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A highly diverse Acetic Acid Bacterial community is involved in Cocoa fermenting process in Côte d'Ivoire

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Abstract

Cocoa fermentation is led by a succession of complex microbial communities where acetic acid bacteria play key roles for cocoa quality at the end of the process. In this study, the molecular diversity of acetic acid bacteria (AAB) involved in cocoa bean fermentation in six major cocoa producing regions in Côte d'Ivoire was estimated. A total of 694 acetic acid bacteria isolated during six days of fermentation was identified using 16S RNA gene analyses. It was revealed that the AAB fermenting community in Côte d'Ivoire consisted of seven (07) species with a predominance of *A. pasteurianus*, and *A. tropicalis*. Moreover, phylogenetic analysis indicated an intraspecific diversity among *A. tropicalis* isolated strains. In addition, it was shown that among the 694 isolates, 222 had a low acetic acid capacity production; while 343 had average capacity and 129 higher capacity. All these data provide new insight about the AAB strains involved in cocoa fermentation in Côte d'Ivoire that could be exploited for selecting appropriate starter cultures to improve this process.

Keywords: Cocoa Fermentation, Acetic acid Bacteria diversity, high Acetic Acid production capacity, Côte d'Ivoire

Résumé

Dans cette étude, la diversité moléculaire des bactéries acétique impliquées dans la fermentation des fèves de cacao de six grandes régions productrices de cacao en Côte d'Ivoire a été estimée. Un total de 694 bactéries acétiques a été isolé pendant six jours de fermentation ont été identifiées à l'aide du gène de l'ARN 16S. Les résultats ont montré que la flore de bactéries acétiques impliquée dans la fermentation du cacao en Côte d'Ivoire était composée de sept (07) espèces avec une prédominance de *A. Pasterianus*, et *A. tropicalis*. De plus, l'analyse phylogénétique a indiqué une diversité intraspécifique parmi les souches de *A. tropicalis*. Par ailleurs, les résultats ont montré que parmi ces 694 isolats, 222 étaient de faibles producteurs d'acide acétique, alors que 343 étaient des producteurs moyens et 129 étaient de forts producteurs d'acide acétique. Toutes ces données fournissent de nouveaux indices sur les propriétés des souches de bactéries acétiques impliquées dans la fermentation du cacao en Côte d'Ivoire et pourraient être exploitées pour la sélection de souches starters nécessaire à l'amélioration de ce processus.

Mots-clés: Fermentation du cacao, diversité des bactéries acétiques, forte capacité de production d'acide acétique, Côte d'Ivoire

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Introduction

The cocoa fermenting process involves different bacteria and fungi species during microbial activities in the pulp to enhance the quality of fermented cocoa (Schwan and Wheals, 2004). The fermenting process in the cocoa pulp is well-defined and successively involve yeasts, during the first hours (24 hours), followed by lactic acid bacteria (LAB), acetic acid bacteria (AAB) (Pereira et al., 2012) and diverse bacteria belonging to the *Bacillus* genus. During this process, yeasts transform sugar into ethanol in the pulp that is metabolized by acetic acid bacteria into acetic acid. Acetic acid bacteria (AAB) that belong to *Acetobacteraceae* constitute an important group of obligate aerobic Gram negative bacteria with ability to oxidize ethanol into acetic acid (Matsushita et al., 1994; Holt et al., 1994). These bacteria that are useful in industrial production of vinegar and various fermenting processes (Adachi et al., 2003) are actively involved in cocoa beans fermentation (Yao et al., 2014). Acetic and lactic acids (produced by lactic acid bacteria) diffuse into the bean and in combination with heat cause the death of the seed embryo as well as the end of fermentation (De Brito et al., 2000). These biochemical changes lead to the formation of molecules precursor involved in development of a characteristic aroma, flavor and color of beans (Afoakwa et al., 2013; Schwan and Wheals, 2004). Therefore, acetic acid bacteria activity during cocoa fermentation is crucial for obtaining high quality fermented cocoa. In this context, AAB species which dominate cocoa fermentation process could determine the quality of fermented cocoa and variability of AAB species may be the source of variable cocoa beans quality (Maura et al., 2016). Despite the fact that the involvement of AAB species in cocoa fermentation has been evidenced in Côte d'Ivoire (Yao et al., 2014) and biochemical characteristic identified (Coulibaly et al., 2018) their molecular diversity has not yet been studied. Cocoa fermentation is a spontaneous microbial process and this nature of cocoa bean fermentation may be the source of micro-

bial diversity, and consequently of variable end-product quality (Maura, et al., 2016). A better monitoring of cocoa fermenting process should imply a thorough knowledge of AAB species diversity in order to control fermented cocoa quality variability. Indeed fermented cocoa variability causes huge economic losses for cocoa-producing countries such as Côte d'Ivoire estimated to 200 billion FCFA/year (ANADER, 2014). The aim of this study was to evaluate the diversity of acetic acid bacteria involved in cocoa bean fermentation within six major cocoa producing regions in Côte d'Ivoire.

Materials and Methods

Cocoa Fermentation conditions and Sampling

Cocoa pods were harvested from six regions of high cocoa producing areas of Côte d'Ivoire including Agneby-Tiassa (6°00' North 4°00' West); Sud-Comoé (5°30' 0 North 3° 15' West), Loh-Djboua (5° 40' North 5° 30' West), Guemon (6° 44' 00" North 7° 21' 00" West), Nawa (5°47' 00 North 6° 36' 00" West) and Indénié-Djuablin (6° 43' 47" North 3° 29' 47" West). Cocoa beans spontaneous fermentation was carried out during 6 days in "National Floristic Center" of Felix Houphouët-Boigny University in traditional conditions using banana leaves as previously described (Koffi et al., 2017). During this process, samples were taken every 12 hours fermentation from the beginning. Moreover, pH and temperature were recorded in the pulp and 200 grams of beans were collected for biochemical and microbiological analysis as previously described (Koffi et al., 2017).

AAB Isolation and enumeration

AAB isolation was performed immediately after sampling by using Duthathai medium according to the process previously described by Coulibaly et al. (2018). Petri dishes were incubated at 30°C for 5 days, and acetic acid bacteria isolates were selected based on the presence of a clear halo around colonies and identified by using Gram staining, catalase and oxidase characteristics. Then, characteristic colonies were enumerated and colony numbers expressed as cfu/g of cocoa pulp-bean mass. Moreover, isolated strains were kept at -20°C in Luria Bertani medium containing 20% of glycerol for further tests.

Acetic acid Production

Acidifying AAB isolates screening was carried out in solid medium based on clear halo diameter in Hestrin-Schramm (HS) medium after incubation (Yao et al., 2014). Briefly, the HS medium containing 0.05 % D-glucose, 0.5 % yeasts extract, 0.3 % casein peptone, 2 % glycerol, 1.5 % calcium carbonate, 1.5 % ethanol (v/v) and 1.5 % agar and supplemented with 0.0016 % bromocresol green was spot inoculated with pure 24 h pre-culture of tested strain and incubated at 30°C for 10 days. Acidifying capacity of each tested isolate was carried out by presence of clear halo around the colony and acidification level of strains was evaluated by measuring of this halo zone diameter.

Molecular identification

The molecular identification of all AAB isolates was carried out in three steps including 16S rRNA gene amplification by PCR, PCR-RLFP and 16S rRNA gene sequencing. 16S rRNA gene amplification by PCR was performed on colony as previously described by Anzai et al. (2000) using primers F27 (5' AGTGGCGGACGGGTGAGTA 3') and R27 (5' CCAACTCCCATGGTGTGACG3') (Anzai et al., 2000). PCR reaction was performed in a final volume of 50 µL mixture containing 1 µL of each dNTP (10 mM), 1.25 U of Taq polymerase (BioLabs, Lyon, France) and 2 µL of each primer (100 µM). PCR consisted of incubating for five (5) minutes at 95°C followed by 35 cycles of 95°C for one (1) minute, 56 °C for one (1) minute and 72 °C for one (1) minute. All PCR reactions were performed in a thermal cycler (Labcycler Senso Quest, Göttingen, Germany). The PCR products were stained with Ethidium bromide and visualized under UV light after gel electrophoresis on 1 % agarose. PCR products were digested with TaqI, AluI and HaeIII restriction enzymes (BioLabs, New England). The enzymatic digestion reaction was performed in a final volume of 20 µL containing 10 µL of PCR product, 2 µL of supplied buffer, 7.6 µL of water and 0.4 µL of restriction enzyme. After incubation at 65 °C for one (1) hour, the digested products were separated by electrophoresis in a 2 % (w/v) agarose. For each restriction group, three strains were selected and sequenced at BIOFIDAL (Lyon, France) using F 27 (5' AGTGGCGGACGGGTGAGTA 3') and R 520 (5'ACCGCGCGGCTGCTGGC 3') (Anzai et al., 2000). The obtained sequences were then compared to the NCBI data Genbank using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences showing a high percentage of identity (= 97%) were considered as belonging to the same species.

Phylogenetic analysis was carried out using the Neighbor-joining method (Saitou and Nei, 1987) with MEGA 6 software (Tamura et al., 2013).

RESULTS

Evolution of cocoa fermentation parameters

The temperature of cocoa mass increased rapidly during the first 24 h to 48 h of fermentation to reach a peak after 36 to 60 h and decreased progressively after this time for all studied areas. However, the higher temperature (46 °C) was obtained in Agneby-Tiassa after 36 h of fermentation and the lower peak of temperature was recorded in Indénié-Djuablin after 24 h of fermentation with a temperature of 39 °C (Figure 1 A). Concerning the pH, values in the pulp at the beginning of the fermentation ranged from 3.7 to 4.5 and decreased to pH 3.5 to 4.0 during the first 36 hours. After this, an increase highly of pH was observed until values ranged to pH 7.1 to 8.0 (Figure 1 B). While, the pH of cocoa beans decreased during fermentation process with values ranged to 6.4 - 6.8 to 6.2 - 5.4. Moreover, no difference was observed between pH values recorded in cocoa fermentation heap of producing areas (Figure 1 C).

Growth dynamic of AAB

Isolation of AAB strains was carried out during each cocoa fermentation for each study region. Evolution of AAB growth dynamic during cocoa fermentations is shown in Figure 2. In generally same evolution of AAB population was observed for the six studied regions with an increase of microbial load at beginning of the process until a peak after around 72 h (Figure 4) and a decrease of AAB population at the end of the fermentation. However, the highest AAB load was recorded in cocoa fermenting mass of Loh-Djiboua with bacterial load estimated to 8.2 Log 10 cfu/g of cocoa beans after 60 h while fermentation of cocoa of Agneby-Tiassa displayed, at the same conditions, the lowest AAB load with values to 7.6 Log 10 cfu/g of cocoa beans (Figure 2). In addition, no lag phase was observed during these fermentative processes for the all

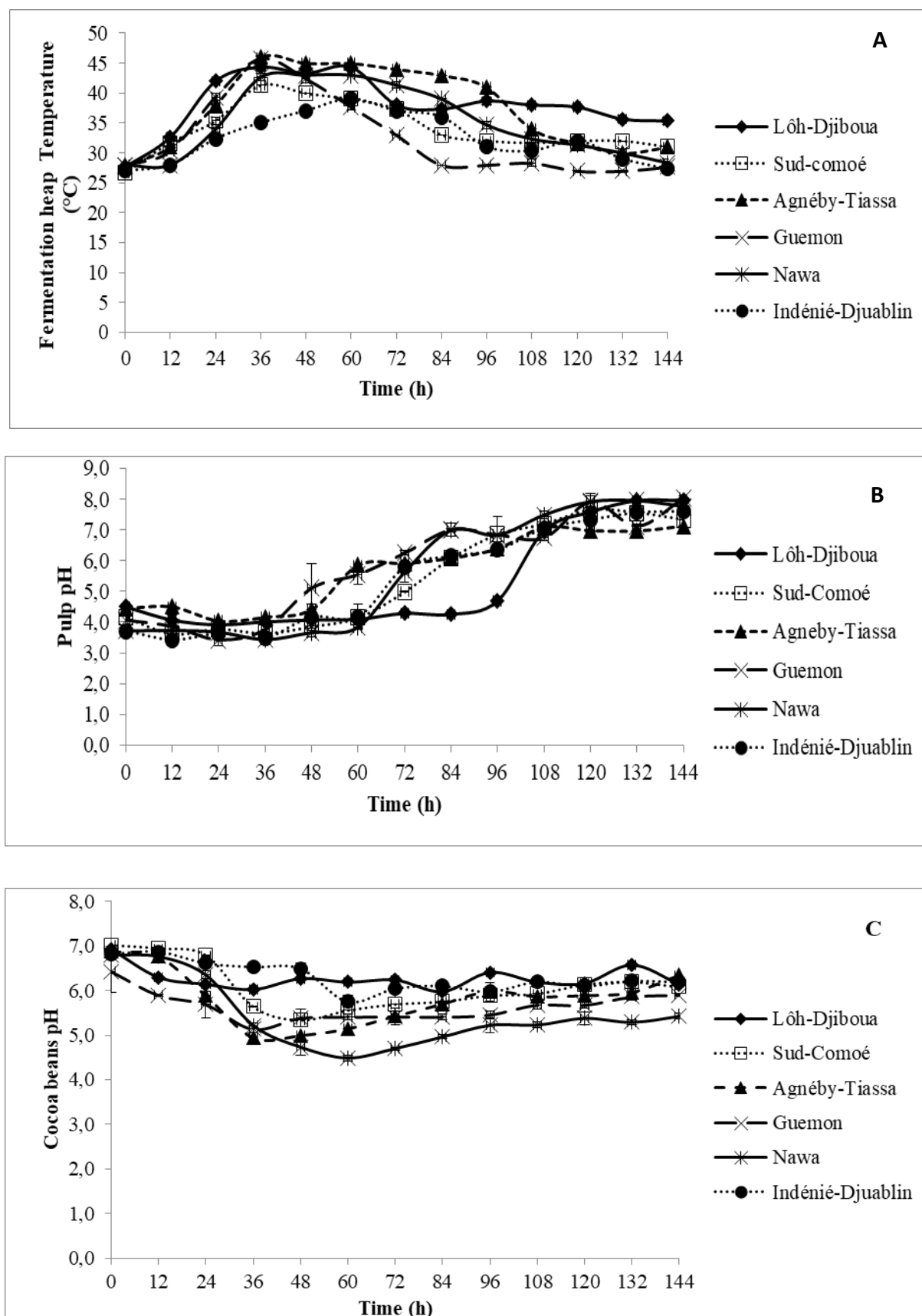


Figure 1: Evolution of physicochemical parameters during the six days of fermentation
 A: Temperature; B: pH of pulp and C: pH of cocoa beans

areas although initial phase of cocoa fermentation are usually unsuitable AAB growth conditions.

Acetic acid production capacity

A total of 694 strains were isolated during of the cocoa fermentation of the six regions. As AAB is essential for fermentation process by the production of acetic acid, all strains were tested for their acetic acid production capacity (Table 1). The results show that although all the 694 strains analyzed have an acidification

capacity, there is a difference in term of amount of acid produced as indicated by the diameter of clear halo around each AAB colony. Among the tested isolates, 222 had a low acetic acid production capacity, 343 have middle capacity and 129 had a high acetic acid production capacity. Moreover, the high number of strains with high acetic acid production capacity was recorded in Agneby-Tiassa, Nawa and Guémon regions with 69, 56 and 51 strains respectively (Table 1).

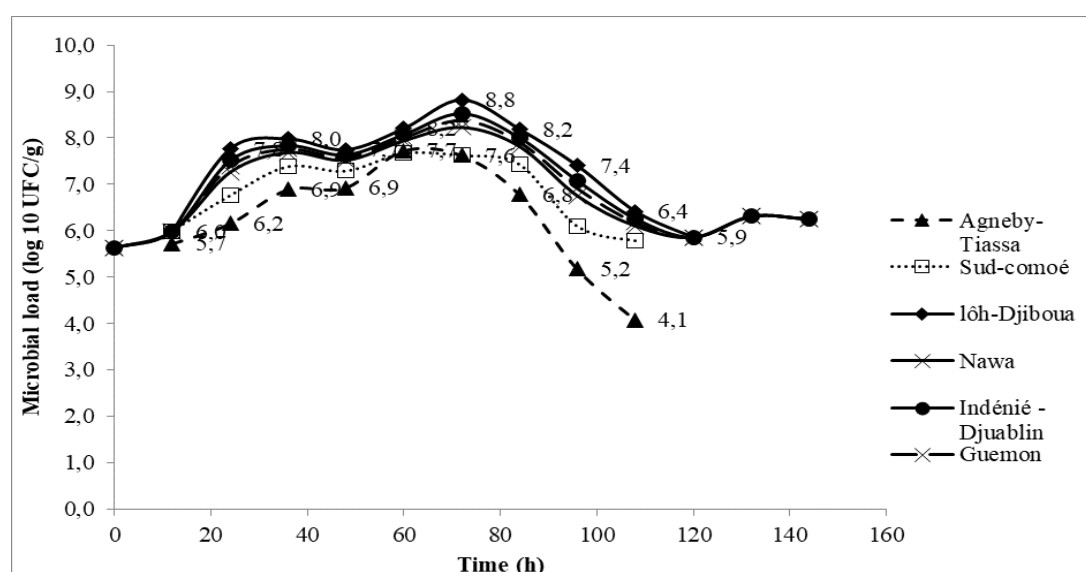


Figure 2: Evolution of AAB load during the six days of fermentation

Table 1: Distribution of isolated strains according to acetic acid production capacity

	Sud-Comoé	Nawa	Agneby-Tiassa	Loh-Djiboua	Indénié - Djuablin	Guémon
Tested strains	107	89	155	166	56	121
High producing-strains [1.7 - 3.1cm]	14 (13.1%)	56 (62.9 %)	69 (44.5 %)	14 (8.5%)	18 (32.1%)	51 (42.2 %)
Middle producing-strains [1.1 – 1.7cm]	56 (52.3%)	31 (34.8 %)	73 (47.1%)	96(57.8 %)	30 (53.6 %)	57(47.1 %)
Low producing-strains [0.4 – 1.1 cm]	37 (34.6 %)	2 (2.3 %)	13 (8.4 %)	56 (33.7 %)	8 (14.3 %)	13 (10.7 %)

Diversity and distribution of the isolated acetic acid bacteria

A total of seven (07) species were identified in the six local regions, including *Acetobacter pastorianus*, *Acetobacter tropicalis*, *Acetobacter ghanaensis*, *Acetobacter syzygii*, *Acetobacter pomorum*, *Acetobacter persici* and *Gluconobacter oxydans* (Figure 3). Of these species iso-

lated in the six cocoa-producing areas, *A. pastorianus* and *A. tropicalis* were the dominant with rate prevailing of 94.8 %. Moreover, these isolates were detected in whole cocoa fermenting heap while *A. pomorum* and *G. oxydans* where isolated in Indénié-Djuablin and in Guémon regions and *A. ghanaensis* was only detected in Sud-Comoé (Table 2)

Table 2: Distribution of AAB genotypes (*Acetobacter* and *Gluconobacter* genera) isolated during cocoa fermentation of six major cocoa producing regions

	Sud-Comoé	Loh-Djiboua	Agnéby-Tiassa	Indénié - Djuablin	Guémon	Nawa
Tested strains	105	165	166	62	93	80
<i>A. pasteurus</i>	80 (76.2 %)	106 (64.3 %)	145 (87.4)	6 (9.7 %)	25 (26.9 %)	32 (40.0 %)
<i>A. tropicalis</i>	21 (20 %)	40 (24.2 %)	04 (2.4)	26 (41.9 %)	13 (14.0 %)	26 (32.5 %)
<i>A. pomorum</i>	3 (2.9 %)	19 (11.5 %)	17 (10.2)	23 (37.1 %)	50 (53.8%)	22 (27.5 %)
<i>G. oxydans</i>	0 (0.0)	0	0	2 (3.2 %)	2 (2.1%)	0
<i>A. pomorum</i>	0	0	0	5 (8.1 %)	3 (3.2 %)	0
<i>A. ghanaensis</i>	01 (0.9 %)	0	0	0	0	0

Discussion

In this study, the diversity of AAB was estimated in order to identify species involved in Ivorian cocoa fermentation in six producing areas and explain the variability of Côte d'Ivoire cocoa beans quality. During the fermentation process, 694 AAB strains were isolated from the six regions. The 16S RNA gene phylogeny of these isolates showed that seven AAB species were involved in cocoa fermentation in the six main cocoa production areas in Côte

d'Ivoire. This diversity is higher than the one described in Ghana where only three species were found (Camu et al. 2007). Our results indicated also that the fermentation process of these cocoa producing areas was dominated by *A. pastorianus*, and *A. tropicalis*. The high prevailing of these species could be due to ability of these strains to withstand the stressful conditions from cocoa fermentation (De Vuyst et al., 2010; Pereira et al., 2012; Samagaci et al., 2016). However, these isolates were detected in whole cocoa fermenting heap while

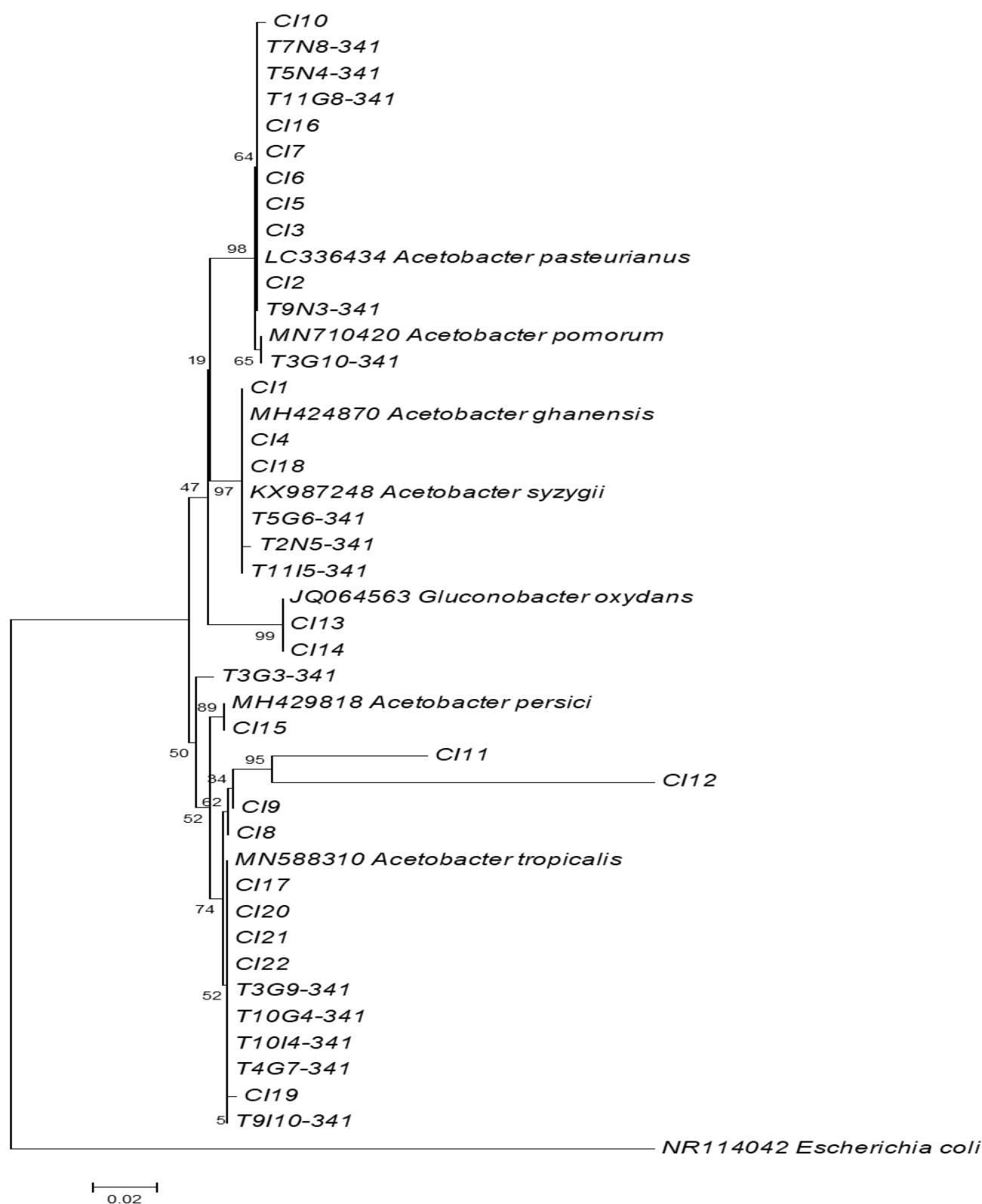


Figure 3: Phylogenetic identification of acid acetic bacteria isolated from cocoa producing zones in Côte d'Ivoire. 16S RNA gene sequences from bacteria species: *Acetobacter tropicalis* (MN588310.1), *A. ghanensis* (MH424870.1), *A. pasteurianus* (LC336434.1), *A. persici* (MH429818.1), *A. pomorum* (MN710420.1), *A. syzygii* (KX987248.1) and *Gluconobacter oxydans* (JQ064563.1) were used for comparison. Sequences from Côte d'Ivoire were labelled CI and T. The tree was constructed by the neighbor-joining method using Mega version 6

G. oxydans and *A. pomorum* were isolated in Indénié-Djuablin and in Guémon regions and *A. ghanaensis* was only detected in Sud-Comoé. This variability in the distribution of AAB species from one region to another was also reported by Visintin et al. (2016). Similar results were shown by Camu et al. (2008) during Ghana cocoa fermentation process. All these data suggested a variability of flora from one region to another and therefore the variability of fermented cocoa quality from one region to another (Barel, 1997). This variability in AAB species distribution could explain the variability observed in fermented cocoa quality in Côte d'Ivoire and shows necessity to select AAB strains with technological properties for using as starter in cocoa fermentation process. Moreover, 16S RNA gene phylogeny indicated diversity within *A. tropicalis* strains implying possible genetic recombination or horizontal gene transfer (Steensels et al., 2014) within this species.

The majority of the isolates (94.8 %) belonged to the *Acetobacter* genus. *Acetobacter* genus is frequently isolated from cocoa fermentation in the world and seemed to be the main AAB genus which is better adapted to fermentations conditions (Ardhana and Fleet, 2003; Coulibaly et al., 2018).

In general, *Acetobacter* is well known to his high acetic acid production capacity more than other genera like *Gluconobacter* and is frequently used in other biotechnological applications such vinegar production and Docosa Hexaenoic Acid (DHA) production (Kerstens et al., 2006). Therefore dominance of *Acetobacter* in AAB population isolated in this study could be interesting since high acetic acid level in the cocoa fermenting heap is the key factor to cocoa quality obtaining. This is the first time, the evolution of cocoa fermentation parameters due to AAB were determined. This study recorded temperatures peak values between 39 and 46 °C at 36 to 60 h of fermentation. Increase of cocoa mass temperature during fermentation process is due to the sugar oxidation in ethanol by yeasts then ethanol to acetic

acid by acetic acid bacteria which are exothermic reactions (Schwan and Wheals, 2004). In this context, intensity of peak of temperature could be due to the intensity of yeast and AAB activities during fermentation process. Consequently, the Agneby-Tiassa region in which the higher temperature was recorded in cocoa mass during fermentation could provide AAB strains with acetic acid production high capacity. Concerning the pH, the values increased during the fermentation process until to pH 7.1 to 8.0 in cocoa pulp while the pH decreased slightly in cocoa beans with values ranging from 6.4 - 6.8 to 6.2 - 5.4. The similar trend was observed by other researchers with pulp pH values at the beginning pH 4 - 4.5 and to pH 7 - 8 at the end of fermentation process (Yao et al., 2014; Coulibaly et al., 2018; Guehi et al., 2010). Generally, low pH observed at the beginning of fermentation process is due to the presence of citric acid. While, the increase of cocoa mucilage pH values is due to the assimilation of initial citric acid by lactic acid bacteria (Kostinek et al., 2008) and yeasts species involved in the fermentation process and diffusion of lactic and acetic acids into the beans (Thompson et al., 2001). This acidification of cocoa beans and increasing pulp temperature allow cotyledon breaks down. This contribute to the activation of endogenous enzymes implicated in development of flavor and color precursors of cocoa (Afoakwa et al., 2013) leading to a better quality of fermented cocoa. The AAB growth dynamics during cocoa fermentation showed that AAB are present during all the fermentation process. Moreover, no lag phase was observed during these fermentative processes. This was probably due to adaptation phenomena of AAB species implicated in cocoa fermentation and suggested that these strains could have exceptional technological properties (Nielsen et al., 2007). These results also indicated that AAB involved in this process could start acetic acid production at early fermenting stage and fully contribute to cocoa quality.

Indeed, acetic acid is one of key metabolites for success of cocoa fermentation process (Thompson et al., 2013).

Moreover, the presence of acetic acid bacteria at the beginning of fermentation process could indicate the ability of these strains to grow in the presence of high acid and low oxygen levels probably due to adaptation phenomenon to cocoa fermentation conditions. This capacity could be an interesting technological property in the context of starter selection for these AAB strains to improve cocoa fermentation process and beans quality.

Acidification is one of the most important properties in cocoa fermentation for development of chocolate aroma (Coulibaly et al., 2018). In this context, a beneficial effect on quality product may occur when fermenting cocoa beans include a population of AAB presenting a high capacity of acidification. Our study recorded 129 isolates with high acid production capacity and the high number of these strains was isolated during Agneby-Tiassa, Nawa and Guémon cocoa fermentation. Because of the importance of acetic acid in cocoa fermentation process, the regions with a high rate of high acid-producing strains could lead to the best cocoa beans quality at the end of fermentation process. In addition, this variability in level of acid production capacities of AAB isolates from one area to another could explain the variability in Côte d'Ivoire cocoa quality (Guehi et al., 2010).

Conclusion

In conclusion, this study showed that AAB involved in cocoa fermentation were highly diverse and able to high acetic acid production capacity. Intraspecific diversity and high acetic acid production capacity observed in some AAB strains in this study could be exploited for selecting starter cultures to improve cocoa fermentation process.

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