strains Isolates; A.R.: acidification rate. A.C. of LAB strains isolates: Acidifying capacity of LAB strains isolates; C.S.A.LAB strains: Cocoa-specific acidifying LAB strains.

LAB were isolated from six Ivorian regions. Thus, these LAB strains were anlysed for their capacity to acidify the fermentative medium. The results showed that the number of isolated LAB strains have unequal distribution depending on the region. San Pédro recording the highest (21.47%) number of isolates (Table 1) unlike Gôh, which has the lowest number (08.45%) of strains. This variability indicated the influence of the local geographical area on the composition of the microflora involved in cocoa fermentation (Schwan and Wheals, 2004). Therefore, this factor could impact on the distribution of their different activities. One of the important activities attributed to LAB strains is to acidify the fermentative medium. As this property is susceptible to play a key role in the fermentative process (Afoakwa et al., 2013), it appears important to screen them based on this ability. Most importantly, this type of metabolic pathway should be determined to identify potential starters among these isolates.

All the 568 isolated LAB microflora which were screened for their acidification capacity showed naturally acidification capacity with different levels of acid production (Table 1). Indeed, this natural capacity for acidification of the fermentative medium by LAB is related to the fact that they are able to exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is lactic acid which is the main metabolite of two broad metabolic categories of LAB. The first metabolic category is represented by the homofermentative LAB. They convert glucose into lactic acid more than 90% by the Embden-Meyerhof (E-M) pathway. The second metabolic group is represented by the heterofermentative LAB producing about 50% of lactic acid as the principle by-product of sugar fermentations (Raimbiault 1995).

It should be noted that a acidification scale has been established based on the acidification capacity of LAB from different fermentation medium such as milk, cheese, wine, fish, meat, sausages, butter and cream (Behannis and Kayanush, 2012; Joko et al., 2014). The recorded lactic acid values (around twenty) ranged between 0.05% and 1.6% and allowed to identify three types of LAB strains according to their capacity of acidification: LAB strains with low acidity capacity (acidification rate (AR) < 0.45%); LAB strains with medium acidity capacity (0.45 < AR ≤1.35%) and those with high acidity capacity (AR ≥ 1.35%).

The results obtained are depicted in Table 1. Based on this classification, three types of LAB strains were observed in the different regions except in Cavally and Tonkpi with respect to the low lactic acid producing strains and the regions of Haut Sassandra and San Pédro with respect to the high producers. In addition, medium acidifying LAB strains dominated with 57.57% compared to 24.12% for high acidifying LAB strains and only 18.31% for low acidifying LAB strains. This diversity in acidifying capacity of LAB strains suggesting a difference in acidification potential and therefore efficiency in acidification by these strains. This could be the main determining factor in the variability of cocoa bean quality as cocoa beans acidification often has negative impact on the final quality of the market cocoa.

Therefore, the cocoa market could ultimately be related to cocoa bean quality which is dependant on selective LAB strains with satisfactory level of acidification. This approach could make it possible to ensure the control of the acid level of marketable beans and thus the control of their quality. With regard to lactic acid, the satisfactory level of acidification can only be obtained with LAB strains that do not have high acidification capacity. Indeed, the low volatility of this acid promotes its accumulation in beans. If this acid is produced in large quantities, it can hardly be eliminated. This will irremediably lead to acidic beans and therefore of poor quality. Thereby, LAB strains with low and medium acidification capacity (431) retained our attention,

Table 2. Influence of temperature on lactic acid production by Cocoa-specific acidifying LAB strains per region.

Regions	Number of LAB strains with acidifying potential ≤1.2% at			
	30°C (%)	35°C (%)	40°C (%)	45°C (%)
Cavally	036 (08.82)	023(09.02)	022(09.73)	17 (16.35)
Gbôklè	078 (19.12)	062(24.31)	058(25.66)	34 (32.70)
Gôh	028 (06.86)	015(05.88)	011(04.87)	06 (05.77)
H.S.	107 (26.23)	081(31.76)	072(31.86)	25 (24.04)
San-Pedro	121 (29.66)	058(22.75)	050(22.12)	16 (15.38)
Tonkpi	038(09.31)	016(06.27)	013(05.75)	06 (05.77)
Total	408 (100)	255 (100)	226 (100)	104 (100)

approximately two-thirds of LAB strains isolated.

Then, a second level of selection has been established to better refine our selection. This selection takes into account the maximum rate of lactic acid produced during the fermentation of cocoa. Studies conducted by Afokawa (2013), Kouame et al. (2015) and Papalexendratou et al. (2013; 2015) on cocoa fermentations, around the world, lead to the identification of a maximum rate of lactic acid produced of 1.2%. Thus, in order to mimic the natural conditions of fermentation, it is important that the selected LAB strains do not produce lactic acid beyond this limit (1.2%). The results of this selection are shown in Table 1. Among the 568 strains of LAB previously isolated, approximately two-thirds (71.83%) produced an amount of lactic acid that not exceed 1.2%. This total of 408 LAB strains applies to all the strains with low acidifying capacity (104) and part of the medium acidifying strains (304 of 327). These strains are considered to be "Cocoa-specific acidifying LAB strains". As high acidity, mainly lactic acid, in cocoa beans produces unsuitable raw cocoa, these 408 Cocoa-specific acidifying LAB strains appear to be the best candidates as potential starter culture to control cocoa bean fermentation.

Influence of temperature and pH on lactic acid production by Cocoa-specific acidifying LAB strains

The pH of cocoa beans during fermentation is critical with regard to the biochemical reactions that take place in the beans (Afokawa et al., 2013). The ability of the 408 Cocoa-specific acidifying LAB strains to maintain their acidifying potential ≤1.2% at temperatures up to 45°C is depicted in Table 2. Only 104 strains maintained their acidifying potential under 45°C of temperature among the 408 strains previously selected.

Cocoa fermentation is generally characterized by high temperature up to 45°C occurring between 48 and 72 h of fermentation (Ho et al., 2014). In view of these results, we can assess that the thermo-tolerance exhibited by these 104 heat-resistant strains should allow them to maintain a satisfactory level of lactic acid production under thermic stress conditions during cocoa fermentation.

On the other hand, the effect of pH variations on these 104 heat-resistant strains, showed that only 24 of them were able to continuously maintain their acid production for pH variations ranging from 3 to 6 (data not showed). These pH values are those prevailing in cocoa beans fermentation within 24 to 72 h of fermentation (Kouame et al., 2015). Table 3 highlights the amounts of lactic acid produced by these 24 cocoa-specific acidifying LAB strains under pH fluctuation.

Regarding the rate of lactic acid produced by these Cocoa-specific acidifying LAB strains, values are ranged from a minimum of 0.03 to a maximum of 1.19 for pH values between 3 and 6 (Table 3). These findings pointed out the fact that these Cocoa-specific acidifying LAB strains did not have the same capacity of adaptation to temperature and pH fluctuations of the fermentative heaps even if they are able to maintain their lactic acid production in the required standards.

Additional activity of isolated Cocoa-specific acidifying LAB strains: citrate degradation and acetoin production

Citrate degradation and acetoin production are important and relevant properties desirable for a good cocoa fermentation (Schwan and Wheals, 2004). Thus, biochemical characterization of the 24 Cocoa-specific acidifying LAB strains showed that they exhibited the property to break down citric acid. Additionally, only six (25%) of them displayed both the ability to degrade citrate and to produce acetoin.

The screening of citrate consumers among these cocoa-specific acidifying LAB strains indicates that all of them are able to breakdown citrate. Citrate metabolism constitutes, an important and particular property, since LAB strains are not usually able to utilize citric acid as carbon source (Kouame et al., 2015). As citrate metabolism is an essential factor for modulating the pH

Table 3. Amount of lactic acid produced by cocoa-specific acidifying LAB strains under pH variation per region.

Regions	Number of LAB strains isolates	Acidifying potential of cocoa-specific LAB (%) at			
		pH_3	pH_4	pH_5	pH_6
Gbôklè	04	0.03±0.014-0.30±0.057	0.04±0.00-0.12±0.02	0.50±0.071-0.80±0.226	1.06±0.057-1.19±0.070
H.S.	03	0.63±0.00-0.86±0.028	0.87±0.01- 1.04±0.00	1.02±0.042-1.10±0.170	1.16±0.030-1.12±0.056
San-Pédro	02	0.65±0.005-0.73±0.006	0.90±0.016-1.09±0.025	1.11±0.028- 1.10±0.150	1.14±0.064-1.16±0.046
Gôh	01	0.05±0.00	0.10±0.00	0.15±0.00	0.16±0.05
Cavally	10	0.08±0.00-0.67±0.002	0.10±0.00- 0.98±0.013	0.20±0.05-1.02±0.06	0.99±0.00-1.18±0.015
Tonkpi	04	0.03±0.00-0.20±0.001	0.10±0.00 0.85±0.113	0.50±0.00-1.06±0.06	0.91±0.001-1.18±0.008
Total	24	0.03±0.00-0.86±0.028	0.04±0.00 -1.09±0.025	0.15±0.00 -1.06±0.06	0.16±0.005 -1.19±0.070

H.S: Haut Sassandra.

and promoting the growth of desirable bacterial flora in fermenting cocoa (Schwan and Wheals, 2004) all these 24 LAB strains should be responsible for the raising of the pH recorded during cocoa beans fermentation.

In addition, acetoin production shows that these six cocoa-specific acidifying LAB strains (T_1Gb_8, T_6C_5, T_6C_{12}, T_7C_8, T_9C_9 and $T_{11}C_5$) were able to metabolized citric acid via pathways pII producing acetoin from citrate. This ketone carbonylic compound (such as acetoin) is crucial for the development of good cocoa flavor. Indeed, Rodriguez-Campos et al. (2012) indicated that acetoin appears to be a precursor of tetramethylpyrazine, an important odor-active component of cocoa flavor. Therefore, these six LAB strains are of interest for the fermentation process as potential starter.

16S rRNA gene PCR and identification of isolates

Amplification of hypervariable regions (V1, V2 and V6) of the 16S gene of six potential starters LAB by colony PCR yielded a DNA fragment with respect to the 500bp (Figure 1). Analysis of these DNA fragments in the NCBI data bank led to the identification of these six LAB (T_1Gb_8, T_6C_5, T_6C_{12}, T_7C_8, T_9C_9 and $T_{11}C_5$) isolates as belonging to *Lactobacillus plantarum* with 99% of similarity. *L. plantarum* has been isolated from many cocoa fermentations around the world (Camu et al., 2007; Pereira et al., 2012).This observation indicates that, these strains play key roles in cocoa bean fermentation. *L. plantarum* species has also been used as starter for cocoa fermentation (Joko et al., 2014; Penia et al., 2013) and are widely used in food industies to make other fermented foods (Joko et al., 2014).

Influence of additional stress on lactic acid production by isolated Cocoa-specific acidifying LAB strains.

In addition to pH and temperature, other factors such as acids (acetic, lactic and citric) and alcohols (ethanol) are key parameters that may strongly influence the performance of the LAB strains in lactic acid production. Thus, the six selected LAB strains (T_1Gb_8, T_6C_5, T_6C_{12}, T_7C_8, T_9C_9 and $T_{11}C_5$) and identified as *Lactobacillus plantarum* were tested to assess the influence of these key parameters on Their ability to maintain lactic acid production.

Effect of acids (acetic and lactic acids) on lactic acid production

In acid conditions induced by lactic, acetic and citric acids, the six selected LAB strains proved to be able to maintain their potential of lactic acid production despite the observed decline. Lactic acid production occurred in medium containing lactic and acetic acid in the same range 0-1.4% for the six strains belonging to *Lactobacillus plantarum* (Figure 2 (A and B)) while this acid production continues up to 1.6% of acetic acid in the medium. The influence of citric acid on lactic acid production was lesser than the other tested acids (Figure 2C). Lactic acid production was slowly decreased with increasing of citric acid concentrations in a range of 0-3%. All *Lactobacillus*

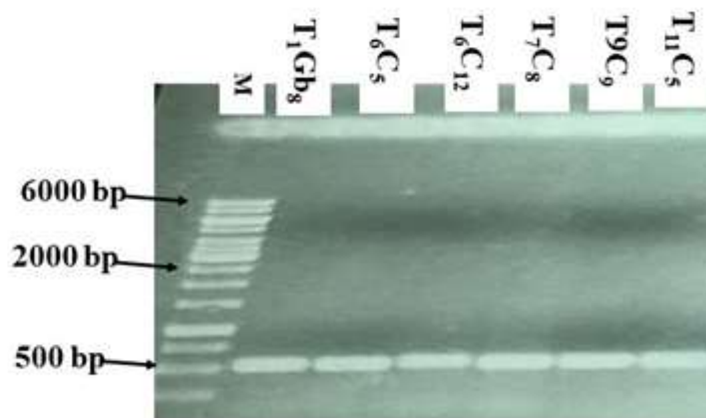

Figure 1. Electrophoretic profile of the PCR products of hypervariable regions of the 16S rRNA gene from the six LAB potential starters. M: molecular weight marker (ladders).

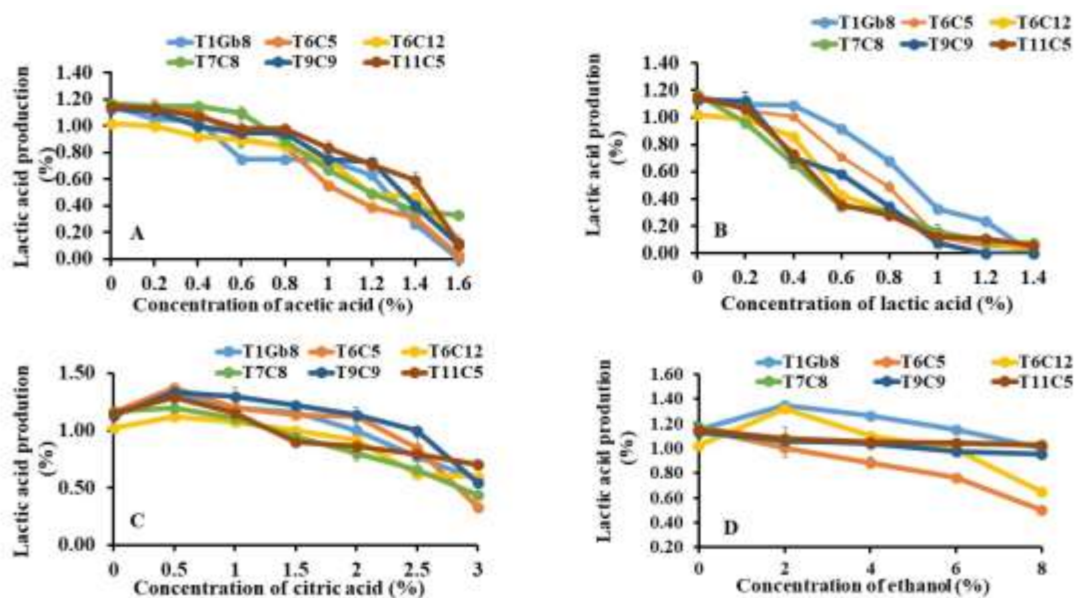

Figure 2. Effect of acetic acid (A), lactic acid (B), citric acid (C) and ethanol (D) on lactic acid production by the six selected LAB strains.

plantarum tested strains maintained their ability to produce lactic acid in proportions ranging from 35 to 66,96% of the initial level with a maximum of lactic acid (between 1.12 and 1.35%) produced under 0.5% of citric acid in the medium. The effect of acids results in a reduction of cytoplasmic pH that is likely to impair lactic acid production (Lopez and Dimick, 1995). This indicates that, compounds present at variable concentrations in the cocoa pulp such acids may be limiting factors for lactic acid production in LAB strains during cocoa fermentation. However, with their ability to maintain lactic acid production despite pH fluctuations between 3 and 6 in cocoa beans fermentation, all the six selected LAB strains should be particularly interesting as starters.

Effect of ethanol on lactic acid production

The influence of ethanol concentration on lactic acid production is shown in Figure 2D. The results suggested

that, all these six studied strains maintained a good level of lactic acid production until 8% of ethanol concentration. This lactic acid poduction ranged between 43.50 and 91.67% of their initial potential production. These results are similar to those described by Behannis et al. (2012) with a decrease in acid produced when ethanol concentration increased from 2.5 to 7.5%. These results are interesting because ethanol is a major metabolite of cocoa pulp fermentation and the maximum rate of ethanol produced by yeasts is around 8% during this process (Lefeber et al., 2012). High concentrations of ethanol reduce cellular vitality and increase cell death resulting in a gradual decline in lactic acid production (Birch et al., 2000). Thus, these six ethanol tolerant isolates (T_1Gb_8, T_6C_5, T_6C_{12}, T_7C_8, T_9C_9 and $T_{11}C_5$) at high ethanol concentration, could both maintain their citrate and acetoin metabolism and their lactic acid production in the required standards to improve the final fermenting cocoa bean quality.

Conclusion

In this study, six LAB strains (T_1Gb_8, T_6C_5, T_6C_{12}, T_7C_8, T_9C_9 and $T_{11}C_5$) involved in Ivorian cocoa fermentation were identified as *Lactobacillus plantarum*. These particular cocoa-specific acidifiying bacteria presented both the required acidifying capacity and in a lesser extent, citrate degradation and acetoin production. All of them were able to maintain their lactic acid production under fermentative stress conditions in the required standards. That make them suit candidate as starter for cocoa fermentation control.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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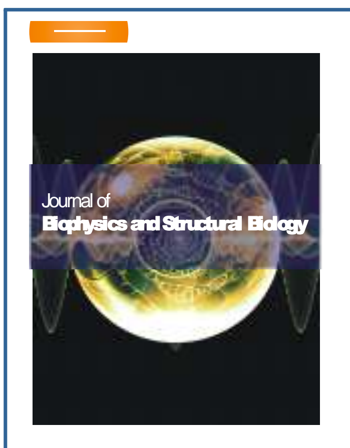

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