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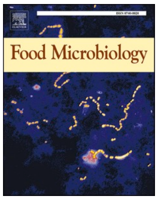

Mapping the functional and strain diversity of the main microbiota involved in cocoa fermentation from Cote d'Ivoire

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ABSTRACT

The variable quality of cocoa produced by farmers is still a problem in the value chain, strongly depending on microbial activities. We analyzed the variability of cocoa microbiota from all twelve producing regions in Cote d'Ivoire, and described the geographical distribution of isolated microbiota, using a mapping. Microbial species were identified by ribosomal genes sequencing, strains were typed by RFLP and their techno-functional capacities were further investigated. Results showed a restricted diversity of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) with respectively 10 and 5 strains. The dominant LAB and AAB strains, notably *Lactobacillus plantarum* 1 A, *Acetobacter pasteurianus* 1 A, *Acetobacter okinawensis* 2 A, and *Acetobacter tropicalis* 3 A, were found in all regions assuming that the acid microbiota was weakly variable. In contrast, the distribution of their functional performance such as acidification capability was variable, stronger in strains from Nawa and Haut-Sassandra regions and weaker in Indenie-Djuablin and San Pedro; this distribution seemed to be random. Moreover, the study also revealed a complex yeasts population showing a wide genetic diversity with 22 species and 45 strains indicating an intraspecific heterogeneity. Strains were generally different from a region to another and the resulting yeasts microbiota was globally variable in the regions. Likewise, the functional capacities such as pectinolytic was weak in *P. kudriazevii* strain 2 K from Gboklè and strong in *P. kudriazevii* strain 2 A from Loh-Djiboua. Additionally, the quality of fermented beans was also variable in the regions. The great variation of yeasts strains in the different regions may be the main microbial factors responsible for variation of the fermented cocoa quality observed.

1. Introduction

Microorganisms play a key role in the fermentation process of cocoa and strongly impact the quality of beans and chocolate (Lefeber et al., 2012; Schwan and Wheals, 2004; Afoakwa et al., 2008; Di Mattia et al., 2017).

An efficient fermentation of cocoa requires the action of a microbial community, mainly including yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and *Bacillus* (Ouattara et al., 2017; Yao et al., 2017; Schwan and Wheals, 2004; Soumahoro et al., 2019) which use the sugar and pectin-rich pulp as substrate to exert their metabolic activity on the outer part of bean. This activity occurs as a sequence of reactions via a microbial succession (Schwan et al., 2014).

During this succession, the firsts stages of the fermentation is characterized by yeasts and LAB growth that transform sugars contained in the pulp into ethanol and lactic acid, respectively (Pereira et al., 2013; Ho et al., 2014; Ouattara et al., 2017). Both microbial groups are also involved in the breakdown of citric acid, raising the pH and creating favorable conditions for the other microbial species (Jespersen et al., 2005; Ouattara et al., 2016; Schwan et al., 2014). Subsequently, ethanol is further oxidized into acetic acid by AAB via an exothermic reaction leading to a sharp raise of temperature up to 45 °C (Lefeber et al., 2011; Schwan et al., 2014; Romero-Cortes, 2012). On the other hand, the pectin is hydrolyzed by yeast and *Bacillus* resulting in a breakdown of the viscous and sticky pulp that flows away (Ouattara et al., 2008, 2011; Samagaci et al., 2015).

The second sequence of the reactions occurs in the inner part of bean. In fact, the metabolite produced from microbial growth, principally acids penetrate deep into beans and lower the inner pH (Schwan and Wheals, 2004). The acidification of the cocoa bean in turn activate pH-dependent enzymes notably aspartic endoprotease and carboxypeptidase that hydrolyze storage proteins (albumin and the vicilin-class-7S) to oligopeptides and amino acids known as the precursors of chocolate aroma (Castro-Alayo et al., 2019; Jinap et al., 2008;

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Voigt et al., 2018). These reactions also result in the development of the main traits of well-fermented cocoa beans, notably the flavor and the brown color as well as the reduction of bitterness and astringency (Afoakwa et al., 2015; Aprotosoaie et al., 2016; Misnawi et al., 2002). Additionally, yeasts, LAB and *Bacillus* from fermenting cocoa, were also found to directly produce aroma compounds (Ouattara et al., 2019, 2020). It clearly appears that, microorganisms involved in the fermentation of cocoa, shape and print the quality of the future chocolate.

As a consequence, the quality of bean and chocolate also depend on the strain or species as well as the properties of microorganisms (Kongor et al., 2016; Lefeber et al., 2012). Hence, the variability of the microbial species fermenting cocoa from different regions remains one of the main factors influencing the variability of products' quality.

Interestingly, many studies carried out independently in different countries, have reported variable microbiota from country to country, although few similar species were also found (Lefeber et al., 2011; Nielsen et al., 2005; Schwan et al., 2014). Moreover, few studies including two countries at the same time, revealed a variation in the microbial diversity in Ghana and Brazil (Garcia-Armisen et al., 2010), and in Cote d'Ivoire and Brazil (Papalexandratou et al., 2011), whereas Bortolini et al. (2016) used high-throughput sequencing technologies (HTS) to define the composition of bacterial communities from fermenting cocoa in Cote d'Ivoire, Ghana and Cameroun.

However, less interest was focused on the variability of the microbial community fermenting cocoa from several areas of the same country. We recently investigated the diversity of LAB (Ouattara et al., 2017) and AAB (Soumahoro et al., 2019) in six cocoa producing regions in Cote d'Ivoire, whereas de ALMEIDA et al. (2019) reported the diversity of yeasts during fermentation of cocoa from two sites in Brazil, but only one microbial group was concerned in each of these studies.

We then hypothesized that, in the same country, there is a delimited geographical area within which the fermentative microbiota of cocoa may be consistent. Studies aiming to identify areas presenting similar microbiota may contribute to labeling and traceability of cocoa quality origin as it is for wine.

In this study, we identified at molecular level, the main microbiota (Yeasts, LAB, AAB and *Bacillus*) fermenting cocoa in twelve producing regions from Cote d'Ivoire, the first cocoa producers with 40% of world's total production (Statistica, 2019) then we analyzed the functional features and the geographical distribution of isolated microbial species and strains.

2. Material and methods

2.1. Location of the cocoa producing regions studied

This study was carried out in twelve regions simultaneously; the location of these regions using Geographical Positioning System (GPS) is indicated in Table S1. The period of fermentations spanned from September 2017 to January 2018, corresponding to a single harvest seasons. One fermentation was conducted in each region in the same harvest season.

2.2. Fermentation and sampling conditions

In each region, one fermentation was conducted in the same harvest season described above. Cocoa pods including mixed genotypes (Forastero, Trinitario and Criollo) were harvested from a high producing cocoa plantation. Fermentations were processed traditionally under local farm conditions. After pods opening and beans removal, heap fermentations of about 200 kg of cocoa beans, were conducted for six days using banana leaves as described by Samagaci et al. (2016). Fermentation conditions such as temperature and pH were monitored at 12 h intervals, these conditions being directly measured on the fermenting heap, at 15 cm depth, with a portable pH-meter and thermometer (Hanna Instruments). At the same time intervals, samples of about 200 g were collected with gloves and placed in sterile bags, put in a cooler box and immediately sent to laboratory under cool conditions for microbiological analyses. The transportation time was less than 6 h.

2.3. Microorganisms isolation and analysis procedure

At laboratory level, each sample was divided into two aliquots. The first aliquot was used to make microbiological analysis notably enumeration and isolation of microorganisms. For this purpose, 25 g of fresh beans were put in 225 mL of 0.1% (w/v) buffered peptone water, pH 7.2 (Oxoid, Basingstoke, United Kingdom) contained in 500 mL sterile flask and allowed to incubate for 1 h at 30 °C at room temperature. The flask was then shaken for 2 min, to obtain a homogenous peptone water containing the microbial cells (initial dilution). Then, 1 mL (1.0 mL) of the microorganisms-enriched peptone water was diluted in 9 mL of 0.1% tryptone salt solution (10-fold dilution). Subsequently, a serial dilution was performed up to 10^{-8}, and 0.1 mL of each dilution was plated onto selective medium depending on the microorganism to be isolated. As isolation and enumeration media, YGC (Yeasts –Glucose-Chloramphenicol) was used for yeasts (Jespersen et al., 2005; Nielsen et al., 2007), MRS (Mans-de Rogossa-Sharpe) for LAB (Ho et al., 2014; Ouattara et al., 2017), NA (Nutrient Agar) for *Bacillus* (Nielsen et al., 2007; Ouattara et al., 2008) and MYGP (Malt extract-Yeasts extract-Glucose- Peptone) for AAB (Soumahoro et al., 2015). All the media for bacteria were supplemented with nystatin (50 μg/mL) to inhibit fungal growth. The cultures were incubated for 48–72 h at 30 °C and enumeration was performed by colony count.

The second aliquot served to measure the inner pH of the beans. The pulp was manually and carefully removed and 50 g of the cotyledons were grinded in 50 mL of distilled water, and then filtered with a sieve. The pH of the filtrate as inner pH was recorded and the total inner acidity was titrated using phenolphthalein as pH indicator and NaOH 0.1 N as neutralizing solution.

The cut test method (index of fermentation) was used to evaluate the quality of fermented beans and assess the efficiency of cocoa fermentations from the different regions (del Boca, 1962; Shamsuddin and Dimick 1986). Results were interpreted basing on the standards of cocoa merchant association, USA (CMA).

2.4. DNA extraction, PCR amplification of the yeasts ribosomal genes and sequences analysis

DNA from yeasts was extracted using the standard phenol/chloroform method as described by Hoffman (1997) and used as template for PCR reactions. These reactions targeted two yeasts ribosomal genes notably the ITS1-5.8 S-ITS2 fragment including the 5.8 S gene with it's flanking ITS (Intragenic Transcribed Spacer) and the second targeted fragment was the D1/D2 region of the 26 S rDNA gene.

All PCR reactions were performed using a thermocycler (Mycycler, Bio Rad, USA) in a total volume of 50 μL containing 1 μL of DNA extract, 1 U Taq DNA polymerase (BioLabs, Lyon, France), 5 μL 10 × magnesium-free buffer, 1.5 mM $MgCl_2$, 0.2 μM of each primer and 200 μM dNTP (Euromedex, France). The ITS1-5.8 S-ITS2 region was PCR amplified, as described by Esteve-Zarzoso et al. (1999) using the eukaryotic universal primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′). The ITS1/ITS2 PCR program was set up as follow: initial denaturation at 95 °C for 5 min, followed by 35 cycles (94 °C for 1 min, 55.5 °C for 1 min, and 72 °C for 2 min) and ending with a final extension at 72 °C for 10 min. The D1/D2 region of 26 S rDNA was PCR amplified, as described by (Hamdouche et al., 2015) using the eukaryotic universal primer gc-NL1 (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCCATATCAATAAGCGGAG- GAAAAG 3′) and the reverse primer LS2 (5′ ATTCCCAAA CAACTCGACTC 3′). The D1/D2 PCR program consisted in one step on hot start at 94 °C for 3 min, followed by 30 cycles (denaturation 95 °C for 1 min, annealing 52 °C for 1 min, and elongation 72 °C for 1min), and a final extension at 72 °C for 10 min. The presence of amplicons as specific yield product from PCR was checked using agarose 0.8% (w/v) gel electrophoresis at 70 V, for 2 h, in 1X Tris Borate EDTA buffer and visualized with ethidium bromide staining and UV transillumination.

2.5. PCR amplification of the bacterial 16 S rRNA genes and sequences analysis

Colony-PCR (not needing DNA extraction) was performed for 16 S ribosomal genes amplification from bacteria (LAB, AAB and *Bacillus*) with a bacterial suspension as template, described by Ouattara et al. (2017). For PCR reactions, different primers depending on the bacterial group were used. Primers (5' -GGYRTGCCTAATACATGCAAGT-3′) and reverse (5′-CCCG- GGAACGTATTCACCGCG-3′) (Ouattara et al., 2017); forward (5′-AGTGGCGGACGGGTGAGTA-3′) and reverse (5′-CCAACTCCCATGGTGTGACG-3′) (Soumahoro et al., 2019); forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and R1492 (5′-TACGGTTACCTTGTTA CGACTT-3′) (Ouattara et al., 2011), were used for 16 S rRNA genes amplification from LAB, AAB and *Bacillus,* respectively. The PCR reactions were run the same manner. These 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. PCR program began with initial denaturation at 95 °C for 4 min, with subsequent 35 cycles, each cycle including: denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min. At last, a 10 min extension at 72 °C was carried out.

Additionally, another fragment of approximately 500 bp representing a partial 16 S rRNA gene was amplified from bacteria. This partial rRNA gene includes the V1, V2 and V6 regions, which are hyper variable (HV) sections of the ribosomal rRNA gene (Gray et al., 1984). This specific region was targeted using the primers forward F27 (5′- AGAGTTTGATCCTGGCTCAG-3′) and R520 (5′-ACCGCGGCTGCT GGC-3′). PCR reactions were performed in the same conditions as described above.

2.6. Molecular identification of microbial isolates and strain typing by RFLP

For molecular identification of yeasts isolates, the PCR-amplified D1/D2 regions of 26 S rDNA were sent for sequencing to BIOFIDAL (Lyon, France) using the PseqD1/D2 primer (5′GGGCCATATCAATAAGC 3′). Sequences were then compared to the basic local alignment search tool (BLAST, blastN) from the NCBI database site (blast.ncbi.nlm.nih.gov/).

For molecular identification of bacterial isolates, the PCR-amplified partial 16 S rRNA genes (variables regions) were purified using the PCR clean-up (Macherey Nagel, Germany) and then sequenced using the primer F27. Identification of microbial isolates was performed from sequence comparison with database described above.

Strain typing was achieved by RFLP using the amplified ribosomal fragments. The RFLP consisted in the use of restrictions enzymes *Hha*I, *Hae*III or *Hinf*I (BioLabs, Lyon, France), for digestion of the amplified ITS1-5.8 S-ITS2 fragments from yeasts whereas *Alu*I, *Hae*III and *Taq*I served as restriction enzymes for digestion of the bacterial 16 S rRNA gene. The digestions were performed in a final 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 (BioLabs, Lyon, France). The reactions were run for 1–2 h at 37 °C for all restriction enzymes except for *Taq*I which was used at 65 °C. Fragments obtained from digestion were slowly separated on a 2% agarose gel in Tris-Borate EDTA buffer at 35 V overnight. Gels were stained with ethidium bromide, visualized by UV transillumination, and digitalized with a gel print system.

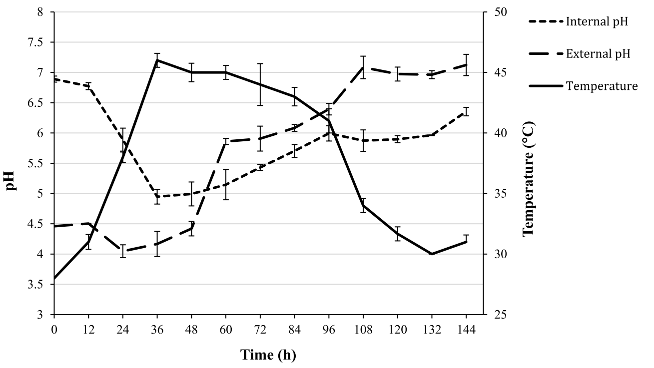

Fig. 1. Evolution of temperature and pH during fermentation of cocoa from Agneby-Tiassa region.

2.7. Acids quantification and enzymes detection

Acidification of fermenting cocoa bean is essential to obtain products with well-developed aroma. The yields of acids were assayed in LAB and AAB based on separation by High Performance Liquid Chromatography (HPLC) as described by Ouattara et al. (2017).

Pectinolytic and citrate lyase, as relevant microbial enzymatic activities required for a good processing of cocoa fermentation, were searched in isolated yeasts and LAB strains, respectively. For pectinolytic activity, a qualitative and semi-quantitative method was used as described by Ouattara et al. (2008) with slight modification consisting in the use of PSM medium (Ouattara et al., 2020).

On the other hand, a qualitative method for citrate lyase detection was applied for isolated LAB strains using KMK medium containing 1% (w/v) milk powder, 0.25% (w/v) casein peptone, 0.5% (w/v) glucose and 1.5% (w/v) agar, supplemented with potassium ferricyanide, iron citrate and sodium citrate as described by (Kempler & McKay, 1980).

3. Results

3.1. Temperature and pH variation during fermentations in all the regions

The temperature and pH are important parameters in cocoa fermentation that directly influence the production of well-fermented bean (Schwan and Wheals, 2004; Voigt et al., 2018).

The monitoring of temperatures in all the twelve fermentations showed the same trend of variation. Hence, only one curve of temperature (from Agneby-Tiassa region) was shown as model (Fig. 1). In Agneby-Tiassa region, the fermentation began at ambient temperature (28.5 ± 0.1 °C) then sharply increased to reach the peak at 46.2 ± 0.6 °C within 36 h, followed by a progressive decrease to 31.4 ± 0.6 °C at the end of the process. Similar curves were obtained from fermenting cocoa in the other regions but differed, in initial and final temperatures, in the peaks of temperature but also in the times at which these peaks occurred (Table 1). The highest peak of temperature (47.3 ± 1.1 °C) was obtained in Cavally fermenting cocoa within 48 h, while the weakest peak was 39.1 ± 0.5 °C, reached after 60 h of fermentation in Indénié-djuablin region (Table 1).

The pH of fermenting cocoa was recorded in the pulp (external pH) and in the cotyledon (internal pH) as described in material and method section. pH variations presented also the same trends in all the regions. Taken as model, the fermenting cocoa from Agneby-Tiassa, displayed a continuous and progressive increase of the external pH from 4.5 ± 0.05 (initial) to 7.1 ± 0.07 (final) (Fig. 1) whiles the initial internal pH in the cotyledons was 6.9 ± 0.03, near the physiological pH. This pH decreased rapidly to a minimum value of 4.9 ± 0.08 after 36 h of fermentation, and then increased slowly and continuously until the end the process (Fig. 1). The fermenting cocoa from other regions showed similar curves of external and internal pH evolution, but the initial and final external

Table 1
Temperatures of fermenting heap cocoa from the different regions.

Regions	Initial temperature (°C) at 0 h	Peak of temperature (°C)	Peak time (h)	Final temperature (°C) at 144 h
Agnéby-Tiassa	28.5 ± 0.1	46.2 ± 0.6	36	31.4 ± 0.6
Cavally	28.1 ± 0.2	47.3 ± 1.1	48	28.7 ± 0.2
Gboklè	29.2 ± 0.2	41.8 ± 0.5	36	29.6 ± 0.5
Gôh	29.7 ± 0.7	43.1 ± 0.2	72	30.6 ± 0.5
Guemon	28.2 ± 0.5	46.6 ± 0.6	36	28.4 ± 0.1
Haut-Sassandra	29.6 ± 0.4	44.3 ± 1.3	48	32.1 ± 1.1
Indénié-djuablin	27.5 ± 0.8	39.1 ± 0.5	60	28.7 ± 0.6
Lôh-Djiboua	28.1 ± 0.2	44.9 ± 0.7	36	28.3 ± 0.4
Nawa	28.9 ± 0.5	43.3 ± 0.4	36	28.4 ± 0.5
San-Pedro	29.2 ± 0.3	40.7 ± 0.9	24	28.9 ± 0.7
Sud-Comoé	27.3 ± 0.6	41.7 ± 0.7	36	31.5 ± 0.3
Tonkpi	28.7 ± 0.6	44.5 ± 0.3	72	29.5 ± 0.4

Measures were done at three different points of the fermenting heap cocoa; the standard deviations between triplicates are presented.

Table 2
External pH of fermenting heap cocoa from the different regions.

Regions	Initial pH at 0 h	Final pH at 144 h
Agnéby-Tiassa	4.5 ± 0.05	7.1 ± 0.07
Cavally	3.9 ± 0.10	7.5 ± 0.11
Gboklè	4.0 ± 0.07	7.7 ± 0.03
Gôh	4.0 ± 0.02	7.7 ± 0.14
Guemon	4.1 ± 0.02	8.0 ± 0.05
Haut-Sassandra	3.7 ± 0.01	7.9 ± 0.10
Indénié-djuablin	3.7 ± 0.03	7.6 ± 0.04
Lôh-Djiboua	4.5 ± 0.08	8.0 ± 0.06
Nawa	3.7 ± 0.04	7.6 ± 0.09
San-Pedro	3.6 ± 0.02	7.3 ± 0.12
Sud-Comoé	4.2 ± 0.09	7.3 ± 0.09
Tonkpi	3.4 ± 0.02	7.0 ± 0.02

Measures were done at three different points of the fermenting heap cocoa; the standard deviations between triplicates are presented.

Table 3
Internal pH of fermenting heap cocoa from the different regions.

Regions	Initial pH at 0 h	Lowest pH reached	Lowest pH time (h)	Final pH at 144 h
Agnéby-Tiassa	6.9 ± 0.03	4.9 ± 0.08	36	6.4 ± 0.21
Cavally	6.7 ± 0.09	5.2 ± 0.12	144	5.2 ± 0.17
Gboklè	6.7 ± 0.17	5.4 ± 0.15	24	6.0 ± 0.07
Gôh	6.7 ± 0.12	5.2 ± 0.04	144	5.2 ± 0.15
Guemon	6.4 ± 0.25	5.2 ± 0.06	36	5.9 ± 0.09
Haut-Sassandra	6.7 ± 0.17	4.8 ± 0.16	60	6.0 ± 0.10
Indénié-djuablin	6.8 ± 0.11	5.8 ± 0.11	60	6.2 ± 0.05
Lôh-Djiboua	6.9 ± 0.08	6.0 ± 0.06	36	6.1 ± 0.16
Nawa	6.8 ± 0.15	4.5 ± 0.12	60	5.4 ± 0.08
San-Pedro	6.7 ± 0.12	6.2 ± 0.22	36	6.5 ± 0.13
Sud-Comoé	7.0 ± 0.06	5.3 ± 0.09	48	6.1 ± 0.05
Tonkpi	6.7 ± 0.14	5.15 ± 0.10	144	5.15 ± 0.09

Measures were done at three different points of the fermenting heap cocoa; the standard deviations between triplicates are presented.

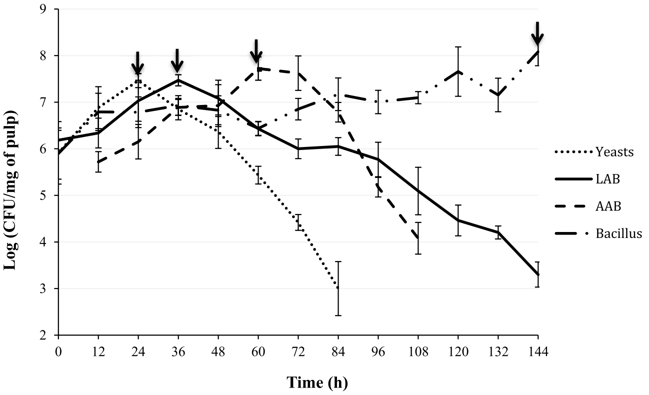

Fig. 2. Growth succession of different microbial groups during fermentation of cocoa from Agneby-Tiassa region.

Table 4
Restriction profiles and identification of yeasts microbiota isolated from cocoa heap fermentation in the twelve regions. The length of the different fragments for each restriction profile can be found in Supplemental Material Table S2.

Species	Unique restriction profiles	Number of isolates
Picha manshurica	4C	11
Saccharomyces cerevisiae	5C; 5D; 2 J; 5G; 5I, 2 K, 5 F	87
Pichia fermentans	1 A; 6 A, 2 A	269
Pichia kudriavzevii	2 A; 2G; 2I; 2 L; 7 A; 7 B; 3D; 2 M; 3H, 2 B, 3 B, 2 K, 5 F	792
Candida aaseri	3 A; 8 A	23
Hanseniaspora uvarum	4 A; 5D; 5H, 5 E	60
Candida intermedia	1C	39
Candida tropicalis	2 F; 7 A, 2 B	38
Pichia.sp	6 B; 2C; 2D; 2 E	32
Candida nitrativorans	8 B	2
Hanseniaspora sp	5 A	11
Pichia kluyveri	1 B; 6D; 2 N, 5 E, 5 F	150
Clavispora lusitaniae	2H	16
Debaryomyces hansenii	3G; 5 B	5
Hanseniaspora opuntiae	5 B	1
Meyerozyma sp/*C carpophila*	3C	27
Kodamaea ohmeri	1D	2
Debaryomyces sp	3 E	17
Candida humilis	3 F	6
Hanseniaspora opuntiae	5H	2
Candida incommunis	1 E	42
Wickerhamomyces anomalus	3 B	29

pH as well as the minimum value of internal pH differed (Table 2 and Table 3). [In these conditions, the different groups of microorganism grew sequentially with yeast peaking at 24 h, followed by LAB (36 h), AAB (60 h) and *Bacillus* (144 h) (Fig. 2). The same succession of microbial growth was observed in all the regions but with different the peak time].

3.2. *Identification and distribution of the different microbial species and strains in all the regions*

A total of 3946 microbial isolates were obtained from this study;

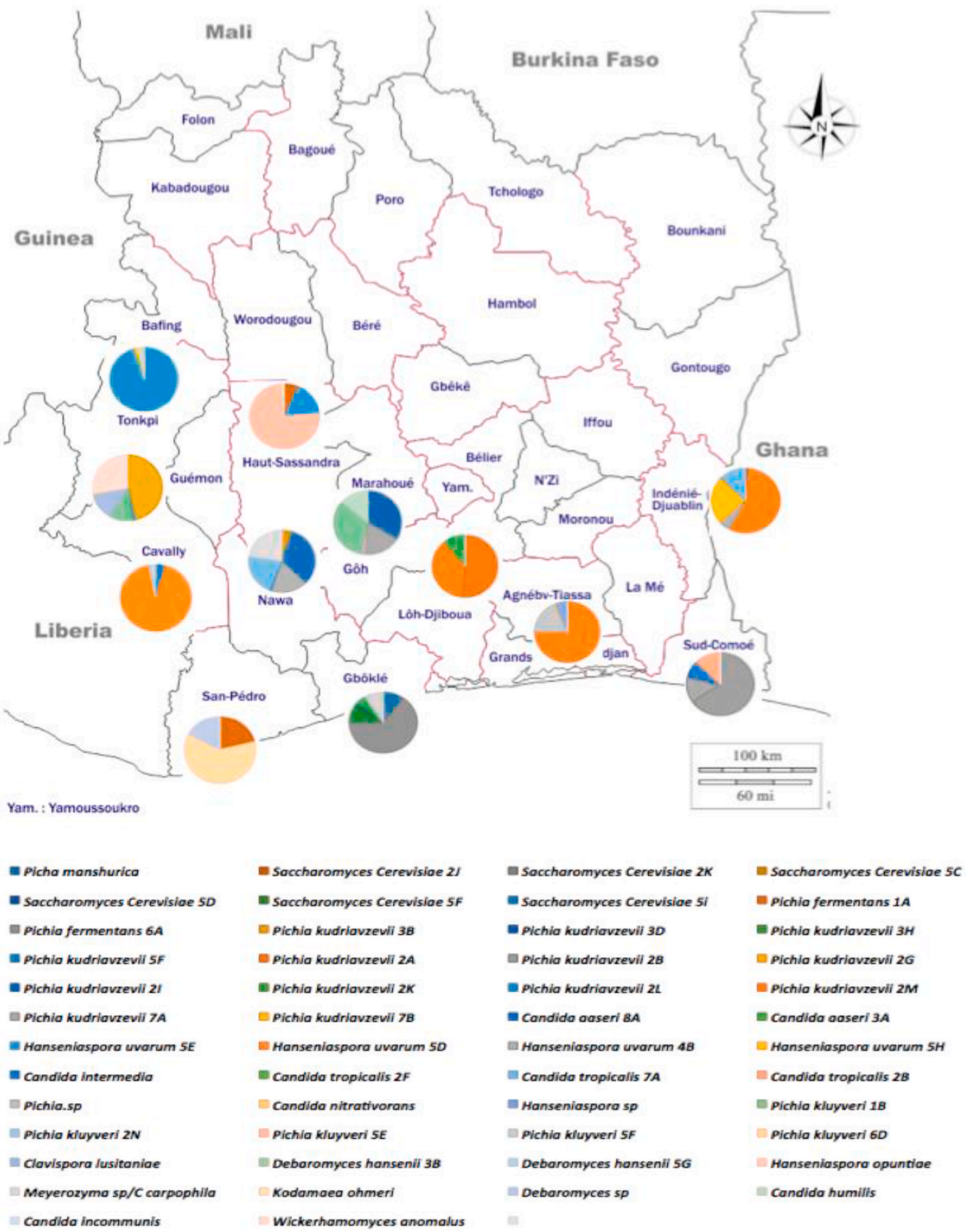

Fig. 3. Geographical distribution of yeast strains involved in cocoa heap fermentation of twelve regions of Côte d'Ivoire.

1661 yeasts, 1243 LAB and 1042 AAB. The molecular identification of microbial isolates at species level consisted first, in a grouping of all isolates basing on the fingerprint generated from RFLP; isolates presenting the same profile were assigned to the same RFLP group. A total of 60 unique restriction profiles each representing one strain were obtained (45 for yeasts, 10 for LAB and 5 for AAB). Then, 1/10 of strains were randomly chosen in each unique restriction group (RFLP group) for sequencing and identification.

3.2.1. *Yeasts*

For yeasts identification, the amplified ITS_1-5.8 S-ITS_2 fragments showed a length polymorphism consisting in 8 different sizes of amplified ITS_1-5.8 S-ITS_2 allowing the classification of the yeasts isolates in at least 8 groups (from I to VIII). To further investigate this apparent genetic heterogeneity, the amplified ITS_1-5.8 S-ITS_2 fragments were digested to generate different RLFP profiles. Hence, the RFLP analysis showed a total of 45 unique RFLP profiles obtained, indicating 45 yeasts strains (Table S2). Furthermore, the identification from D1/D2

Table 5

Restriction profiles and identification of LAB microbiota isolated from cocoa heap fermentation in the twelve regions.

RFLP Group	Length of Amplified 16 S rADN	Restriction profiles (bp)		Number of isolates	Species
		*Hae*III	*Taq*I		
1 A	1400	580 + 460+300	700 + 350+200	875	*Lactobacillus plantarum*
2 A	1400	1100 + 250	700 + 400+250	247	*Leuconostoc mesenteroides*
3 A	1400	600 + 460+320	750 + 550+200	15	*Lactobacillus casei*
4 A	1400	1150 + 300	750 + 600	4	*Weissella paramesenteroides*
5 A	1400	600 + 450+280	700 + 450+200	30	*Lactobacillus curieae*
6 A	1400	750 + 350+280	700 + 360+250	62	*Fructobacillus pseudoficulneus*
7 A	1400	1100 + 300	750 + 450+150	1	*Weissella cibara*
8 A	1400	600 + 450+250	650 + 450+250	1	*Enterococcus faecium*
9 A	1400	600 + 450+300	700 + 400+200	7	*Lactobacillus fabifermentens*
10 A	1400	650 + 450+300	750 + 400+200	1	*Enterococcus casseliflavus*

bp: base pairs; *Hae*III, *Taq*I: restriction enzymes.

sequencing of the strains revealed 22 yeasts species (Table 4). In general, we observed that a unique profile (or strain) corresponded to one species, with no obvious intraspecies variation; this concerned up to 13 out of 22 yeasts species (Table 4). In contrast, the other yeasts species included more than one strain or unique restriction profile, notably *Saccharomyces cerevisiae* (7 strains), *Pichia fermentans* (3 strains), *Pichia kluyveri* (5 strains), *Pichia kudriavzevii* (13 strains), *Candida aaseri* (2 strains), *Hanseniaspora uvarum* (4 strains), *Candida tropicalis* (3 strains) and *Pichia* sp. (4 strains) (Table 4).

To analyze the distribution of the isolated microbiota, we first focused on the dominant species notably *Pichia kudriazevii, Pichia fermentans, Pichia kluyveri* and *Saccharomyces cerevisiae* representing more than 85% of the total yeasts isolates and 60% of yeasts strains. As a general trend, the species *Pichia kudriazevii* was the most distributed yeasts species since it was present in 10 out of 12 studied regions (Fig. 3). However, the *Pichia kudriazevii* strains were different from a region to another region; in other words a given strain located in a specific region could not be found in other regions; although few exceptions were observed with the *Pichia kudriazevii* strain 2 A found in the regions of Loh-Djiboua, Agneby-Tiassa and Indenié Djuablin and *Pichia kudriavzevii* strain 7 A present in Nawa and Indenié-Djuablin regions (Table 7). These latter regions also recorded the presence of the same *Candida tropicalis* strain 7 A (Table 7). The distribution of the different yeasts strains from the other dominant and non-dominant species followed the same general trend (described above), as strains were different from region to region (Fig. 3). For instance the species *Pichia kluyveri* was found in 5 regions, but in each region the strain of *P. kluyveri* differed (with different RFLP profiles); strain 1 B in Guemon, strain 5 E in Haut-Sassandra, strain 5 F in Gboklè, strain 6D in San-Pedro and strain 2 N in Gôh (Table 7).

Additionally, it was possible to find different strains of the same species in the same region, as this is the case for *Saccharomyces cerevisieae* strain 2 K and *Saccharomyces cerevisieae* strain 5I present in Gboklè. Likewise, several different strains of *Pichia kudriazevii* could be found in a given region (Table 7).

3.2.2. LAB

A 16 S gene fragment with a size of approximately 1400 bp was PCR-amplified from LAB isolates. The RFLP analysis from restriction of amplified 16 S gene gave 10 unique restriction profiles and allowed classifying the strains in 10 RFLP groups (Table 5). Each RFLP group representing a strain corresponded to a single species as revealed the sequence analysis of hypervariable section of amplified 16 S gene (Table 5). Regarding the distribution of the LAB strains, it appeared that *Lactobacillus plantarum* strain IA was present and predominated in all the different cocoa producing regions (Fig. 4). Thus all the 12 regions could be defined as a single area presenting the same dominant LAB strain (*Lactobacillus plantarum* strain 1 A). This species was most generally found in association with *Lactobacillus casei* strain 3 A and to a lesser extent *Lactobacillus curiae* strain VA that were found in several regions (Fig. 4).

3.2.3. AAB

The PCR-amplification of the 16 S gene from AAB gave a 1300 bp fragment, approximately. The fingerprinting from this fragment using RFLP gave 5 different restrictions profiles allowing the grouping of the strains into 5 RFLP groups (Table 6). The identification of the strains from each RFLP group revealed that the profile IIA included two species (*Acetobacter okinawensis* and *Acetobacter ghanensis*) whereas the profile IIIA was composed of *Acetobacter tropicalis* and *Acetobacter senegalensis* strains. The other group or strains corresponded each to a single species (Table 6).

Analyzing the distribution of AAB species and strains in the different regions, it appeared that *Acetobacter pasteurianus* strain 1 A, one of the dominant AAB strains, was consistently found in all the regions (Fig. 5). Like LAB, the same AAB strains could be found in several regions. However, unexpectedly, some strains in the restriction group IIA were identified as *Acetobacter okinawensis*, while other strains of the same restriction group 2 A were identified as *Acetobacter ghanensis*. Additionally, the restriction group 3 A contained two species notably *Acetobacter tropicalis* and *Acetobacter senegalensis* (Table 6). These unexpected results were not further considered.

In sum, LAB and AAB displayed a restricted species and strain diversity with a relative homogenous distribution through the regions whereas yeasts population showed a wider genetic diversity with a microbiota that was never the same from a region to another region.

3.3. Distribution of functional capacities of the isolated microbiota

Functional properties impart to microorganisms, their value in food technology. In cocoa fermentation, the properties such as production of pectinolytic enzymes, carbon metabolism and acids secretion are crucial for quality product (De Vuyst and Weckx, 2016; Schwan et al., 2014; Soumahoro et al., 2015; Yao et al., 2014).

The results showed that a large rate (roughly 78%) of yeasts isolated was pectinolytic enzyme (PE) producer. These isolates presented different level of enzyme production as indicated by the halo diameter, which ranged between 0.5 and 3 cm. We further considered the strains presenting high performance of enzyme production with a halo diameter over 1.5 cm. On this basis, strong PE producers were dominant in seven (7) regions, representing more than 56% of yeasts isolates while less than 43% of yeasts producing PE were found in five (5) regions (Table S3). The unequal distribution of yeasts pectinolytic strains in the regions was particularly evidenced with a high rate (>69%) of strong EP producers in Nawa and Guemon regions, and a weak proportion (only 5.72%) in Loh-Djiboua region (Table S3). Moreover, strains of the same species were observed to present different level of enzymes production; even having the same RFLP profile some strains could not present similar level of PE production (data not shown).

The analysis of carbon metabolism showed that, almost all yeasts and

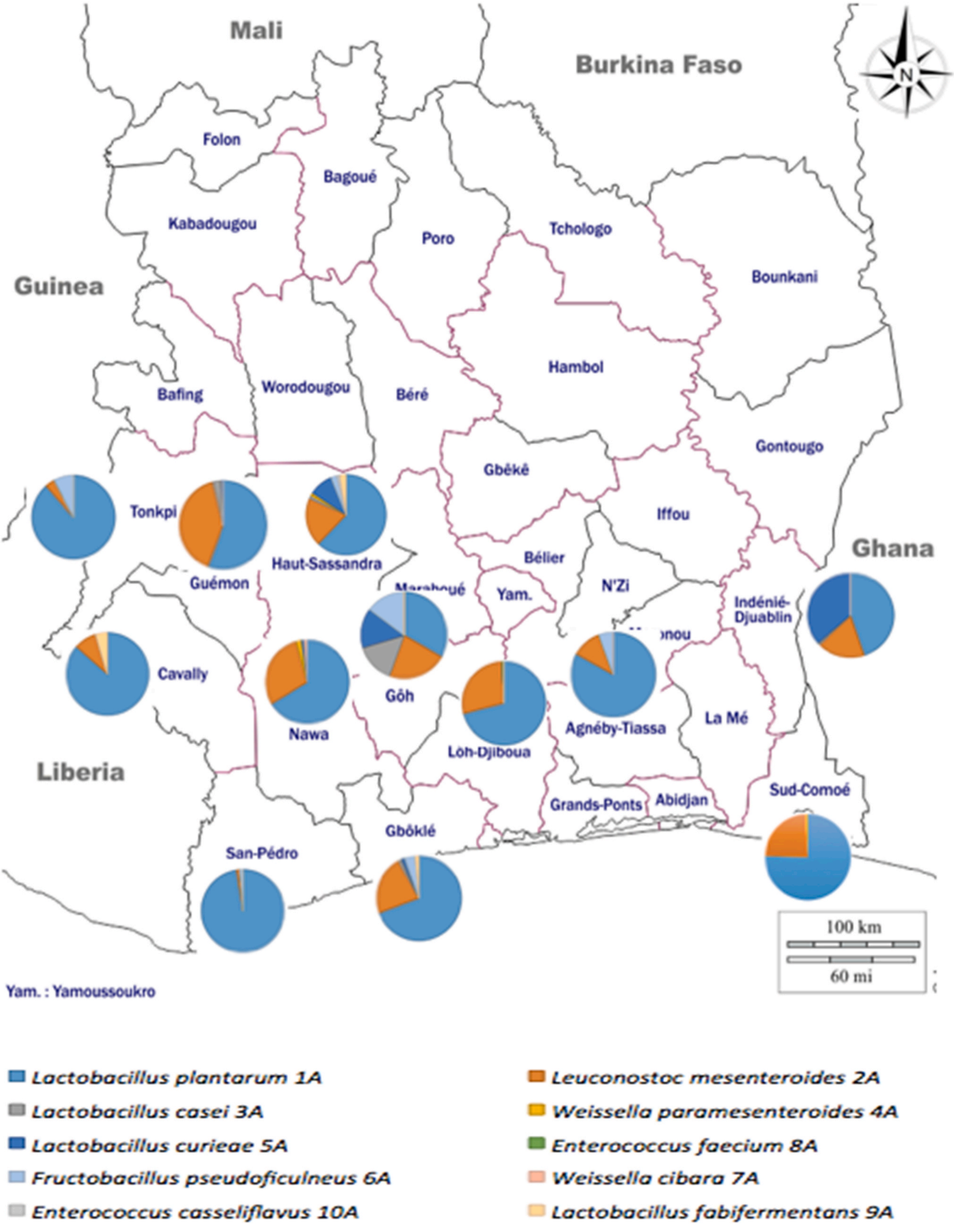

Fig. 4. Geographical distribution of LAB strains involved in cocoa heap fermentation of twelve regions of Côte d'Ivoire.The pie chart in each region presents the proportion of the different strains composing the microbiota. This proportion was calculated as percentage (number of isolates of a strain/number of total isolates) × 100.

Table 6

Restriction profiles and identification of AAB microbiota isolated from cocoa heap fermentation in the twelve regions.

RFLP Group	Length of Amplified 16 S rADN	Restriction profile (bp)		Number of isolates	Species
		*Taq*I (bp)	*Hae*III (bp)		
1 A	1300	500 + 380+300 + 120	550 + 300+180 + 150	471	*Acetobacter pasteurianus*
2 A	1300	500 + 380+150 + 130+140	1000 + 250	255	*Acetobacter okinawensis Acetobacter ghanensis*
3 A	1300	650 + 380+270	550 + 300+180 + 150	274	*Acetobacter tropicalis* *Acetobacter senegalensis*
4 A	1300	800 + 380+120	550 + 300+180 + 150	22	*Acetobacter malorum*
5 A	1300	380 + 200+160 + 130 +120 + 110+100 + 100	550 + 300+180 + 150	20	*Gluconobacter oxydans*

bp: base pairs; *Hae*III, *Taq*I: restriction enzymes.

LAB isolates have the capacity to metabolize glucose, fructose and sucrose the main sugars contained in the pulp. However, citrate metabolism was found to be unequally distributed in isolated LAB population. The general proportion of LAB citrate positive never exceeded 70% except in Haut-Sassandra region (83%), the lowest rate being 17.35% obtained from Nawa region (Table S3). Additionally, the capacity of LAB strains to produce acid from glucose was analyzed focusing on maximum acid production. LAB strains presenting a production more than 8 g/L were considered as high performers. The proportion of such isolates ranged from 32.50% (San Pedro) to 59.43% (Nawa) (Table S3). Concerning AAB, apart from acetic acid production, no capacity was detected among the functional properties analyzed. An unequal distribution of acetic acid producers through the regions was also observed (Table S3).

3.4. Quality of fermented beans

Results in Table 8 showed that "Good Fermented" quality was achieved only in four regions notably Agneby-Tiassa, Indenié-Djuablin, Loh-Djiboua and Cavally. From these regions where Good Fermented quality was obtained, *Pichia kudriazevii* strain 2 A was the dominant yeasts species in the three firsts regions whiles *Pichia kudriazevii* strain 2 M dominated in the latter. As lactic acid bacteria, *Lactobacillus plantarum* strain IA, was the dominant species in all the four regions. Moreover *Acetobacter pasteurianus* strain IA (in Agneby-Tiassa and Loh-Djiboua) and *Acetobacter okinawensis* (in Cavally and Indenié-Djuablin) were the dominant acetic acid bacteria species. We also observed that this microbiota was in general also predominant in the other regions where "Fair Fermented" quality was recorded. Hence, strong correlation could not be found between the microbiota and the quality of fermented beans in the conditions of our study.

Table 7

Yeasts strains distribution in the sampled regions.

Species	Strains (Profiles)	Lôh-Djiboua	Sud-Comoé	Agnéby-Tiassa	Guemon	Nawa	Indénié-Djuablin	Haut-Sassandra	Gbôklè	San-Pedro	Tonkpi	Cavally	Gôh
Saccharomyces cerevisiae	2j	–	–	–	–	–	–	20	–	9	–	–	–
	2 k	–	–	–	–	–	–	–	84	–	–	–	–
	5c	–	–	–	–	6	–	–	–	–	–	–	–
	5 d	–	–	–	–	–	1	–	–	–	–	–	–
	5f	–	–	–	–	–	–	–	11	–	–	–	–
	5i	–	–	–	–	–	–	–	–	–	–	6	–
Pichia fermentans	1a	109	–	–	–	–	–	–	–	–	–	–	–
	6a	–	121	–	–	–	–	–	–	–	–	–	–
Pichia kluyveri	1 b	–	–	–	13	–	–	–	–	–	–	–	
	2n	–	–	–	–	–	–	–	–	–	–	–	18
	5e	–	–	–	–	–	–	91	–	–	–	–	–
	5f	–	–	–	–	–	–	–	11	–	–	–	–
	6 d	–	–	–	–	–	–	–	–	61	–	–	–
Pichia kudriavzevii	3 b	–	–	–	59	–	–	–	–	–	–	–	–
	3 d	–	–	–	–	–	–	–	7	–	–	–	–
	3 h	–	–	–	–	–	–	–	–	–	–	–	67
	5f	–	–	–	–	–	–	–	11	–	–	–	–
	2a	**122**	–	**87**	–	–	**64**	–	–	–	–	–	–
	2 b	–	**25**		–	–	–	–	–	–	–	–	–
	2 g	–	–	–	–	–	–	–	–	–	–	–	35
	2i	–	–	–	–	60	–	–	–	–	–	–	–
	2 k	–	–	–	–	–	–	–	84	–	–	–	–
	2L	–	–	–	–	–	–	–	–	–	117	–	–
	2 m	–	–	–	–	–	–	–	–	–	–	125	–
	7a	–	–	–	–	**34**	**5**	–	–	–	–	–	–
	7 b	–	–	–	–	–	22	–	–	–	–	–	–
Candida aaseri	8a	–	1	–	–	–	–	–	–	–	–	–	–
	3a	22	–	–	–	–	–	–	–	–	–	–	–
Hanseniaspora uvarum	5e	–	–	–	–	–		18	–	–	–	–	–
	5 d	–	–	–	–	–	1	–	–	–	–	–	–
	4 b	1	–	–	–	–	–	–	–	–	–	–	–
	5 h		–	–	–	–	–	–	–	–	4	–	2
Candida tropicalis	2f	–	–	–	1	–	–	–	–	–	–	–	–
	7a	–	–	–	–	**28**	**11**	–	–	–	–	–	–
	2 b	–	25	–	–	–	–	–	–	–	–	–	–

The numerals indicate the number of isolates. (–) Indicates not found.

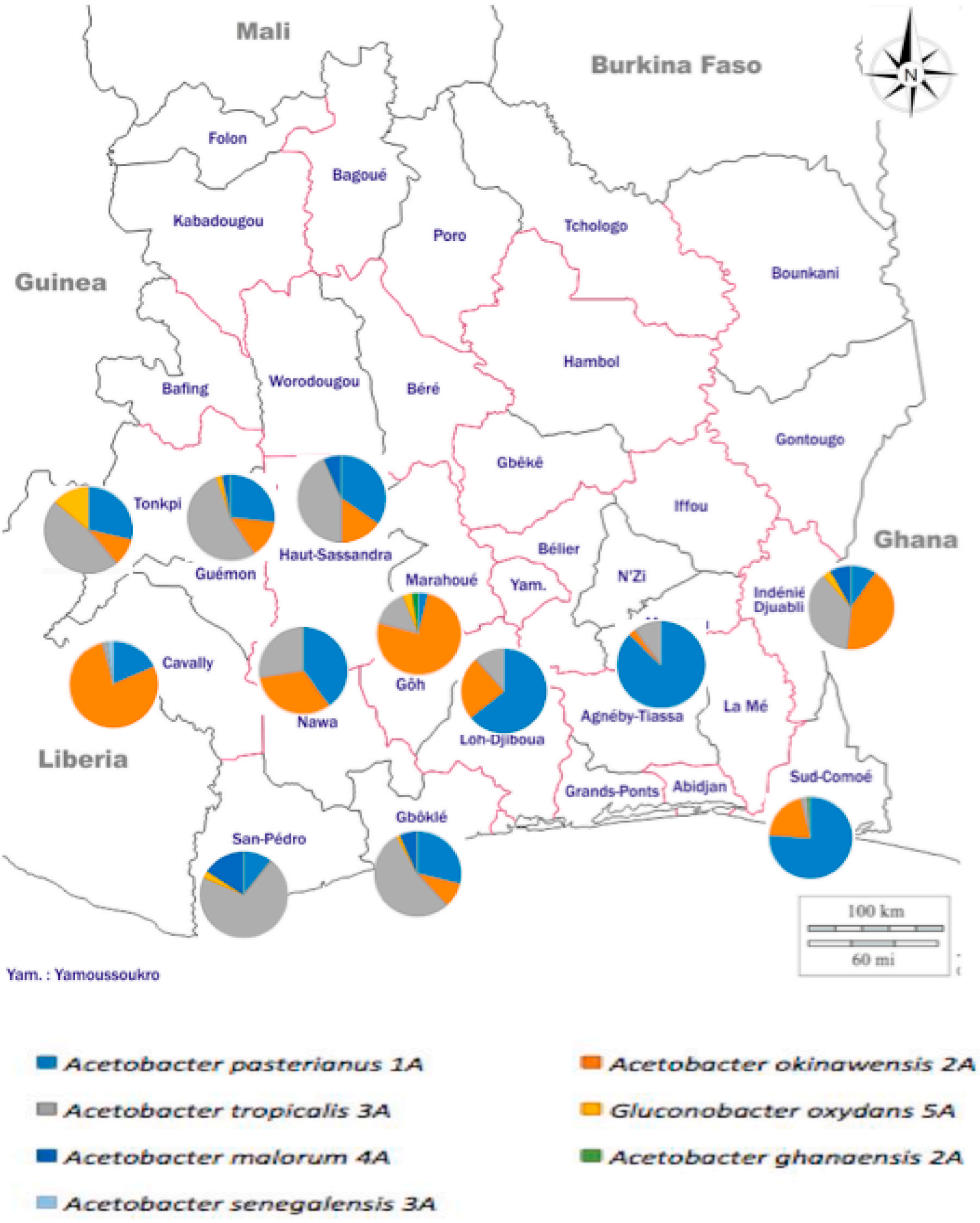

Fig. 5. Geographical distribution of AAB strains involved in cocoa heap fermentation of twelve regions of Côte d'Ivoire.The pie chart in each region presents the proportion of the different strains composing the microbiota. This proportion was calculated as percentage (number of isolates of a strain/number of total isolates) × 100.

4. Discussion

In this study, the microbiota involved in Ivorian cocoa fermentation through twelve producing regions, has been extensively analyzed both at genetic and functional level, and the geographical distribution of the isolated microbiota was mapped.

From analysis of fermentation conditions, it was observed that, pH and temperature keep the same trends of variation in all the regions analyzed. The temperature increased in the first half-fermentation then decreased in the second half; this thermal increase being known as resulting from exothermic reactions from ethanol oxidation into acetic acid (Voigt et al., 2018). External pH of beans increased continuously to the end of the process, due to citrate metabolism (Ouattara et al., 2017) while internal pH undergo a decrease to reach a minimum, due to acid penetration into bean (Camu et al., 2007). The similar features of fermenting cocoa from all regions reveals that cocoa fermentation is a natural biological system that basically works the same way whatever the geographical location. As to support this observation, the same trend

Table 8
Quality of fermented beans from the different regions.

	Percentage of beans (%) after fermentation											
	Agneby-Tiassa	Cavally	Gbôklè	Gôh	Guemon	Haut-Sassandra	Indénié-Djuablin	Loh-Djiboua	Nawa	San Pedro	Sud-Comoe	Tonkpi
Brown	90	91	88	88	83	82	95	96	84	89	84	87
Slaty	2	3	2	8	5	7	0	0	4	1	5	6
Purple	4	3	6	3	9	3	4	2	8	5	7	5
Other	4	3	4	1	3	8	1	2	4	5	4	1
QUALITY	GF	GF	FF	FF	FF	FF	GF	GF	FF	FF	FF	FF

The quality of fermented and dried cocoa bean was assessed using the standard method of cut test as described by Shamsuddin and Dimick. (1986), with a sample size of 300 beans. Numerals are indicated as percentage. GF Good Fermented; FF Fair Fermented, according to the standards of cocoa merchant association, USA (CMA).

of variation of cocoa fermentation conditions was also reported in the other countries (de Melo Pereira et al., 2013; Nielsen et al., 2007; Schwan and Wheals, 2004).

However, strong differences were observed in the peaks of temperature, minima of pH and the time of their occurrence. For instance, a high peak of temperature (47 °C) was reached at 48 h of fermentation in Cavally region corresponding to AAB count of 7.79 (±0.41) log (UFC/g of pulp), whereas the peak of temperature in Indenié-Djuablin (39 °C) occurring at 60 h was relatively weak, corresponding to AAB population of 6.58 (±0.24) log (UFC/g of pulp). The differences in temperature peaks may probably result from differences in microbial activity notably AAB activity known to be responsible for the raise of temperature (Camu et al., 2007; Soumahoro et al., 2015). However, other factors such as genotypes, mixing frequency and climate may interfere with the peak of temperature (Schwan and Wheals, 2004). The significance of temperature peak was recently discussed by Barel (2013) reporting that higher is the peak of temperature reached during fermentation, better is the quality of the cocoa products. Although, this gives an insight into the probable variation of cocoa products quality from region to region, the peak of temperature only could not be sufficient to infer such conclusion. Another important parameter is the decrease of internal pH (acidification), which reached the lowest value (4.5) in Nawa region after 60 h of fermentation. Acidification of internal cocoa bean is responsible for induction of proteolysis that leads to the formation of specific aroma precursors of chocolate (Jinap et al., 2008; Voigt et al., 2018). The timing in which this acidification occurred in this study was also variable, from 6 h in Agneby-Tiassa, Guemon and Lôh-Djiboua, to 144 h in Tonkpi. It was previously reported that, the formation of aroma precursors are strongly dependent on the time course of bean acidification during the fermentation process, due to the pH dependency of proteolysis (Schwan and Wheals, 2004; Voigt et al., 2018), and impact the final quality of cocoa. The differences in key parameters (peak of temperature, minimum pH, timing of acidification) observed from the different regions suggest that each fermentation evolves singularly probably toward a typical quality. This also point out the need to standardize this process for a quality labeling.

However, since it is well-assessed that the pH and temperature variations during cocoa fermentation result from microbial activity, we analyzed the growth of microbiota from all the regions. To this regards, LAB and AAB reached the highest count of bacterial population (8.58 log CFU/g and 9.16 log CFU/g, respectively) in fermenting cocoa from Nawa, where the lowest pH value of beans (efficient acidification) was observed. The high count of LAB and AAB has undoubtedly produced important quantity of lactic acid and acetic acid, the mains acids responsible for bean acidification during cocoa fermentation (Ouattara et al., 2014; Yao et al., 2014; Voigt et al., 2018). On the other hand, similar high bacterial count of LAB and AAB was obtained in Indenié-Djuablin regions (8.50 log CFU/g and 9.20 log CFU/g, respectively) but this resulted in a poor acidification of beans with an internal pH value that never dropped below 5.8. From these observations one could assume that, a well-processed fermentation may not necessarily depend on the microbial load, but rather on the functional performance of the microbial strains that may be most determinant in this process.

Furthermore, investigations on genetic diversity of microbiota isolated from Ivorian cocoa revealed a wide diversity of yeasts population with at least 22 species and 45 strains, each defined by a unique restriction profile. These strains were more generally different from a region to another region, indicating that in each region, the yeasts microbiota is specific. Previously, Jespersen et al. (2005), reporting the genetic diversity of yeasts microbiota from fermenting cocoa in Ghana, by chromosome length polymorphism, indicated that several different strains are involved in the fermentations. The variation of yeast microbiota observed from region to region in our study may be an important factor of cocoa quality variation. Indeed the quality of beans varied considerably from region to region.

In contrast, LAB and AAB presented a restricted diversity with a limited number of species and strains 10 and 7, respectively. Many studies have also reported the restricted diversity of LAB and AAB in cocoa ecosystem (Bortolini et al., 2016; Lefeber et al., 2011). Additionally, we observed that, each species corresponded to a unique RFLP profile representing a strain, indicative of a lack or a weak intraspecific variability. Moreover, regarding strains distribution, the same LAB and AAB strains were found in different regions, strongly contrasting with yeasts microbiota that was specific in each region. This suggests that all the twelve regions may function as a single cocoa ecosystem for LAB and AAB.

On the other hand, we unexpectedly found that the RFLP used in this study could not distinguish between strains from *Acetobacter okinawensis* and strain from *Acetobacter ghanensis.* This method could not either differentiate *Acetobacter tropicalis* strains from *Acetobacter senegalensis* strains. This may be indicative of a limited discriminatory power of the RFLP used in the conditions of our study, since only a portion of the genome (ribosomal genes) was targeted. Generally, typing methods targeting the whole genome notably RAPD are more resolutive (Saghrouni et al., 2013; Tabit, 2016). To this point of view, the apparent genetic diversity of the microbiota found in this study may be underestimated, since the cocoa microbiota is complex. Furthermore, different strains belonging to the same species displayed different functional performance, as the technological traits of these microorganisms is strain dependent. This may indicates that the control of the cocoa fermentation using starter culture should be considered at strain level but not at species level.

From our results, only 33.33% of regions produced “Good Fermented” cocoa. Previous study reported that, only 22–27% of 300 fermented cocoa samples collected randomly from both Ivorian ports had the “Good Fermented” quality (Dembele et al., 2009). This gives an insight into the problem of quality from spontaneous cocoa fermentation in Cote d’Ivoire, the world’s leading producer.

Furthermore, we could not strongly correlate the dominant microbial strains with the quality of fermented cocoa. This is probably due to the complexity of the microbiota from which same species never means same performance. Additionally, interferences of other minor species or other non-microbial factors may counterbalance the activity of useful microbial strains during spontaneous cocoa fermentation. This evidences the need to achieve a fine cocoa fermentation with a defined microbial culture at strain level.

5. Conclusion

This study constitutes the widest and the most extensive analysis of Ivoirian cocoa microbiota since it includes not only all the majors microbial groups but also the largest number of cocoa producing regions ever targeted in a study.

Yeasts microbiota involved in Ivorian cocoa fermentation showed a great genetic heterogeneity and a yeasts microbiota that was never the same in general from a region to another region; this may be an important factor responsible for variation on cocoa quality observed in the different producing region. In contrast, due to their restricted diversity, LAB and AAB species did not vary considerably in the different regions since the same strains were found in several regions. On the other hand, the functional performances seemed to be randomly distributed in the regions, with Nawa and Cavally regions recording the highest rate of acidifying strains, while strong PE producers were found in seven other regions.

Declaration of competing interest

Authors do not have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103767.

External pH is pH of pulp; internal pH is pH of nibs. Measures were triplicate and error bars indicated standard deviation.

Enumeration was performed using two plates per dilution; two successive dilutions were used. Arrows indicate the different peaks. Analyses were duplicate and error bars indicated standard deviation.

The pie chart in each region presents the proportion of the different strains composing the microbiota. This proportion was calculated as percentage (number of isolates of a strain/number of total isolates) × 100.

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