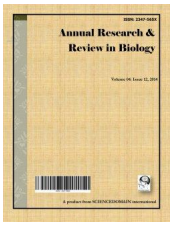

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Assessment of the Diversity of Lactic Acid Bacteria Involved in Cocoa Fermentation of Six Main Cocoa Producing Regions of Côte d'ivoire

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Authors' contributions

This work was carried out in collaboration between all authors. Author SLN designed the study, Author ECA performed the experiment. Authors SLN, ECA and GGD managed the draft of the manuscript. Authors ECA and HDO wrote the protocol, and wrote the first draft of the manuscript. Authors GGD and SLN managed the literature searches and performed the analysis. All the authors read and approved the final manuscript.

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ABSTRACT

Aims: The variability of lactic acid bacteria (LAB) species involved in cocoa bean fermentation would cause inconsistency in the quality of cocoa. The aims of this study is to investigate the physicochemical parameters of cocoa bean fermentation in order to assess the activity and the molecular diversity of LAB involved in cocoa fermentation from six other regions of Côte d'Ivoire.
Place and Duration of Study: Laboratory of Biotechnology, UFR Biosciences, University Félix Houphouet-Boigny (Côte d'Ivoire), between October 2016 and September 2017.
Methodology: Spontaneous heap fermentations were conducted in six cocoa producing regions during 6 days. Physicochemical analysis of cocoa mass such as temperature, pH, titratable acidity and reducing sugars were carried out. In addition, LAB isolation was performed using plate culture on MRS medium and their fermentative type as well as their profile were determined. In addition, LAB species were determined by restriction profile analysis of the 16S gene.
Results: a total of 568 LAB were isolated from cocoa fermentation. Biochemical and morphological

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identification of these germs revealed the clear dominance of the bacilli form (81.16%) and the heterofermentative type (over 80%) with facultative heterofermentative type recording more than half (54.4%) of the isolated population. Their molecular identification by sequencing the hypervariable zone of the 16S rDNA gene of a few representatives from each restriction group revealed 08 species with a predominance of *Lactobacillus plantarum* (76.76%) and *Leuconostoc mesenteroides* (15.31%) associated with minority species. This species diversity could be exploited for selecting appropriate starter cultures.

Conclusion: This diversity of LAB species could be responsible for the variability of cocoa quality in Côte d'Ivoire.

Keywords: Cocoa beans; Côte d'Ivoire; fermentation; lactic acid bacteria; molecular diversity.

1. INTRODUCTION

Lactic acid bacteria (LAB), Gram-positive and catalase–negative bacteria, are useful microorganisms for humans [1]. They are a heterogeneous group composed of cocci and bacilli which grow under micro aero-tolerant to strictly anaerobic conditions [2]. These germs are highly prevalent in habitats rich nutrient such as soil, water, manure, animal skin, feces and plant matter [3]. The use of LAB has been of great importance in food technology. It is basically used to improve aroma, texture, flavor, shelf live, nutritional value or digestibility of fermented foods products and beverages such as dairy foods, fermented sausages etc. LAB can achieve these various functions because they synthesize a broad range of compounds including organic acids, bacteriocins, vitamins antimicrobial agents, aromatic compounds and exopolysaccharides (EPS) among others [4].

A wide variety of LAB have been identified in food fermentation, including mainly *Lactobacillus, Leuconostoc, Weissella, Streptococcus*, and *Pediococcus* species. During cocoa fermentation, LAB strains undergo either the homo-fermentative or the hetero-fermentative pathway. LAB strains like *L. lactis*, *L. delbrueckii* and *L. casei* uses the homo-fermentative pathway to metabolize one molecule of hexose sugar such as glucose to two molecules of lactic acid and two molecules of ATP whiles, strains like *L. amylovorus*, *L. manibotivorans* ferments one molecule of glucose to one molecule of lactic acid, one molecule of ethanol or acetate, one molecule of CO_2, and only one molecule of ATP [5].

So in cocoa fermentation, LAB use pulp sugars to produce mannitol, lactic and acetic acids [5-6]. These germs also metabolize the citric acid contained in the cocoa pulp to produce aromatic compounds such as acetaldehyde, diacetyl, acetoin, 2,3-butanediol and offer favorable conditions for the other bacteria growth notably *Bacillus* and acetic acid bacteria (AAB) in cocoa fermentation. The lactic acid and mannitol they produce could serve as extra energy sources for AAB species [7]. LAB species are important for a successful microbial succession during cocoa bean fermentations. Actually, they form the link between the ethanol and flavor-producing yeast fermentation and the acetic acid-producing AAB fermentation [8]. This importance of LAB in cocoa fermentation explain why these germs were mostly studied in order to assess their diversity and identify different key species which could be exploited for selecting appropriate starter cultures in cocoa fermentation. Species involved in cocoa fermentations from several countries have been identified. These studies have shown that LAB species involved in cocoa fermentation differ from country to another country and also from local region to another [9-10-11-12]. This species variability would be responsible for the inconstency of fermented cocoa bean quality in Côte d'Ivoire as well as the hazardous and spontaneous nature of this fermentation. To fix this problem, scientists have suggested to use starters to improve this process [13-14-15]. Thus, authors [8] indicate that LAB accelerates the fermentation process by producing metabolites such as organic acids, aromatic compounds. Also, Penia et al. [16] showed that *Lactobacillus plantarum* improved the fermentation index and could significantly reduce the fermentation time. Authors [17] found a positive effect on the quality of fermented cocoa. The addition *of Lactobacillus fermentum* in the fermentation mass of cocoa led to high quantity of fully fermented beans (98.43%). In addition, the controlled fermentation trials, with convincing results, were performed with microbial cocktails always including a strain of LAB [13-18].

This underlines the fundamental role of LAB in this fermentation process and points out the

need to identify the different species involved in the fermentation of cocoa from the main ivorian cocoa-producing regions. So investigations must be carry out in the different cocoa-producing areas. From Côte d'Ivoire, the first cocoa producer/exporter, on 17 cocoa producing regions, only LAB strains involving in cocoa fermentation with varieties of cocoa (Forastero, Trinitario and Criollo) of six local regions, including Agnéby-Tiassa, Guemon, Indénié-Djouablin, Lôh-Djiboua, Nawa, Sud-Comoé were studied in order to assess molecular diversity of LAB citrate degradation strains and to select appropriate starter [12]. Perfect knowledge of the diversity of these strains is necessary for all the ivorian producing regions as none information exists from the others local regions. The aims of this study is to investigate the physicochemical parameters of cocoa bean fermentation in order to assess the activity and the molecular diversity of LAB involved in cocoa fermentation from six other regions of Côte d'Ivoire.

2. MATERIALS AND METHODS

2.1 Fermentation Condition and Sampling

Cocoa pods constituted of mixed genotypes (Forastero, Trinitario and Criollo cultivars) were harvested at farms from six cocoa producing regions of Côte d'Ivoire notably 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). The spontaneous cocoa bean fermentation was performed in National Flowers Center of Félix Houphouët-Boigny University (temperature around 28-30°C and humidity 60-65%), in traditional conditions by heap fermentation during 6 days. The fermenting mass (50 kg) set on banana leaves and covered with banana leaves (Fig 1). The fermenting heap was mixed and 100 g beans were collected in sterile Stomacher bag, at the start and each 12 h of fermentation. A total of 13 samples of each fermenting cocoa according to the locality were collected for physicochemical and microbial analysis.

2.2 Physicochemical Analysis

2.2.1 Temperature and pH determination

The temperature was recorded directly on mass fermenting with a precision thermoter (ALLA, France). The pulp pH was determined according to [19] method. Ten (10) g of the pulp make as was weighed using a scale (SARTORIUS) and then, 90 mL of distilled water was added. The extract obtained was homogenized and centrifuged at 4500 rpm (LABOFUGE) for 10 min. The supernatant obtained was used to read the pH by introducing a previously calibrated pH-meter electrode.

2.2.2 Cocoa pulp titratable acidity determination

Titratable acidity was determined according to [19] method. Cocoa beans were mixed in distilled water and filtrate. Titration was carried out by pouring drop by drop a solution of NaOH (0.1. N) (V1) in 5 mL of filtrate in the presence of phenolphthalein (2 drops) until obtaining a pink color (indicating the end of the titration). Titratable acidity was expressed milliequivalents (meq) for 1 g of fresh matter by the following relation:

Each treatment was tested in triplicate

$$Titrable\ acidity\ \left(\frac{meq}{g}\right) = \frac{N \times V_1 \times 100}{m \times V_0}$$

V0: volume (mL) of the collected filtrate,
V1: volume (mL) of poured NaOH,
N: normality of NaOH (0.1),
m: mass of the sample

2.2.3 Reducing sugars extraction and determination

The extraction of the water-soluble sugars from the pulp was carried out according to [20] method. Thus, 5 g of cocoa beans were homogenized in 50 mL distilled water at 60°C for a few min until cooled at ambient temperature. The mixture was then filtered on Watman paper (Whatman, Ø 185 mm) and the filtrate was supplemented until 100 mL with distilled water.

The quantification of the reducing sugars was carried out according to the method described by [21]. To 100 µL of water-soluble sugar previously extracts were added 200 µL of DNS (3,5 dinitrosalycilic acid). Then, the solution was heated during 5 min. After cooling, 2 mL of distilled water were added and the optical density was obtained with a spectrophotometer (PIOWAY, Singapore) at 540 nm against a blank. A standard curve was established with glucose

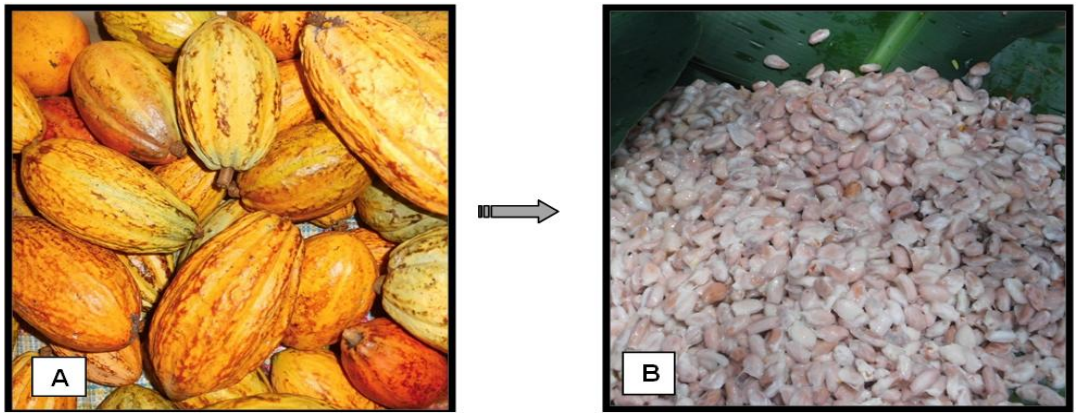

Fig. 1. Cocoa pods before slicing (A) and fresh cocoa beans on banana leaves (B)

solution (1 mg/mL) and the amount of reducing sugars of each sample was obtained from the regression equation established using the standard curve.

2.3 Biochemical Characterization of LAB Strains Isolates

2.3.1 Isolation and numeration of LAB strains

The enumeration and isolation of LAB was carried out using the decimal dilution method [22]. A mass of 25 g of fermented beans was added to 225 mL of buffered peptone water in a 500 mL sterile flask, and shaken for 2 to 5 min at room temperature to obtain homogenous sample containing the bacteria (initial dilution). One milliliter (1.0 mL) of the bacteria-enriched peptone water was diluted in 9 mL of 0.1% tryptone salt solution which was the first dilution and then, from this solution, a serial dilution was performed up to 10^{-8}. This serial dilution was only plated onto the rich elements composition medium of Man-Rogosa-Sharpe (MRS) agar (Oxoid) supplemented with 50 µg/mL of nystatin to inhibit fungal growth [22-23]. Plates were incubated at 30°C for 48–72 h, under anaerobic conditions, for subsequent colony counts and bacterial enumeration expressed as CFU per g of cocoa (ISO 4833). LAB were identified as being Gram-positive, oxidase and catalase negative, and unable for sporulation. These isolates were stored at −80°C in MRS buffer medium supplemented with 20% (v/v) glycerol, in Eppendorf tubes, for further investigations. Each treatment was tested in triplicate.

2.3.2 Fermentative type of LAB isolates

The fermentative type was performed according to the method described by [24]. Ten (10) mL of MRS medium with either glucose or gluconate as sole carbon source were placed in test tubes with Durham tubes. After autoclaved at 121°C for 20 min and cooling, the broths were inoculated with one colony of LAB and Incubated at 30°C for 2-7 days (Venticell, Medcenter, Germany). In the case of gas production from glucose, the isolate is strictly heterofermentary. If gas produced from gluconate, the isolate is facultative heterofermentary, otherwise, the isolate is said to be strictly homofermentary.

2.3.3 Carbon metabolism of LAB isolates

The carbon metabolism of bacterial strains was evaluated by [25] method. This study was performed in a modified MRS medium containing the appropriate carbohydrate at 2% as sole carbon source, and supplemented with 0.005% of bromocresol purple. The carbohydrates tested were glucose, fructose and sucrose that are known to be the sugars contained in the cocoa pulp [26]. The medium was inoculated with the strain to be tested and then incubated at 30°C for 48 h (MMM Medcenter, Germany). The capacity of strains to metabolize the carbon source is assessed by the change of medium color from purple to yellow due to pH lowering, comparatively to the negative control.

2.4 Identification of LAB Species Isolates

2.4.1 PCR amplification of 16S ribosomal RNA genes (16S rRNA gene)

A genotypic approach toward the 16S rRNA genes in all the isolates was performed to discriminate and identify LAB microorganisms isolated previously. This approach is now generally accepted as the best target for studying

phylogenetic relationships [5-27]. For this purpose, a multiple alignment (https://npsaprabi.ibcp.fr/NPSA/npsa_clustalw.html) of 16S rRNA genes from various species of LAB enabled us to design the forward (5′-GGYRTGCCTAATACATGCAAGT-3′) and reverse (5′-CCCGGGAACGTATTCACCGCG-3′) primers. These specific primers anneal to the most highly conserved 5′ and 3′ regions of the 16S rRNA gene, respectively, and after PCR they generate an amplicon of approximately 1400 bp. To perform the PCR reactions, bacteria grown for 24 h on agar plates were suspended in 100 µL of sterile distilled water and the resulting suspensions were used as DNA templates. PCR amplification was carried out in a Sensoquest Labcycler, as described previously [28]. Reactions were performed in a final volume of 50 µL containing 1 µL of bacterial suspension, 1.25 U of Taq DNA polymerase (Biolabs, Lyon, France), 5 µL of 10× standard buffer; 1 µL deoxynucleoside triphosphate (10 mM), 2 µL of each primer (10 µM) (Eurofins Genomics, Allemagne) and 38.75 µL of water. After an initial denaturation at 95°C for 4 min, reactions were run for 35 cycles, each cycle comprising: denaturation at 95°C for1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. Finally, a 10 min extension at 72°C was carried out. The presence and yield of specific PCR products were monitored using agarose 0.8% (w/v) gel electrophoresis at 70 V, for 2 h, in 1× Tris Borate EDTA buffer and visualized with ethidium bromide staining and UV transillumination.

2.4.2 16S rRNA gene restriction and sequence analysis

The 1400 bp PCR products were directly digested with two restriction enzymes, HaeIII and TaqI, in separate reactions. The digestions were carried out for one hour at 37°C for restriction enzyme HaeIII and at 65°C for TaqI in afinal volume of 20 µL containing 12 µL of PCR product, 2 µL of commercially supplied incubation buffer, 5 µL of water and 1 µL (10 U) of the restriction enzyme (Biolab, Lyon, France). Digestion products were run on a 2% agarose gel in Tris-Borate EDTA buffer at 35 V overnight. Gels were stained with ethidium bromide, visualized by transillumination, and digitalized with a gel print system. Strains were grouped according to their restriction profiles. For further identification, at least four representative strains from each restriction group were randomly chosen for amplification of the hyper-variable region of the 16S rRNA genes, about 500 bp, using the primers F27 (5′AGAGTTTGATCCTGGCTCAG-3′) and R520 (5′-ACCGCGGCTGCTGGC3′) [29]. PCR reactions were performed in the same conditions as described previously. PCR products were purified using the PCR clean-up (Macherey Nagel, Germany) and then sequenced using the primer F27. The basic local alignment searchtool (BLAST,blast N) from the NCBI database site (blast.ncbi.nlm.nih.gov/) was used to find the closest sequences relative to the amplified 16S RNA genes in order to identify our LAB strains. The phylogenetic tree was constructed from the partial sequences of RNA 16S genes alignment using the maximum likelihood method [30].

2.5 Data Analysis

The collected data were formatted with the Office Excel 2013 software. Descriptive parameters including standard deviations, averages of the parameters studied (temperature, pH, titratable acidity, reducing sugar) were calculated using the XLstat 2017 software. Sequences of the strains were compared with each other using the Clustal W in MEGA6 software to see the phylogenetic links between the different species of lactic acid bacteria isolated.

3. RESULTS

3.1 Change in Environmental Condition During Cocoa Bean Fermentation

3.1.1 Temperature and hydrogen potential (pH)

Fig. 2 shown the temperature and pH variations that occur during the different fermentations. Concerning the temperature (Fig. 2A), the study revealed an inside variation in the fermenting cocoa mass ranged from an initial of 29°C to a maximum of (40-48°C) obtained after 24 to 72 h of fermentation. Then the temperature decreased progressively, dropping at 30°C at the end of process for all the studied areas. However, the highest temperature peak (47°C) was obtained in the Cavally region after 48 h of fermentation, while the lowest peak was recorded in the San-Pédro area after 24 h of fermentation. Moreover, it also observed that Gbôklè and San-Pedro reached maximal temperature faster than Haut-Sassandra and Cavally, and Gôh and Tonkpi after 24, 48 and 72 h of fermentation, respectively.

Unlike the temperature that droped at the end of fermentation, the pH raised progressively during this process, to 3.4 at the beginning of the process up to 7.7 at the end (Fig. 2B). However, during the first 72 h of this fermentation, the pH of cocoa pulp remain acid with values ranging between 4.4 – 5.6.

3.1.2 Titratable acidity

The titratable acidity variation of cocoa pulp was similar for the six regions (Fig. 3). However, where Gôh region recorded its higher titratable acidity after only 12 h of fermentation time the other five regions (Gbôklè, San-Pedro, Haut-Sassandra, Cavally and Tonkpi) take longer than 24 h. The higher acidity value (4.43 meq/g of cocoa beans) was obtained in San-Pedro while the lower acidity value (1.96 meq/g of cocoa beans) was recorded in Cavally. Regions of Gôh and Tonkpi were recorded the same maximum acidity value (2.06 meq/g of cocoa beans) but at different times of fermentation around 12 h and 24 h, respectively (Fig. 3).

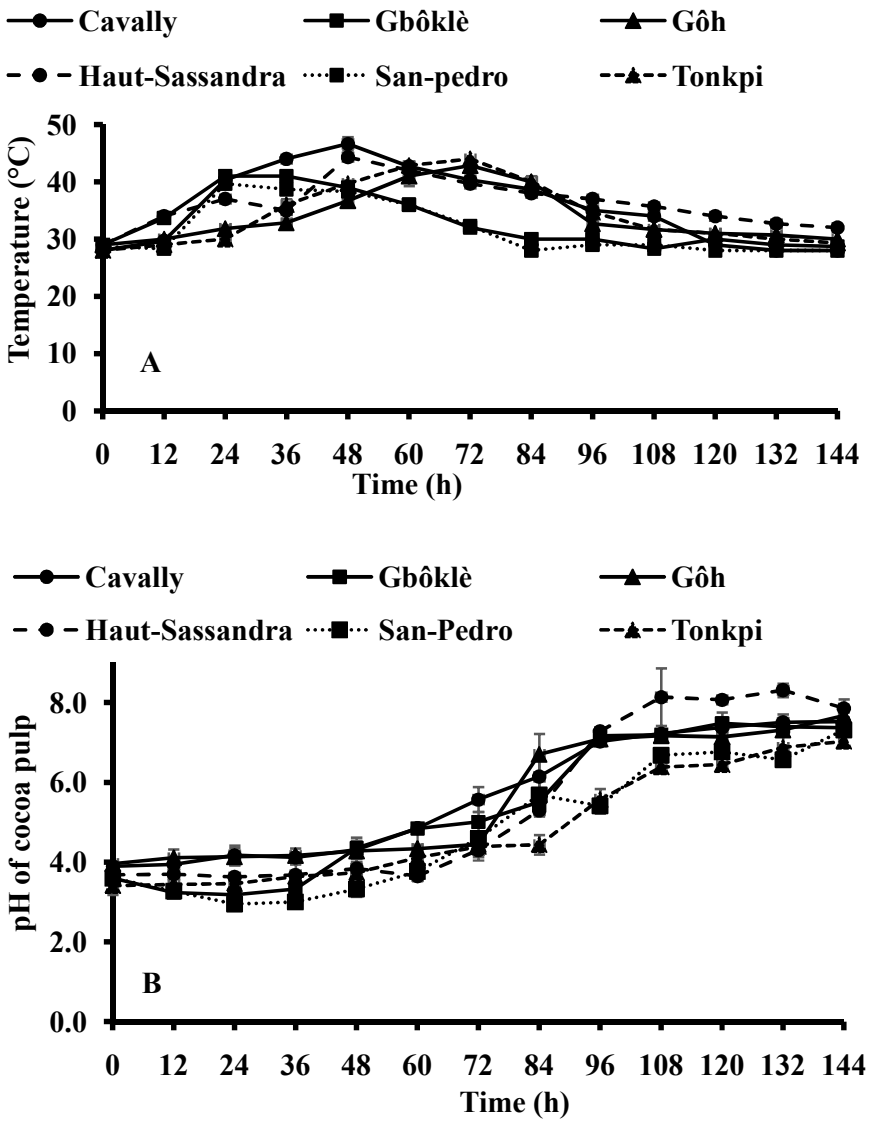

Fig. 2. Evolution of temperature (A) and pulp pH (B) during cocoa heap fermentation
The temperature and pH are presented as means and standard deviation

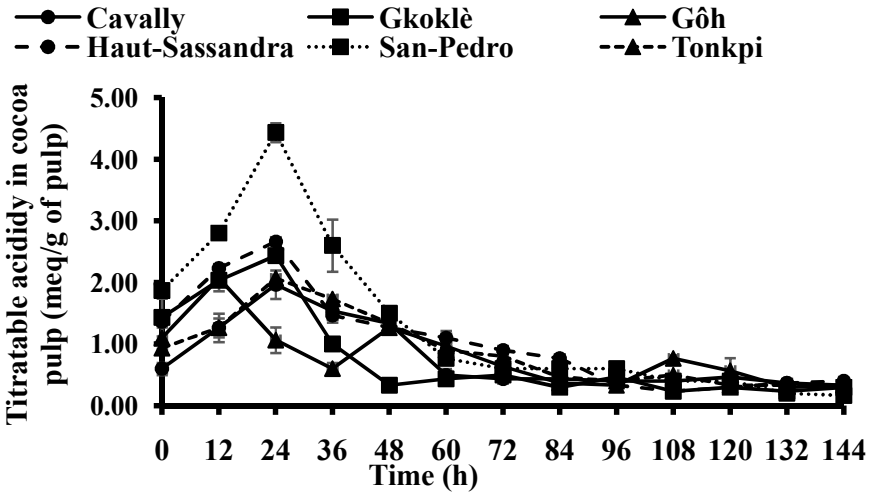

Fig. 3. Evolution of titratable acidity of cocoa pulp during cocoa heap fermentation
Titrable acidity is presented as means and standard deviation

3.1.3 Sugar reducing of cocoa pulp

The evolution of the reducing sugars contained in the cocoa pulp during the fermentation is illustrated in Fig 4. A decrease in the amount of sugar followed by a stabilization until the end of fermentation is observed. At the beginning of fermentation, the amount of sugar varies from 56 to 72 mg/g of beans and drops to about 10 mg/g of beans at the 84th h then stabilizes until the 144th h.

3.2 Isolation and Biochemical Identification of LAB Strains

3.2.1 Growth dynamics of LAB

The dynamic of LAB's population obtained from numeration during fermentation of cocoa from six major producing regions of Côte d'Ivoire is depicted in Fig 5. In general, these strains exhibited a similar growth profile. Indeed, these strains are present at the beginning of the fermentation at varying loads between 4.42 and 6.51 log CFU / g of beans for Gôh and San Pedro regions, respectivly. Then, the LAB microbial population of Gôh region rapidly increased to a maximum load of 6.95 log CFU/g of beans, after 24 h of fermentation time unlike Cavally, Gbokle and Tonkpi regions which took 36 hours to reach a maximum load between 6.87 and 8.14 log CFU / g of beans and Haut Sassandra 48 hours for a maximum load of 7.32 log CFU / g of beans. Subsequently, LAB population decreased gradually until reaching undetectable levels at the end of fermentation. However, the occurrence time of LAB during these six cocoa fermentations is 24 h – 72 h.

3.2.2 Morphology of LAB isolates

Biochemical and morphological identification of LAB strains isolated from the six cocoa producing regions studied, revealed the presence of Lactococci (107 strains) and Lactobacilli (461 strains) in (Table 1) with a clear dominance of the bacilli form (81.16%).

There is also an unequal distribution of these forms according to the regions. In fact, San-Pedro region recorded the highest proportion (25.59%) of bacilli form against 6.94% for Gôh region, while the cocci form dominates (37.38%) in the region of Haut-Sassandra unlike San-Pedro region (03.75%).

3.2.3 Fermentative type and profile

The fermentative type of the different LAB strains isolated from cocoa fermentation of the six cocoa producing regions studied is also presented in Table 1. The gas production from glucose or gluconate by LAB (Table 1) led to group these seeds in three fermentative types: Those who produced gas from glucose are strict heterofermentative, while those who produce it from gluconate are facultative heterofermentative. Finally strict homofermentative are unable to produce gas from gluconate. Overall, the heterofermentative type dominates (over 80%) the homofermentative type in all the studied regions with the facultative Hetero-fermentative type recording more than half (54.4%) of the isolated population.

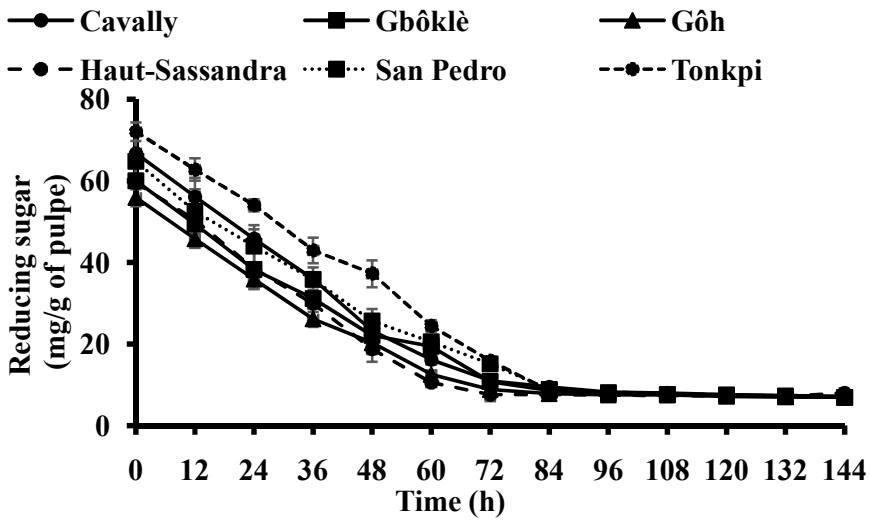

Fig. 4. Evolution of reducing sugars of cocoa pulp during cocoa heap fermentation
Reducing sugar is presented as means and standard deviation

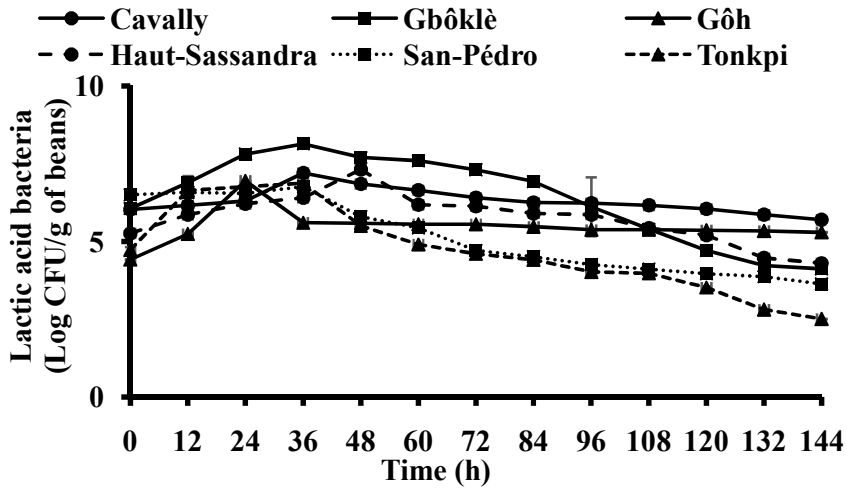

Fig. 5. LAB growth dynamic during cocoa heap fermentation
LAB population is presented as means and standard deviation

With regard to the fermentative profile, the use of the different studied sugars by these germs resulted in the turn of the fermentative medium from purple to yellow. So All the tested isolates were able to use both glucose, fructose and sucrose as substrate to produce acid.

3.2.4 Species of lactic acid bacteria involved in cocoa fermentation from the six regions of Côte d'Ivoire

The presumptive isolates of LAB: Gram positive, oxidase and catalase negative isolates, were stored in cryostubes containing MRS broth supplemented with 20% glycerol at -20°C for subsequent analyzes. The number of LAB isolates varied from one region (Table 2). San-Pedro area recorded the highest level of isolates (122), while in Gôh region 48 LAB were isolated. A total of 568 LAB were isolated for all regions.

The species of LAB isolates from the cocoa fermentation of the six studied regions were determined by sequencing the hypervariable zone of the 16S rDNA gene of a few representatives from each restriction group. The LAB flora involved in the fermentation of cocoa in Côte d'Ivoire consists essentially of four genera divided into 8 species (Table 2).

Table 1. Distribution of lactic acid bacteria (LAB) strains isolates according to their form and their fermentative type per region

Regions		Cavally	Gbokle	Goh	Haut sassandra	San Pedro	Tonkpi	Total
Total isolates		**104** (18.31%)	**087** (15.32%)	**048** (08.45%)	**109** (19.19%)	**122** (21.48%)	**098** (17.25%)	**568** (100%)
Form	Cocci	**12** (11.21%)	**23** (21.49%)	**16** (14.96%)	**40** (37.38%)	**04** (03.75%)	**12** (11.21%)	**107** (18.84%)
	Bacilli	**92** (19.95%)	**64** (13.87%)	**32** (06.94%)	**69** (14.97%)	**118** (25.59%)	**86** (18.65%)	**461** (81.16%)
Fermenta-tive type	Homo-Fermentative strict	**06** (05.56%)	**16** (14.82%)	**33** (30.56%)	**15** (13.89%)	**17** (15.74%)	**21** (19.43%)	**108** (19%)
	Hetero-Fermentative strict	**23** (15.24%)	**37** (24.50%)	**06** (03.97%)	**46** (30.46%)	**18** (11.92%)	**21** (13.91%)	**151** (26.58%)
	Facultative Hetero-fermentative	**75** (24.27%)	**34** (11.00%)	**09** (02.91%)	**48** (15.53%)	**87** (28.15%)	**56** (18.14%)	**309** (54.40%)

Table 2. LAB species isolated from cocoa fermentation and their RFLP group

Genera	Species	RNA (16S) Pb	Restriction fragment Hae III (Pb)	Restriction fragment Taq I (Pb)	Number of isolates	RLFP group
Lactobacillus	*Lactobacillus plantarum*	1400	580+460+300	700+350+200	436	I
	Lactobacillus Casei	1400	600+460+320	750+550+200	10	III
	Lactobacillus curieae	1400	600+450+280	700+450+200	14	V
	Lactobacillus fabifermantens	1400	600+450+300	700+400+200	7	VII
Leuconostoc	*Leuconostoc mesenteroides*	1400	1100+250	700+400+200	87	II
Weissella	*Weissella paramesenteroides*	1400	1150+300	750+600	2	IV
Enterococcus	*Enterococcus feacium*	1400	600+450+250	650+450+250	11	VI
	Enterococcus casseflavus	1400	650+450+300	750+400+200	1	VIII

A comprehensive study on the distribution of these LAB species in the different studied regions led to Fig 6. The majority species in all these six regions are *Lactobacillus plantarum* with a rate of 76.76% followed by *Leuconostoc mesenteroides* (15.31%). The minority species consist of *Lactobacillus curieae* (2.46%), *Enterococcus faecium* (1.93%), *Lactobacillus casei* (1.76%) *Lactobacillus fabifermentans* (1.23%), *Weissella paramesenteroides* (0.35%) and *Enterococcus casseliflavus* (0.17%) (Fig 6). Globally, *Lactobacillus plantarum* dominates the lactic flora involved in cocoa fermentation in the six regions studied. Furthermore, the hyper-variable region of the 16S RNA gene sequence analysis revealed very weak intraspecies variation (< 2%) inside the different restriction groups (Fig 7).

4. DISCUSSION

4.1 Physicochemical Parameters of the Fermental Mass of Cocoa

Microbial fermentation is an indispensable process for high quality chocolate from cocoa bean raw material [31]. The growth and development of the microbiota involved in natural cocoa bean dependent fermentation is closely related to fermentative conditions such as temperature, pH, oxygen, suga [7-31]. Thus, four fermentative parameters were studied to assess the activity of the germs involved in cocoa fermentation. The temperature study of cocoa mass fermented revealed an increase of this one during the first three days followed by a decrease until the end of the fermentation. The increase in temperature would be due to the growth of yeasts which convert sugars into ethanol and the growth of acetic acid bacteria (AAB) which oxidize ethanol into acetic acid by exothermic reaction [6-31]. In our work the temperature peaks were between 40 and 47°C. These results are similar with those of Vinicius et al. [32] who obtained 46°C during cocoa fermentation. Authors [10] noted a temperature increase of the fermentation heap up to 50°C. It should be noted that the highest temperature was obtained during the fermentation of cocoa from Cavally, the strains resulting from this fermentation would have an interesting characteristic as much as they could possibly carry out their activity up to 47°C. The acidic pH observed at the beginning of fermentation increases gradually to reach alkaline pH at the end of fermentation. Similar results have been obtained in several works

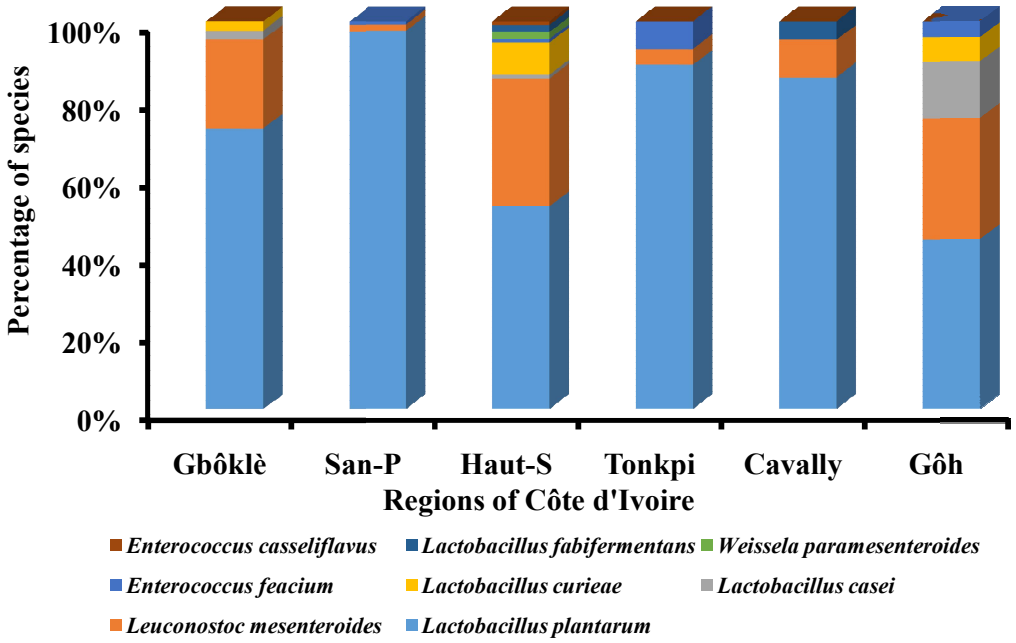

Fig. 6. Distribution of lactic acid bacteria in different cocoa producing by regions

San-P: San-Pedro ; Haut-S : Haut-Sassandra

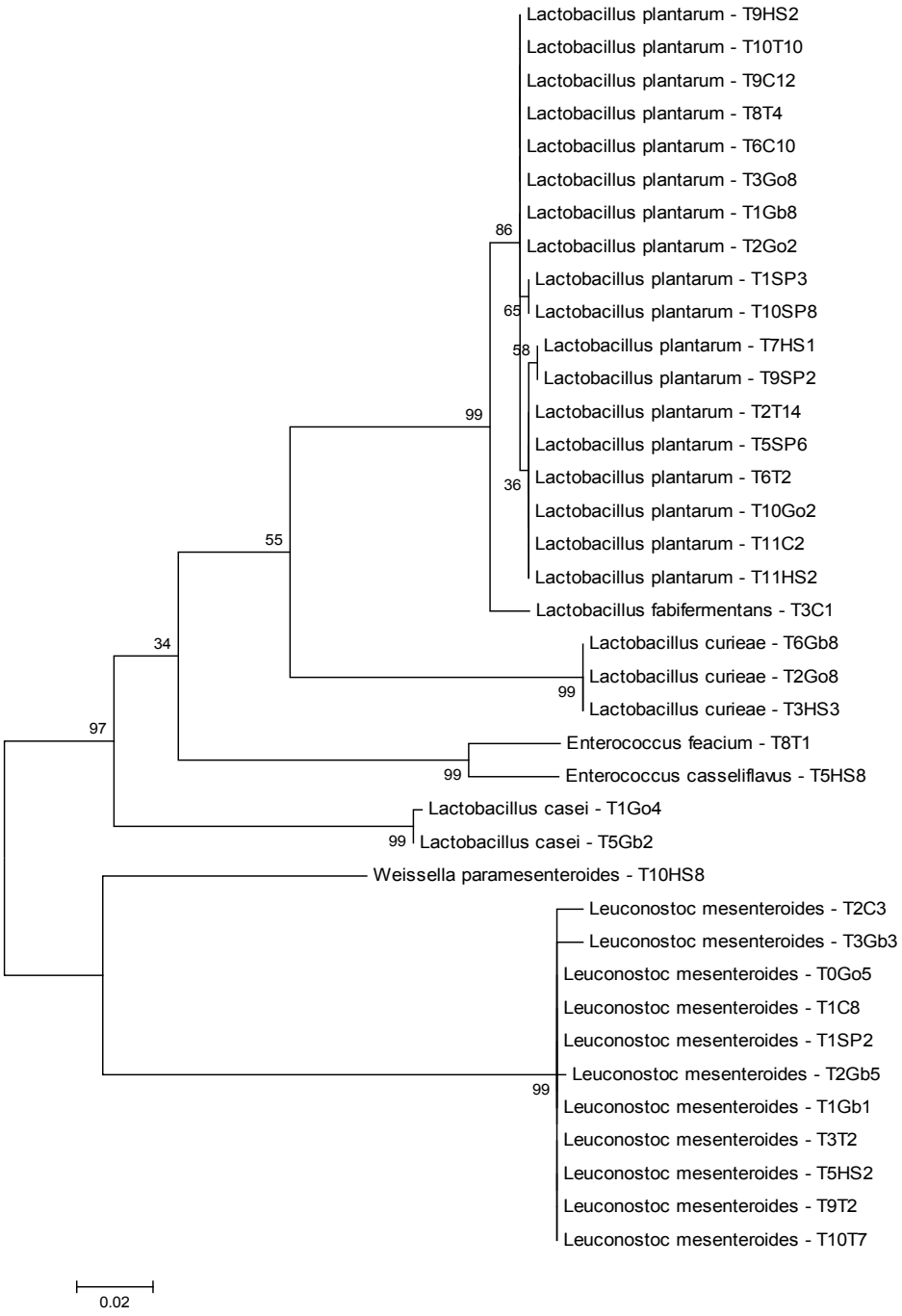

Fig. 7. Phylogenetic tree of LAB strains isolated from Ivorian cocoa fermentation

The partial sequences of the hyper variable region of the 16S rRNA genes were aligned and the evolutionary distribution was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (-1929.0880) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 38 nucleotide sequences. All positions with less than 100% site coverage were eliminated. That is, fewer than 0% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 442 positions in the final dataset. Evolutionary analyses were conducted using Clustal W in MEGA6.

including those of authors [33-34] in Côte d'Ivoire and Mahazar et al. [35] in Malaysia. This result is due to the degradation of citric acid contained in cocoa pulp by yeasts and LAB [12-15]. Indeed, metabolites (acetic and lactic acids) resulting from the degradation of citric acid diffuse inside the cotyledons for the activation of endogenous enzymes and / or to volatilize under heat and mixing of the fermenting mass [31]. In addition, yeast and LAB use the carbon substrates contained in the pulp to produce organic acids and ethanol which translates the decrease of sugar during the fermentation of the six studied regions. A similar evolution of reducing sugars during cocoa fermentation has been reported by [9-36]. The increase in titratable acid observed at the beginning of the fermentation is due to the activity of LAB and AAB. Indeed, these germs use sugars present in the cocoa pulp to produce organic acids such as lactic and acetic acids. These organic acids will diffuse inside the cotyledons and cause the decline of its pH [9]. This decrease in the pH of cotyledons promotes the triggering of biochemical reactions inside the seed to produce precursors of chocolate aroma [37]. On the other hand, the change in temperature and pH of fermented cocoa mass influences the microbial growth [31].

4.2 LAB Dynamics During Cocoa Fermentation

The dynamics of LAB population obtained during the fermentation shows that these germs are present throughout cocoa fermentation with different proportions. For the majority of the studied regions, the maximum bacterial population was obtained after 36 h of fermentation with values ranging from 10^6 to 10^8 CFU/g of beans. This strong growth at the beginning of fermentation is due to the presence of sugars including glucose, fructose and sucrose in fresh cocoa pulp and anaerobic conditions created by the abundance of cocoa pulp. Also, in the early hours of the cocoa fermentation, the temperature oscillates between 30 and 40°C and the pH is acidic. All these factors promote the growth of LAB, hence the strong population of LAB observed during this period. The same growth dynamics of LAB was observed during the fermentation of cocoa in Mexico [6-22]. Our results corroborate those obtained by [9] who observed a strong growth of these bacteria (10^6-10^8 CFU / g of beans) during the first hours of fermentation (36-48 h). The decrease in LAB population could be explained by the reduction of carbohydrates in cocoa pulp after 48 h of fermentation and an increase of the oxygen content in the medium as well as the temperature of the fermentation mass.

4.3 Biochemical Characteristics of Isolated LABs

It should be noted that LAB isolated from cocoa fermentations from the six regions are characterized by morphological diversity. Indeed they present themselves in two forms, namely bacilli and cocci. The bacilli dominate the flora of the six regions with percentages between 63.3% and 96.72%. This predominance of bacilli has already been demonstrated by authors [11-38-39]. These bacteria are distinguished into three groups according to their fermentative type; strict homofermentative, strict heterofermentative and facultatif heterofermentative. Facultatif heterofermentative dominate the lactic flora for all regions with 54.4% followed by strict heterofermentative (26.58%) and strict homofermentive (19%). These values are close to those of Kostinek et al. [11] who obtained 20.7% strict heterofermentive; 22.28 strict homofermentive and 57% of facultatif heterofermentive. The Gôh region is dominated by strict homofermentative LAB with a percentage of 68.75%. Strict homofermentative species produce only lactic acid from carbon substrate. This acid could be used by AAB of the genus *Acetobacter* to produce aromatic compounds such as acetoin and contribute to the acidification of cotyledons. This acidification will contribute to death of the embryo and induce the activation of enzymes responsible for the development of chocolate precursors [16-35]. But it is important to point out that lactic acid is not volatile and high concentrations of this residual acid will impart a sour flavor to the chocolate [40]. Therefore, cocoa from Gôh region could give chocolate acid notes. The heterofermentative species produce in addition to lactic acid, acetic acid. Fermentation carried out with this type of bacteria would be more advantageous as much as they produce less lactic acid. Also, the production of acetic acid is a very interesting characteristic because it triggers the reactions responsible for the development of the aroma chocolate [31]. The regions of Cavally, Gboklê, Haut-Sassandra, San-Pedro and Tonkpi dominated by facultatif heterofermentative LAB could produce good quality beans. The fermentative type diversity of LAB would be at the origin of the variability of the quality cocoa beans.

4.4 Diversity of LAB Isolates

Genetic diversity has been observed in LAB isolated from cocoa fermentation from the six studied regions of Côte d'Ivoire. Indeed, this study showed LAB isolates are subdivided into eight species whith a predominance of *Lactobacillus plantarum* (76.76%) in all regions. This finding confirms the dominance of the facultatif heterofermentative type observed in the studied regions as *Lactobacillus plantarum* species are known to be facultatif heterofermentative type. *Lactobacillus plantarum* is an facultative heterofermentative species capable of fermenting sugars via the glycolysis pathway or the pentose phosphate pathway. This species has a homolactic or heterolactic fermentation profile [41]. The predominance of this species could be explained by its adaptability in different ecological niches thanks to high number of sugars transport system [41] but also its ability to resist in acid and ethanol stresses [42]. Our results corroborate those of [11-12] who reported a predominance of *Lactobacillus plantarum* during cocoa fermentation in Côte d'Ivoire. Also this species was found mainly in the Democratic Republic [43] and in Nigeria [10]. On the other hand, Vinicius et al. [32] reported a predominance of *Lactobacillus fermentum* in Ghana and Ashokkumar et al. [44] in India. The predominance of *Lactobacillus plantarum* in all the studied regions, would be beneficial for leading to good quality beans by its ability to produce metabolites such as lactic acid, acetic acid and acetoin [13-22]. It should be noted this species has already been successfully used as starter in controlled cocoa fermentation [13-16]. *Lactobacillus plantarum*, is also capable of producing antimicrobial substances, which play an important role in bacterial ecology [6]. The minority species namely, *Lactobacillus casei, Lactobacillus fabifermentans, Weissella paramesenteroides, Enterococcus faecium, Enterococcus casseliflavus* and *Lactobacillus curieae* would provide specific cocoa from the regions where they have been isolated. *Lactobacillus fabifermentans* derived from Cavally and Haut-Sassandra regions is a new species isolated from the spontaneous cocoa fermentation in Ghana by De Bruyne et al. [45]. *Enterococcus casseliflavus* isolated from cocoa fermentation from Sassandra has already been found in Ghana by [6]. *Lactobacillus casei* has many properties such as lactic acid and protease production. It is used in the manufacture of cheese [46], yogurt [47] and saussice [48]. This germ could contribute to flavor development in cocoa due to proteolytic activitie. *Leuconostoc mesenteroids, weissella paramesenteroids* and *Enterococcus feacium* have low acidity [49] but are able to inhibit the growth of food-borne pathogens by the production of bacteriocins [50-51]. Also these microorganisms are known for their ability to produce volatile compounds such as ethanol and acetoin [49]. Their use as starters could help to obtain the aromatic character of cocoa sought and guaranty its safety. Thus, the strains of *Lactobacillus plantarum* and *Leuconostoc mesenteroides*, which are the most abundant species in this study whith a great biochemical diversity, constitute some potential ways concerning starters research for the control of fermentative cocoa process still spontaneous in Côte d'Ivoire. This diversity of LAB species would be responsible for the variability of cocoa quality in Côte d'Ivoire.

5. CONCLUSION

The results of this study clearly highlight the molecular diversity of LAB involved in the fermentation of six Ivorian cocoa producer regions, with a predominance of *Lactobacillus plantarum* and *Leuconostoc mesenteroides* among the eight distinct species identified. This diversity of LAB species, varying one local region to another, can explain the variability of cocoa beans quality in Côte d'Ivoire. Moreover, the minority species make as identified would make as provide specific cocoa from the regions where they have been isolated. So, the great diversity of LAB species would contribute to resolve the problem of the variability of cocoa beans quality which cause huge economic loss in Côte d'Ivoire.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kandler O, Weiss N. Genus lactobacillus beijerinck 1901, 212 AL. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG. (Eds). Bergey's manual of systematic bacteriology (8th ed.). Baltimor. 1986;2:208-234.
2. Axelsson L. Acid lactic bacteria: classification and physiology. In: Salminen S, Wright AV, Ouwehand A. edition 3 lactic acid bacteria: Microbiological and Functional Aspects. Marcel Dekker, New York. 2004;1-66.

3. Desmazeaud M. Les bactéries lactiques du lait: Les groupes microbiens d'intérêt laitier. Hermier J, Lenoir J, Weber F. (Eds) Centre de formation permanente et de perfectionnement des cadres des industries du lait. 1992;9-60.
4. Desmazeaud M. Les bactéries lactiques dans l'alimentation humaines : utilisation et innocuité. Cah Agric. 1996;5:331-343.
5. Vanddamme P, Pot B, Gills MP, Kerters K, Swings J. Polyphasic taxonomy a consensus approach to bacterial systematics. Microbiol Rev. 1996;60: 407-438.
6. Camu N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama JS, Vancanneyt M, De Vuyst L. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Appl Env Microbiol. 2007;73:1809-1824.
7. Lefeber T, Janssens M, Moens F, Gobert W, De Vuyst L. Interesting starter culture strains for controlled cocoa bean fermentation revealed by simulated cocoa pulp fermentations of cocoa-specific lactic acid bacteria. Appl Env Microbiol. 2011;77:6694-6698.
8. Lefeber T, Janssens M, Camu N, De Vuyst L. Kinetic analysis of strains of lactic acid bacteria and acetic acid bacteria in cocoa pulp simulation media toward development of a starter culture for cocoa bean fermentation. Appl Env Microbiol. 2010;76:7708-7716.
9. Ardhana M, Fleet G. The microbial ecology of cocoa bean fermentations in Indonesia. Int J Food Microbiol. 2003;86:87-99.
10. Kostinek M, Ban-Koffi L, Ottah-Atikpo M, Teniola D, Schillinger U, Holzapfel WH, Franz C. M. A. P. Diversity of predominant lactic acid bacteria associated with cocoa fermentation in Nigeria. Curr Microbiol. 2008;56:306-314.
11. Visintin S, Alessandria V, Valente A, Dolci P, Cocolin L. Molecular identification and physiological characterization of yeasts, lactic acid bacteria and acetic acid bacteria isolated from heap and box cocoa bean fermentations in West Africa. Int J Food Microbiol. 2016;216:69-78.
12. Ouattaraa Hadja D, Honoré Ouattara G, Michel Droux, Sylvie Reverchon, William Nasser, Sébastien L Niamke. Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: Species diversity and citrate lyase production. International J Food Microbiol. 2017;256:11-19.
13. Schwan RF. Cocoa fermentation conducted with a defined microbial cocktail inoculum. Appl and Env Microbiol. 1998;64:1477-1483.
14. Lefeber T, Papalexandratou Z, Gobert W, Camu N, De Vuyst L. On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. Food Microbiol. 2012; 30:379-392.
15. Crafack M, Mikkelsen MB, Saerens S, Knudsen M, Blennow A, Lowor S, Takrama J, Swiegers JH, Petersen GB, Heimdal H, Nielsen DS. Influencing cocoa flavour using Pichia kluyveri and Kluyveromyces marxianus in a defined mixed starter culture for cocoa fermentation. Int J Food Microbiol. 2013;167:103-116.
16. Penia KMTA, Suryani L, Affifah M. Improvement of cocoa beans fermentation by LAB starter addition. J Med Eng. 2013;2:274-278.
17. Nga Thi Tuyet Mai, Thi Loan Ho, Nhat Thanh Tran. Fermentation of cocoa bean with addition of lactic acid bacteria. Khon Kaen Agr J. 2014;4:211-217.
18. Crafack M, Hanna Keul, Carl Emil Eskildsena, Mikael A Petersen, Sofie Saerens, Andreas Blennow, Mathias Skovmand-Larsen, Jan H Swiegers, Gert B Petersend, Hanne Heimdal d, Dennis S Nielsen. Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. Food Res Int. 2014;63: 306–316.
19. AOAC official methods of analysis. Association of official analytical chemists ed., Washington DC. 1990;684.
20. Agbo NG, Uebersax MA, Hosfield GL. An efficient extraction technique of sugars from dry edible beans (*Phaseolus vulgaris* L.) and estimation HPLC. Annales de l'Université National. 1985;21:167-187.
21. Bernfeld D. Amylase β et α, In method in enzymology 1, Colowick SP, and Kaplan NO, Academic Press. 1955;149-154.
22. Pereira GVM. Microbiological and physicochemical performance of cocoa bean fermentations carried out in different systems and screening of yeast and bacteria strains toward development of a

stater culture. Dissertação Apresentada à Universidade Federal de Lavras. 2012;178.
23. Ouattara Djeneba H, Honoré G Ouattara, Jacques N Adom, Bernadette G Goualié, Gisèle A Koua, Ginette G Doué and Sebastien L Niamke. Screening of lactic acid bacteria capable to breakdown citric acid during Ivorian cocoa fermentation and response of bacterial strains to fermentative conditions. Br Biotechnol J. 2016;10(3):1-10.
24. Harrigan WF, Mc Cance ME. Laboratory Methods in Food Dairy Microbiol. J Basic Microbiol. 1976;18:226-227.
25. Dicks LM, Van Vuuren HJJ. Microbiol Methods. 1987;6:273-275.
26. Afoakwa E, Kongor J, Takrama F, Budu A. Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans. Int Food Res J. 2013;3:1215-1222.
27. Rossello-Mora R, Amann R. The species concept of prokaryotes. FEMS Microbiol. Rev. 2001;25:39–67.
28. Ouattara HG. Caractérisation biochimique et moléculaire des bacillus fermentaires du cacao et de leurs enzymes pectinolytiques : Recherche de potentiel starter, Thèse de Doctorat. Université de Cocody-Abidjan. 2010;154.
29. Anzai Y, Kim H, Park J, Wakabayashi H. Phylogenetic affiliation of the pseudomonas based on 16S rRNA sequence. Int. J. Syst. Evol. Microbiol. 2000;50:1563–1589.
30. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6/ molecular evolutionary genetics analysis version 6.0. Mol Biol and Evol. 2013;30:2725-2729.
31. Schwan RF, Wheals AE. The microbiology of cocoa fermentation and its role in chocolate quality. Crit Rev Food Sci Nutr. 2004;44:205-221.
32. Vinícius de Melo Pereira Gilberto, Maria Gabriela da Cruz Pedrozo Miguel, Cíntia Lacerda Ramos, Rosane Freitas Schwan. Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. Appl Env Microbiol. 2012;78:5395–5405.
33. Guehi ST, Dabone S, Ban-Koffi L, Kra DK, Zahouli GI. Effect of turning beans and fermentation method on the acidity and physical quality of raw cocoa beans. Adv. J Food Sci Technol. 2010;2:163-17.
34. Ouattara HD, Ouattara GH, Goualie GB, Kouame ML, Niamke LS. Biochemical and functional properties of lactic acid bacteria isolated from Ivorian cocoa fermenting beans. J Appl Biosci. 2014;77:6489-6499.
35. Mahazar NH, Sufian NF, Meor Hussin AS, Norhayati H, Mathawan M, Rukayadi Y. Candida sp. as a starter culture for cocoa (*Theobroma cacao* L.) beans fermentation. Int Food Res J. 2015;22:1783-1787.
36. Pereira GVM, Magalhães KT, Almeida EG, Coelho ISC, Schwan RF. Spontaneous cocoa bean fermentation carried out in a novel-design stainless steel tank: Influence on the dynamics of microbial populations and physical–chemical properties. Int J Food Microbiol. 2013;161:121-133.
37. Voigt J, Lieberei R. Biochemistry of cocoa fermentation. In R. F. Schwan, GH Fleet (Eds.), Cocoa and coffee fermentations. Boca Raton: CRC Press. 2014;103-225.
38. Illeghems K, De Vuyst L, Papalexandratou Z, Weckx S. Phylogenetic analysis of a spontaneous cocoa bean fermentation metagenome reveals new insights into its bacterial and fungal community diversity. Plos One. 2012;7:38040-38040.
39. Papalexandratou Z, Lefeber T, Bahrim B, Lee OS, Daniel FAHM, De Vuyst L. Hanseniaspora opuntiae, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentations, underlining the importance of these microbial species for a successful cocoa bean fermentation process. Food Microbiol. 2013;28: 1326-1338
40. Afoakwa EO, Paterson A, Fowler M, Ryan A. Flavor formation and character in cocoa and chocolate: A Crit Rev Food Sci Nutr. 2008;48:840-857.
41. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MW, Stiekema W, Lankhorst RM, Bron PA, Hoffer SM, Groot MN, Kerkhoven R, De Vries M Ursing B, De Vos WM, Siezen RJ. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci USA. 2003;100:1990-1995.
42. Giraud E, Lelong B, Raimbault M. Influence of pH and initial lactate concentration on the growth of

Lactobacillus plantarum. Appl Microbiol Biotechnol. 1991;36:96-99.

43. Lagunes Galvez Sandra, Gérard Loiseau, José Luis Paredes. Study on the microflora & biochemistry of cocoa fermentation in the Domican Republic. Int J Food Microbiol. 2007;114:124-130.
44. Ashokkumar C, Baskaran D, Gnanalaksshmi KS. Study of microflora involved in cocoa bean fermentation in tamil nadu. Int J Agric Sci Res. 2017;7: 83-92.
45. De Bruyne Katrien, Nicholas Camu, Luc De Vuyst, Peter Vandamme. *Lactobacillus fabifermentans* sp. nov. and *Lactobacillus cacaonum* sp. nov., isolated from Ghanaian cocoa fermentations. Int J Syst Evol Microbiol. 2009;59:7–12.
46. Dimitrellou D, Kandylis P, Sidira M, Koutinas AA, Kourkoutas Y. Free and immobilized *Lactobacillus casei* ATCC 393 on whey protein as starter cultures for probiotic Feta type cheese production. J Dairy Sci. 2014;97(8):4675-4685.
47. Sidira M, Saxami G, Dimitrellou D, Santarmaki V, Galanis A, Kourkoutas Y. Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool. J Dairy Sci. 2013;96(5):3369-3377.
48. Sidira M, Kandylis P, Kanellaki M, Kourkoutas Y. Effect of curing salts and probiotic cultures on the evolution of flavor compounds in dry fermented sausages during ripening. Food Chem. 2016;201:334-338.
49. De Paula Aline T, Ana Beatriz Jeronymo-Ceneviva, Svetoslav D Todorov, Ana Lúcia B Penna. The two faces of *Leuconostoc mesenteroides* in Food Systems. Food Rev Int. 2014;31(2):147-171.
50. Nes IF, Diep DB, Ike Y. Enterococcal bacteriocins and antimicrobial proteins that contribute to niche control. In: Gilmore MS, Clewell DB, Ike Yet al, editors. Enterococci: From commensals to leading causes of drug resistant infection [internet]. Boston: Massachusetts Eye and Ear Infirmary; 2014 Available:http://www.ncbi.nlm.nih.gov/books/NBK190428/.
51. Favaro L, Basaglia M, Casella S. Bacteriocinogenic potential and safety evaluation of non-starter *Enterococcus faecium* strains isolated from home made white brine cheese. Food Microbiol. 2014; 38:228-239.

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