DIVERSITY OF MICROBIAL STRAINS INVOLVED IN COCOA FERMENTATION FROM SUD-COMOÉ (COTE D'IVOIRE) BASED ON BIOCHEMICAL PROPERTIES

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Abstract

The cocoa pulp fermentation is one of keys step for obtaining a quality chocolate. However, microflora involved in this process is complex and variable that leads to inconsistent production efficiency on cocoa quality. Here we investigate the diversity of microflora implicated in Sud-Comoé (area of Côte d'Ivoire) cocoa fermentation based on their biochemical properties. During fermentation, yeasts, Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (AAB) reached maximum load at 36, 24 and 72 h respectively and decreased rapidly while the rate of *Bacillus* load increased throughout the fermentative process to reach a peak at 144 h. Among the *Bacillus* strains, 62.22, 87.5 and 33.7 % were able to produce pectinolytic enzymes, acid and citrate lyase respectively. Concerning yeasts strains, 25.43 % showed pectinolytic enzymes production capacity and all of these isolates were able to ferment glucose and fructose while 52 % were able to produce gas from sucrose. Population of LAB was predominant by homofermentative group with proportion at 85.96 % while AAB were dominated by genera *Acetobacter* with 88.78 %. Therefore, this study shows the diversity of biochemical properties of tested microbial strains indicating a diversity of microbiota involved in Sud-Comoé cocoa fermentation.

Introduction

The microbial fermentation is one of major step in the post harvest processing of cocoa beans and it stays essential in the formation of cocoa flavor (Pereira *et al.,* 2012; Hii *et al.,* 2009). The microbial activity in the cocoa pulp is a well-defined microbial succession in which numerous microbial species, mainly yeasts and bacteria including *Bacillus*, acetic acid bacteria (AAB) and lactic acid bacteria (LAB) are involved (Schwan et Wheals, 2004; Ouattara *et al.,* 2008). At the onset of cocoa beans fermentation, yeasts liquefy pulp by production of pectinolytic enzymes and produce ethanol from carbohydrates (Jespersen *et al.,* 2005). *Bacillus* genera take part also in the degradation of pulp according to the study of Ouattara *et al.* (2008). LAB convert sugars and organic acids mainly into lactic acid but in addition acetic acid, gas (CO_2) and ethanol for other species. Acetic acid bacteria oxidize the ethanol by exothermic reaction, initially produced by the yeasts, into acetic acid when air comes in fermentation heap (Nielsen, 2006). Ethanol and acetic acid diffuse into the beans, and this, in combination with the heat produced by this exothermic bioconversion and causes death of seeds embryo as well as the end of fermentation. This initiates biochemical changes in the beans, leading to the formation of precursor molecules for the development of a characteristic chocolate aroma (Quesnel, 1965; Thompson *et al.,* 2001). Therefore, specific microbial flora involved in fermentation could be determining the final quality of the cocoa and chocolate thereof. So, a perfect knowledge of these microorganisms is necessary to improve this process.

Several studies have reported that yeast, AAB, LAB and *Bacillus* are generally implicated in all cocoa bean fermentation, they demonstrated however existence of diversity of genera and species which dominated according to the country (Jespersen *et al.,* 2004; Lefeber *et al.,* 2011; Schwan et Wheals, 2004; Lagunes-Galvez *et al.,* 2006; Ardhana et Fleet, 2003). Some studies, for example, show that *Hanseniaspora guilliermondii and P. membranifacians* are dominant yeasts species in Ghana (Nielsen *et al.,* 2006) while *Candida krusei* is predominant in Nigeria (Jespersen *et al.,* 2004) and *Candida tropicalis* in Indonesia *(*Ardhana and Fleet, 2003). Other studies reported that *Lb. plantarum* and *Lb. acidophilus* are dominant LAB in Brazil and in Dominican Republic respectively. Concerning the AAB, *A. pasteurianus*, *A. ceti* and *A. tropicalis* are dominant in fermented cocoa heap in many countries (Schwan and Wheals, 2004; Ardhana and Fleet, 2003) while *Acetobacter lovaniensis* is predominant in Dominican Republic (Lagunes-Galvez *et al.,* 2006). This diversity could be owing to origin of

cocoa pods (Lima *et al.,* 2011) since the microbial flora contaminated the sterile carbohydrate-rich cocoa pulp and would come mainly from pods (Nielsen *et al.,* 2005; 2007; Camu *et al.,* 2008 ; Papalexandratou *et al.,* 2011). Moreover, suggesting that cocoa beans fermentation is spontaneous according to Romero-Cortes *et al.* (2012), the microbial flora could differ from country to country, from farm to farm even from season to season.

In Côte d'Ivoire, a few studies have been conducted to investigate the microbial flora associated with Ivoirian cocoa fermentation (Papalexandratou *et al.,* 2011; Hamdouche *et al.,* 2015), but remain localized in fermentation field experiments or concern some pods cocoa. Yet, in Côte d'Ivoire, different regions produced cocoa with certainly different qualities due probably to the diversity of microbial flora. In this way, we identify the microbial population implicated in fermentation of cocoa cultivated in the region of Sud-Comoé (south-east) in order to make preliminary studies of characterization.

Material and methods
Cocoa beans fermentation conditions and sampling
The cocoa pods were harvested at farms from Sud-Comoé, area situated in south-east (geographic coordinates 5°30' 0 North -3°15'West) of Côte d'Ivoire. The spontaneous coca bean fermentation was performed in National Flowers Center of the university Felix Houphouet-Boigny in traditionally conditions by heap fermentation during 6 days. The fermenting mass (100 kg) set on banana leaves and covered with banana leaves were constituted of mixed genotypes (Forastero, Trinitario and Criollo cultivars). At the start of the fermentation (0 h) and after each 12 hours of fermentation, temperatures and pH of inside fermenting cocoa bean were measured directly at 15 cm depth on the fermenting heap (Papalexandratou *et al.,* 2011). Therefore, the fermenting heap was turning and 200 g of beans were collected in Stomacher bag for microbial analyzed.

Plating and enumeration of cocoa fermenting heap flora
Culture dependent approach was performed immediately after sampling. Therefore, 225 ml of 0.1% (wt/vol) buffered peptone water (Oxoid, Basingstoke, United Kingdom) were added to 25 g of pulp and beans in a sterile stomacher bag and vigorously shaken for 2 min to obtain an uniform homogenate. Samples (1.0 mL) of the homogenate were serially diluted 10-fold in 0.1 % trypton salt, from which aliquots (0.1 mL) were plated on different selective agar media. Samples were then incubated for 1 to 4 days in a standard incubator (Jouan, St. Herblain, France) at 30 °C for monitoring, isolation, and enumeration (by recording the number of CFU) of specific groups of microorganisms responsible for fermentation. Yeasts

medium was constituted by Malt Yeast Peptone Glucose agar (3 % yeast extract, 3 % malt extract, 5 % peptone (Himedia), 1 % glucose, 100 mg/L chloramphenicol (Sigma, Paris, France), pH 5.6) while Lactic acid bacteria (LAB) medium was constituted by Man-Rogosa-Sharpe (MRS) agar (Oxoid) supplemented with 50 Pg/mL of nystatin to inhibit fungal growth. Acetic acid bacteria (AAB) were isolated on Duthathai and Pathom-Aree (2007) medium consisted of 0.5 % of D-glucose, 1 % of yeasts extract, 1 % of peptone, 2 % of glycerol, 1.5 % of potato and 4 % of ethanol (v/v)). This medium was supplemented with 0.0016 % of bromocresol green as color indicator and nystatin (50 pg/mL). *Bacillus* medium was constituted by nutrient agar supplemented with 50 Pg/mL of nystatin (Lefeber *et al.,* 2012).

Then, morphologically different colonies were picked up from a suitable dilution of each sample on MYGP, MRS, Duthathai and Pathom-Aree (2007) and nutrient agar media, purified through subculturing and stored at -20°C in the same medium supplemented with 25 % (vol/vol) glycerol.

Screening of yeast and *Bacillus* pectinolytic activity strains

The screening of pectinolytic yeast and *Bacillus* strains was carried out by the method, described by Ouattara *et al.* (2008). The screening basal medium containing 0.28% $NH_4 SO_4$, 0.6 % K_2HPO_4, 0.01 % $MgSO_4$, 0.2 % KH_2PO_4 and 1.7 % agar with pH adjusted to 6 was supplemented with 0.02 % yeast extract and 1% pectin as sole carbon source. After sterilization, 20 mL of medium were cooled in plate and five wells of 0.5 cm in diameter with 2 to 3 mm in depth were made aseptically in the medium. Optical density of 1 at 600 nm was then reached with pure yeast and Bacillus cultures, suspended separately in saline trypton. For each strain, wells were subsequently loaded with 7 μL of the suspension. All the wells of the same plate were inoculated with a single suspension. After incubation at 30 °C during 24 to 48 h, the solid culture medium was flooded with a solution of iodine and potassium iodide (5 g potassium iodide + 1 g iodine + 330 ml distilled water) to reveal clear zones around the wells, indicating pectinolytic activity (Soares *et al.,* 1999).

Fermentative capacity of yeast strains

The fermentative capacity of sugars study was performed according to Wickerham (1951). In this study, D-glucose, D-maltose, D-fructose, D-sucrose, D-lactose and D-galactose were tested and the culture medium was containing 0.5 % yeast extract and 2 % of tested carbohydrate. Briefly, 10 μL of the preculture ($OD_{600} = 0.5$) were used to inoculate 10 mL of sterilized medium contained in 25 mL tube in which a Durham tube was inserted.

Cultures were incubated at 30º C for 48 h to 3 week. The presence of gas in Durham tubes indicates that the isolates ferment the carbohydrate.

Acidification capacity of *Bacillus* strains

Evaluation of acidification capacity of Bacillus isolates was carried out by the method of Andelib and Nuran (2009) using the HS medium (2 % glucose, 0.3 % peptone, 0.5 % Yeast extract, 1.5 % $CaCO_3$, 1.2 % agar) supplemented with 0.0016 % bromocresol green as color indicator. A volume of 4 mL of the HS medium contained in 10 mL tubes was seeded with strains and incubated at 30 °C during 48 hours. Acid production was monitored by formation of yellow area in the tube with or no gas production.

Citrate metabolism capacity of *Bacillus* and LAB strains

The citrate metabolism capacity was evaluated on Kempler and McKay medium containing 1% milk powder, 0.25 % casein peptone, 0.5 % glucose and 1.5 % agar. After sterilization, the medium was supplemented with 1 mL K_3FeCN (10 %) solution and 1 mL of solution containing iron citrate (2.5 %) and sodium citrate (2.5 %) (Kempler and McKay, 1980). Then, the culture medium was spot inoculated with pure 24 h pre-culture of bacterial strain and incubated at 30 °C for 24 hours for *Bacillus* and 48-72 h for LAB. The citrate metabolism was monitored by the presence of blue colonies on the plate after incubation.

Acidification capacity and fermentative type of LAB

Fermentative type and acidification capacity of bacterial strains were evaluated as described previously by Dicks and Van Vuuren (1987) modified by MRS medium containing the appropriate carbohydrate at 2 % as sole carbon source, 1.7 % agar and supplemented with 0.005 % of bromocresol purple. Each strain was cultivated in 7 mL of MRS agar by central sting in the medium and then incubated at 30 °C for 72 hours in anaerobic conditions. The carbohydrates tested were glucose, fructose and sucrose that are known to be the sugar contained in the cocoa pulp (Afoakwa *et al.,* 2013). A negative control was prepared in the same conditions and not inoculated with the microbial culture. Acidification capacity was monitored by the change of medium color due to pH lowering, comparatively to the negative control. The fermentative type was determined by ability of strains to produce gas from carbon source. Indeed, the presence of gas at the bottom of the tube accompanied by yellow zone indicates heterofermentative LAB type.

Screening of acetic acid bacteria by evaluation of acidification capacity

Acidification capacity was evaluated in solid medium as previously described by Andelib and Nuran (2009). Briefly, Hestrin-Schramm (HS) agar supplemented with 0.0016 % bromocresol green, was spot inoculated with pure 24 h pre-culture of bacterial strain and incubated at 30 °C for 10 days. Acid production in solid medium was monitored by formation of yellow zone around the spot. Acidification capacity of strains was evaluated each day by measuring of the yellow zone diameter.

Overoxidation capacity

The test of overoxidation was performed in HS broth medium containing 0.05 % glucose and 1 % acetic acid or lactic acid as carbon source. Addition of acetic acid or lactic acid to the HS broth provokes the change of the green medium color into yellow (Lisdiyanti *et al.,* 2000). One fresh colony of acetic acid bacteria was inoculated in 3 mL of HS broth and then incubated at 30 °C for 48 hours in aerobic atmosphere. Over oxidation of acetic acid or lactic acid into CO_2 and H_2O was assessed by the change of medium color back to green, due to pH rising.

Results and discussion
Fermentation conditions and microbial growth

Figure 1 shows the changes in inside temperature and pH values of cocoa heap fermentation. The initial temperature was approximately 27 °C and increased to reach 41 °C after 36 h followed by a slight decrease to a value of 31 °C after 144 h of fermentation.

Concerning the pH, the value that was 4.2 at the beginning of the fermentation process decreased to approximately pH 3.6 after 24 h. After this, the pH value increased rapidly and reached to a maximum pH value of pH 7.3 after 144 h of fermentation (Fig. 1).

The same evolution of temperature and pH was reported in other studies (Jespersen *et al.,* 2005; Nielsen, 2007; Papalexandratou and De Vuyst, 2011; Guehi *et al.,* 2010). The increase in temperature during fermentation is due to the growth of yeasts which convert sugars into ethanol and the growth of acetic acid bacteria which oxidize ethanol into acetic acid (Camu *et al.,* 2007). The decrease of temperature after 36 h is due to the decrease of acetic acid and yeasts population. Moreover, optimal temperature observed in this work was weak than others reported by many studies in which the temperature reached 50 °C after 48 -72 hours (Shawn and Wheal, 2004). This could impact the quality of fermenting Sud-Comoé cocoa seeing that high increase of temperature is essential for death of the seed embryo and initiation of biochemical changes in the beans, leading to the formation of precursor molecules for the development of a characteristic flavour and

colour of the beans (Hansen *et al.,* 1998, Thompson *et al.,* 2001). The increase of inside cocoa heap pH after 24 h was due to the decrease of pulp citric acid concentration in cocoa pulp which was metabolized by LAB and yeasts strains (Thompson *et al.,* 2001). As previously observed by Yao *et al.* (2014) and Guehi *et al.* (2010), the pH value at the end of fermentation in this study is also high than those reported in others country (Schwan, 1998; Schwan and Wheals, 2004; Camu *et al.,* 2007) confirming the particularity of Côte d'Ivoire cocoa fermentation suggested by Yao *et al.* (2014).
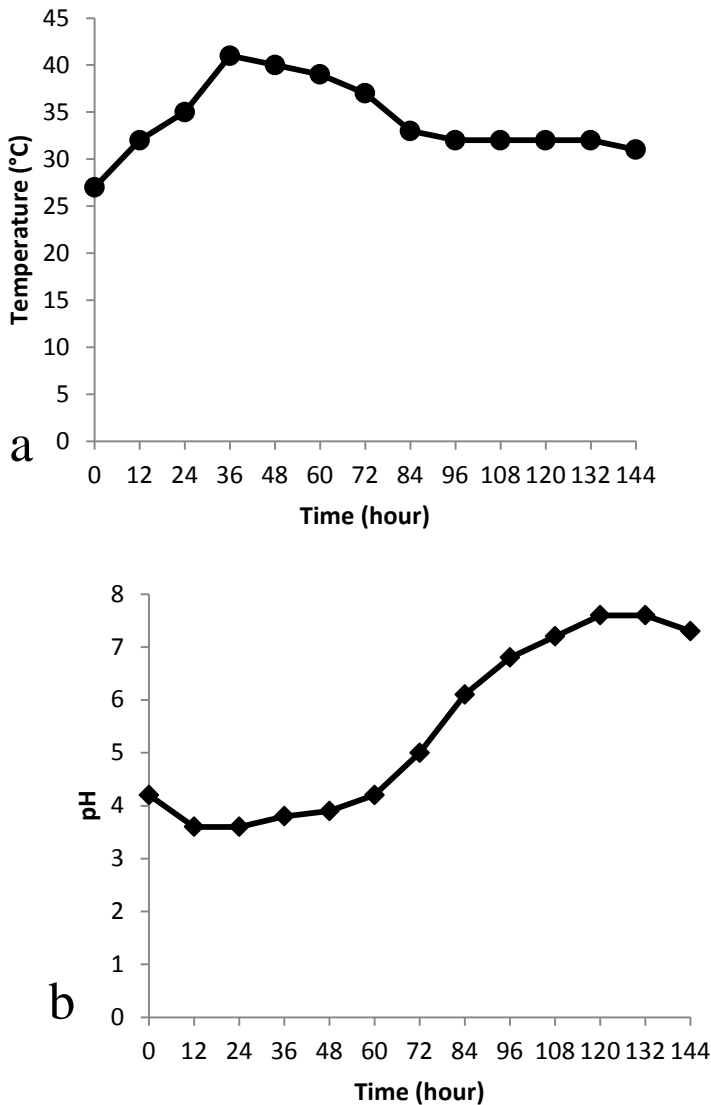

Figure 1: Evolution of temperature (a) and pH (b) during Sud-Comoé cocoa fermentation

The numeration of microflora on selective agar media revealed the succession of yeasts, LAB, AAB and *Bacillus* during Sud-Comoé cocoa beans fermentation as expected (Fig. 2). Yeasts, LAB, AAB and Bacillus are present at the beginning of the process while AAB strains emerged after 12 hours of fermentation and reached a peak at 72 hours with bacterial load of 7.67 Log_{10} UFC/g of cocoa beans. After 84 hours, a decrease was observed. Yeasts population estimated to 6.3 Log_{10} UFC/g increased rapidly to a maximum at 7.23 Log_{10} UFC/g at 36 hours. After this, yeasts population decreased to a microbial load at 1.38 Log_{10} after 84 hours (Fig. 2). LAB counts reached their peak (6.9 Log_{10} UFC/g of cocoa beans) after 24 hours of fermentation and decreased rapidly until the end of the process with bacterial load estimated to 2.54 Log_{10} UFC/g of cocoa beans. Our results are not similar to those observed in Sud-Comoé (Côte d'Ivoire) cocoa fermentation in which yeasts, LAB and AAB reached a peak at 24 h for yeast and LAB and at 72 h for AAB with microbial load estimated to 4.24, 7.22 and 7.11 Log_{10} UFC/g (of cocoa beans) respectively (Yao *et al.,* 2014, Ouattara *et al.,* 2014, Samagaci *et al.,* 2014). These differences in results could suggest different cocoa qualities from area to area in Côte d'Ivoire.

Concerning the *Bacillus*, the bacterial load estimated to 5.89 Log_{10} UFC/g at the beginning increased progressively to reach a peak (8.1 Log_{10} UFC/g) at the end of the process, contrary to other microorganisms (Fig. 2).

The microbial succession observed in our study is consistent with those reported by other authors (Shwan and Wheals, 2004; Kresnowati *et al.,* 2013; Papalexandratou *et al.,* 2011) and is due to the composition of cocoa pulp, according to other studies. Indeed, the initial acidity of the pulp (pH 3.6), due to citric acid, together with low oxygen levels, favor colonization by yeasts that are able to utilize pulp carbohydrates under both aerobic and anaerobic conditions (Passos *et al.,* 1984). Additionally, as ethanol amount and aeration increased, the fermenting mass becomes favorable for growth of acetic acid bacteria. On the other hand, the decrease in AAB, yeasts and LAB populations is probably due to their inhibition by the high temperature in the cocoa mass and a high concentration of organic acids has been synthesized (Lagunes-Galvez *et al.,* 2007; Camu *et al.,* 2012).

The increase in the *Bacillus* population is due to the increase in aeration, pH value of cocoa pulp and a rise in temperature to about 40 °C in the cocoa mass during the later step of fermentation (Shwan and Weals, 2004). Indeed, many *Bacillus* spp. are thermotolerant and others grow well at elevated temperatures. This evolution of *Bacillus* population is consistent with those reported in other studies (Shwan and Wheal, 2004; Schwan *et al.,* 1995; Schwan *et al.,* 1990). The predominance of *Bacillus* during fermentation suggests that these microorganisms were important in this process to obtain quality cocoa.
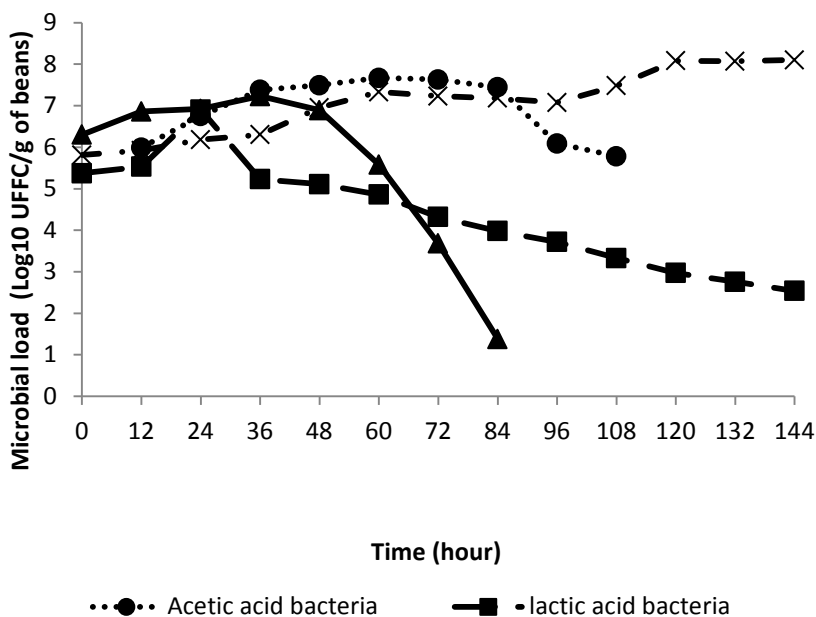
Figure 2: Dynamic of microbial flora during Sud-Comoé cocoa fermentation

Screening of pectinolytic activity strains

      Two hundred and thirty two (232) yeast strains were isolated during Sud-Comoé cocoa beans fermentation and were tested for pectinolytic activity. Among these isolates, 59 (25.43 %) showed pectinolytic enzymes production capacity as revealed by clear zone around the well with halo diameters comprised between 1 and 3 cm. Moreover, out of the 59 strains, 33 (51.56 %) showed the most important enzymes production capacity, 22 produced middle enzymes amount and 9 showed low enzymes production capacity with halo diameter ranged from 2-3 cm, 1.5-1.9 cm and 1-1.4 cm, respectively. The capacity of yeast strains to produce pectinolytic enzymes is according to the previously studies (Samagaci *et al.,* 2004; Shwan and Wheal, 2004; Ho *et al.,* 2014). However, the proportion of pectinolytic yeasts isolated from Sud-Comoé cocoa fermentation is relatively low even if these isolates are mainly high producer.

      Concerning Bacillus strains, 144 isolates were tested for their capacity to produce pectinolytic enzymes. Thus, 71 (62.22 %) were able to produce pectinolytic enzymes with halo diameters ranging from 1 to 3.4 cm. However, only 4 (6.75 %) among the producers showed a most important enzymes production capacity while 28 (37.84 %) were middle capacity and high proportion (55.4 %) showed a low capacity. It is well known that production of pectinolytic enzymes is one of the key factors for cocoa fermentation process (Schawn, 1998; Schawn and Wheals, 2004, Ouattara *et al.,* 2011). Indeed, pectinolytic enzymes activity allowed increase of aeration

and therefore emergency of the bacterial strains such as AAB implicated in development of characteristics chocolate aroma (Schawn and Wheals, 2004; Ho *et al.,* 2014).

At day, yeasts are better known to their capacity for pectinolytic enzymes production during cocoa fermentation contrary to *Bacillus* (Shwan and Wheal, 2004). Yet, Ouattara *et al.* (2008) have demonstrated that *Bacillus* is able to produce mainly varieties of pectinolytic enzymes contrary to yeast strains. In all case, this diversity of pectinolytic activity strains contributed probably to the quality of Sud-Comoé cocoa.

Metabolism capacity of pectinolytic activity yeast strains

Among pectinolytic yeasts isolates, 25 strains were tested to evaluate their fermentative capacity from 6 sugars (D-glucose, D-fructose, D-saccharose, D-maltose, D-lactose and D-galactose). The fermentative capacity of each strain was revealed by the presence of gas in Durham tube after incubation. The results showed that all strains tested produced gas from glucose and fructose (Table I). Additionally, 13 strains were able to ferment sucrose. Moreover, the results showed 4 groups according to the number of fermenting sugars (Table I). It well known that, yeasts produced ethanol and gas from sugars (Shwan and Wheal, 2004). Therefore, these results suggested probably a diversity of yeast strains with pectinolytic enzymes and ethanol production capacity isolated from Sud-Comoé fermentating cocoa beans. So, this diversity could be beneficial for cocoa quality.

Table 1: Fermentative capacity of pectinolytic activity yeast strains

Groups	Isolates	Glucose	Fructose	Saccharose	Maltose	Lactose	Galactose
I	T1AB25	+	+	+	+	+	+
	T0AB5	+	+	+	+	+	+
	T1AB17	+	+	+	+	+	+
	T1AB18	+	+	+	+	+	+
	T0AB18	+	+	+	+	-	+
II	T1AB24	+	+	+	+	-	+
	T1AB16	+	+	+	+	-	+
	T1AB34	+	+	+	-	+	+
	T0AB7	+	+	+	-	+	+
III	T4AB10	+	+	-	-	-	+
	T5AB10	+	+	-	-	-	+
	T7AB15	+	+	-	-	-	-
IV	T7AB16	+	+	-	-	-	-
	T0AB4	+	+	-	-	-	-
	T1AB38	+	+	-	-	-	-

+ : gas production ; - absence of gas in Durham tube

Bacillus sp isolates technological properties

All of 112 *Bacillus* strains were analyzed for their capacity to catabolism the citrate and to produce acid from glucose metabolism. The results show that 98 (87.5 %) of them were able to produce acid from glucose with or no gas. This results were similar to that observed by Nakamo *et al.* (1997) and Combet-Blanc *et al.* (1999) who reported that *Bacillus* are able to produce acetic and lactic acids. In view of importance of these acids mainly acetic acid in chocolate quality, the presence of *Bacillus* strains with acids production capacity could improved the cocoa quality of Sud-Comoé area even if type and amount of acids produced by each strains will be evaluated.

Moreover, 38 (33.7 %) of the tested strains were able to catabolize citrate because of the presence of blue colonies on KMK agar medium. Our results are consistent with those reported by Willecke and Pardee (1970) who showed capacity of some *Bacillus* strains to metabolize citrate by production of citrate lyase enzyme. These results suggesting that *Bacillus* strains contributed also to promote the growth of bacterial population is essential for obtaining of good fermenting cocoa and chocolate thereof.

LAB metabolism capacity

A total of 144 LAB strains characterized as Gram positive, oxydase negative, catalase negative and cocci or rods shaped were selected from MRS agar plates during the cocoa beans fermentation process. All of these isolates were tested for their ability to produce gas from glucose metabolism in order to identify homofermentative and heterofermentative strains. The results show that a high proportion (85.96 %) among these isolates were homofermentative because of absence of gas in the tube after incubation while only 16 (14.03 %) strains were heterofermentativ. The predominance of homofermentative LAB strains were also reported by other studies (Kostinek *et al.,* 2008; Ouattara *et al.,* 2014). It is well known that homofermentative LAB strains convert sugars almost exclusively into lactic acid while heterofermentative strains are able to produce lactic and acetic acids (Raimbiault, 1995). It is also known that lactic acid is less volatile and confer to fermented and dried beans a final acidity that deteriorate their quality (Schwan and Wheals, 2004) on contrary to acetic acid which is desirable for development of precursors chocolate aroma (Biehl *et al.,* 1993; Jinap, 1994). Therefore, heterofermentative strains producing other acids more volatile such as acetic acid, may be more interesting and desirable, but this type of strain remains in minor proportions in spontaneous Sud-Comoé cocoa fermentation as also showed by our results.

Moreover, all the 114 strains were able to metabolize glucose and fructose regarding the change of medium color due to pH lowering

comparatively to the control. It was also observed that, 113 (99.12 %) strains were able to ferment sucrose (Table 2) and 94 (82.46 %) showed citrate metabolizing capacity on KMK medium due to the presence of blue colonies on the plates. Citrate was reported to be a significant component of the pulp. It may compose 1-3% of the pulp composition on wet basis and its presence is normally associated with the acidity of the pulp with fermenting pH value around 3.9 (Shwan and Wheal, 2004), not favorable for growing of bacterial population such as AAB. So, elevation of the pH value through catabolizing of citrate is necessary for success of the fermentation process and this diversity of strains with citrate metabolism capacity observed in this study can contribute probably to emergency of other bacteria implicated in this process.

Table 2: Metabolism capacity of LAB strains

Sugars	Number (%) of LAB metabolizing sugar
Glucose	114 (100 %)
Fructose	114 (100 %)
Saccharose	113 (99,12 %)
Citrate	94 (82,46 %)

Characterization of AAB isolates

Acetic acid bacteria (AAB) strains were identified as Gram negative; short rod-shaped, catalase positive, oxidase negative and obligatory aerobic. These bacteria formed colonies surrounded by characteristic yellow area on isolation medium. During Sud-Comoé cocoa beans fermentation, a total of 107 AAB strains were isolated. Among these strains, 95 (88.78 %) were able to further oxidise acetic acid and lactic acid into CO_2 and H_2O and subsequently classified as *Acetobacter* while 12 (11.21 %) strains were not able to this over oxydation; these strains belong therefore to *Gluconobacter* genus.

All the 107 strains were then tested for the acidification capacity and showed naturally acidification capacity but with different levels of acid production. Indeed, among the tested isolates, 14 strains showed the most important acidification capacity with clear halo diameter ranging from 1.7 to 3.1 cm. Moreover, 56 strains produced middle acidity with halo diameter in the range 1.1-1.7 cm and 37 isolates showed weak acidification capacity.

As it is well known that microbial strains with acidification capacity are desirable for the production of cocoa beans and chocolate of quality, therefore, the acidification capacity, mainly acetic acid production, is an interesting technological property which contribute efficiently to the final cocoa quality (Schawn and Wheal, 2004; Romero-Cortes *et al.,* 2012).

Conclusion

This study demonstrates a microbial flora succession including yeasts, LAB, AAB and *Bacillus* sp. during Sud-Comoé cocoa fermentation. These data indicate also that majority of the LAB and AAB isolates were homofermentative and *Acetobacter* genera respectively. Moreover, *Bacillus* genera are presents through fermentation and have some interesting technological properties suggesting importance of these bacteria in this process. So, their using as starter to improve the cocoa fermentation process could be considered.

References:
Afoakwa, E. O, Kongor, J. E., Takrama, J. F. and Budu, A. S. Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans. International Food Research Journal, 20(3): 1215-1222, 2013.
Andelib, A. Y. and Nuran, D. A. Isolation of cellulose producing bacteria from wastes of vinegar fermentation. In: Proceedings of the World Congress on Engineering and Computer Science Vol I, San Francisco, USA. pp. 978-988, 2009.
Ardhana, M., and Fleet, G. The microbial ecology of cocoa bean fermentations in Indonesia. International Journal of Food Microbiology, 86:87–99, 2003.
Biehl, B., J. Voigt, H. Heinrichs, V. Senjuk and Bytof, G. pH-dependent enzymatic formation of oligopeptides and amino acids, the aroma precursors in raw cocoa beans. In: Lafforest, J. (Ed.), Proceedings in XIth International Cocoa Research Conference. Cocoa Producers' Alliance, Yamassoukro, Ivory Coast, pp. 717-722, 1993.
Camu, N., T. De Winter, K. Verbrugghe, I. Cleenwerck, P. Vandamme, J. S. Takrama, M. Vancanneyt and L. De Vuyst. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Applied and environmental microbiology, vol. **73**, pp. 1809–1824, 2007.
Camu N, DeWinter T., Addo S. K., Takrama J. S, Bernaert H., De Vuyst L. Fermentation cocoa beans: influence of microbial and polyphenol concentrations on the flavour of chocolate. Journal of Science of Food and Agriculture 88: 2288-2297, 2008.
Combet-Blanc Y., Dieng M. C., Kergoat P. Y. Effect of organic complex compounds on Bacillus thermoamylovorans growth and glucose fermentation. Applied and environmental microbiology, 65 (10): 4582-4585, 1999.
Dicks L. M. and Van Vuuren H. J. J. Microbiology Methods, 6: 273-275, 1987.

Duthathai, F. and W. Pathom-Aree. Application of chemical dyes as colour indicator for selective isolation of acetic acid bacteria. Research Journal of Microbiology, 2: 885-888, 2007.

Guehi, S.T., S. Dabone, L. Ban-Koffi, D.K. Kra, and G.I. Zahouli. Effect of turning beans and fermentation method on the acidity and physical quality of raw cocoa beans. Advance Journal of Food Science and Technology, 2:163-171, 2010.

Hamdouche Y, Guehi T, Durand N, Kedjebo K. B. D., Montet D., Meile J-C. Dynamics of microbial ecology during cocoa fermentation and drying: Towards the identification of molecular markers. Food Control, 48: 117-122, 2015.

Hansen, C. E., del Olmo M. and Burri C. Enzyme activities in cocoa beans during fermentation. Journal of the Science of Food and Agriculture, 77:273–281, 1998.

Hashim, P., Selamat J., Muhammad S. K. S. and A. Ali. Effect of mass and turning time on free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. Journal of the Science of Food and Agriculture, 78:543–550, 1998.

Hii, C. L., Law C. L., Cloke M., Suzannah S. Thin layer drying kinetics of cocoa and dried product quality. Biosystems Engineering, 102, 153–161, 2009.

Ho V.T.T., Zhao J., Fleet G. Yeasts are essential for cocoa bean fermentation. International Journal of Food Microbiology. 174 72–87, 2014.

Jespersen L., Nielsen D. S., Hønholt S., Jakobsen M. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast Research 5: 441-453, 2005.

Jinap S. Organic acids in cocoa beans – a review. ASEAN Food J. 9: 3–12, 1994.

Kempler G. M., McKay L. L. Improved medium for detection of citrate-fermenting Streptococcus lactis subsp. diacetylactis. Applied and Environmental Microbiology, 39:926–927, 1980.

Kostinek M., Ban-Koffi L., Ottah-Atikpo M., Teniola D., Schillinger U., Holzapfel W. H., Franz C. M. A. P. Diversity of Predominant Lactic Acid Bacteria Associated with Cocoa Fermentation in Nigeria. Current Microbiology , 56:306–314, 2008.

Kresnowati M. T. A. P., Suryani L., and Affifah M. Improvement of Cocoa Beans Fermentation by LAB Starter Addition. Journal of Medical and Bioengineering, Vol. 2, No. 4. 2013.

Lagunes-Galvez, S., G. Loiseau J. L. Paredes M. Barel and J.-P. Guiraud. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. International Journal of Food Microbiology, 114:124–130, 2007.

Lefeber, T., W. Gobert, G. Vrancken, N. Camu and L. De Vuyst. Dynamics and species diversity of communities of lactic acid bacteria and acetic acid bacteria during spontaneous cocoa bean fermentation in vessels. Food Microbiology. 28, 457–464, 2011.

Lefeber, T., Z. Papalexandratou, W. Gobert, N. Camu and L. De Vuyst. 2012. On-farm implementation of starter culture for improved cocoa bean fermentation and its influence on the flavor of chocolate produced thereof. Food Microbiology. 30:379-392, 2012.

Lima, L. J. R., M. H. Almeida, M. J. R. Nout and M. H. Zwietering. Theobroma cacao L., "the food of the gods": quality determinants of commercial cocoa beans, with particular reference to the impact of the fermentation. Critical Reviews in Food Science and Nutrition, 52:731–761, 2011.

Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata. Systematic study of the genus *Acetobacter* with descriptions of *Acetobacter indonesiensis* sp. nov., *Acetobacter tropicalis* sp. nov., *Acetobacter orleanensis* (Henneberg 1906) comb. nov., *Acetobacter lovaniensis* (Frateur 1950) comb. nov., and *Acetobacter estunensis* (Carr 1958) comb. nov. Journal of General and Applied Microbiology, 46:147–165, 2000.

Nakano M. M., Dailly Y. P., Zuber P., Clark D. P. Characterization of anaerobic fermentative growth of Bacillus subtilis: identification of fermentation end products and genes required for growth. Journal of Bacteriology, p; 6749-6755, 1997.

Nielsen, D. S. The microbiology of Ghanaian cocoa fermentations. Thesis, Department of Food Science, the Royal Veterinary and Agricultural University Denmark, 111 p. 2006.

Nielsen, D. S., O. D. Teniola, L. Ban-Koffi, M. Owusu, T. Andersson and W.H. Holzapfel. The microbiology of Ghanaian cocoa fermentations analysed using culture dependent and culture independent methods. International Journal of Food Microbiology. 114, 168–186, 2007.

Nielsen, D. S., S. HØnholt, T. Debrah and L. Jespersen. Yeasts populations associated with Ghanaian cocoa fermentations analyzed using denaturing gradient gel electrophoresis (DGGE). Yeast, 22:271-284, 2005.

Ouattara D. H., H. G. Ouattara, B. G. Goualié, L. M. Kouame and S. L. Niamke. Biochemical and functional properties of lactic acid bacteria isolated from Ivorian cocoa fermenting beans. Journal of Applied Biosciences, 77:6489– 6499, 2014.

Ouattara, H. G., S. Reverchon, S. L. Niamke and W. Nasser. Molecular identification and pectate lyase production by Bacillus strains involved in cocoa fermentation. Food Microbiology. 28:1-8, 2011.

Ouattara, H.G., L. Ban-Koffi, G.T. Karou, A. Sangare, S.L. Niamke, and J.K. Diopoh. Implication of Bacillus sp. in the production of pectinolytic enzymes during cocoa fermentation. World Journal of Microbiology and Biotechnology. 24: 1753-1760, 2008.

Papalexandratou, Z., G. Vranckena, K. De Bruyne, P. Vandamme and L. De Vuyst. Spontaneous organic cocoa bean box fermentations in Brazil are characterized by a restricted species diversity of lactic acid bacteria and acetic acid bacteria. Food Microbiology 28:1326–1338, 2011.

Papalexandratou, Z., De Vuyst, L. Assessment of the yeast species diversity of cocoa bean fermentations in different cocoa-producing regions using denaturing gradient gel electrophoresis. FEMS Yeast Research. 11, 564e574, 2011.

Passos, F. M. L., D. O. Silva, A. Lopez, C. L. L. F. Ferreira, and W. V. Guimara˜es. Characterization and distribution of lactic acid bacteria from traditional cocoa bean fermentations in Bahia. Journal of Food Science, 49:205–208, 1984.

Pereira, G. V. M., M. G. C. P. Migue, C. L. Ramos and R. F. Schwan. Microbiological and Physicochemical characterization of small scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. Applied and Environmental Microbiology, 78:5395-5405, 2012.

Quesnel, V. C. Agents inducing the death of the cacao seeds during fermentation. Journal of the Science of Food and Agriculture, 16:441–447, 1965.

Raimbiault M. Importance des bactéries lactiques dans les fermentations du manioc. In transformation Alimentaire du Manioc. T. Agbor Egbe, A. Brauman, D. Griffon, S. Trèche (éd ORSTOM), 1995.

Romero-Cortes, T., V. Robles-Olvera, G. Rodriguez-Jimenes and M. Ramírez-Lepe. Isolation and characterization of acetic acid bacteria in cocoa fermentation. African Journal of Microbiology Research, 6. (2). 339-347, 2012.

Samagaci L., H. G. Ouattara, B. G. Goualié and S. L. Niamke. Growth capacity of yeasts potential starter strains under cocoa fermentation stress conditions in Ivory Coast. Emirates Journal of Food and Agriculture. 26 (10): 861-870, 2014.

Schwan, R. F. Cocoa fermentation conducted with a defined microbial cocktail inoculum. Applied and Environmental Microbiology, 64:1477-1483, 1998.

Schwan, R. F., Lopez, A., Silva, D. O., and Vanetti, M. C. D. Influência da frequência e intervalos de revolvimentos sobre a fermenta¸cao de cacau e qualidade do chocolate. *Rev. Agrotr´op.*, **2**:22–31, 1990.

Schwan R. F. and A. E. Wheals. The microbiology of cocoa fermentation and its role in chocolate quality. Critical Reviews in Food Science and Nutrition, vol. 44, pp. 205-220, 2004.

Schwan, R. F., A. H. Rose, and R. G. Board. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. Journal of Applied Bacteriology-Symposium. Suppl. 79:96S–107S, 1995.

Soares, M. M. C. N., R. Da Silva and E. Gomes. Screening of bacterial strain for pectinolytic activity: characterization of the polygalacturonase produced by *Bacillus* sp. Revista Microbiology 30:229-303, 1999.

Thompson S. S., K. B. Miller and A. S. Lopez. "Cocoa and coffee" in *Food microbiology: Fundamentals and frontiers*, 2nd ed, M. P. Doyle, L. R. Beuchat, and T. J. Montville, Ed. Washington, American Society Microbiology Press, 2001, pp. 721-733, 2001.

Wickerham L.J. Taxonomy of yeasts. VS. Dept. Agric washinton. D. C. Tech. Bull., 1029, 1-56, 1951.

Willecke K. and Pardee A. Inducible transport of citrate a Gram positive bacterium *Bacillus subtilis.* Journal of biological chemistry, vol. 246 (4) p. 1032-1040, 1970.

Yao, W., H.G. Ouattara, B. Goualie, S. Soumahoro and S. Niamke. Analysis of some functional properties of acetic acid bacteria involved in Ivorian cocoa fermentation. Journal of Applied Biosciences. 75:6282–6290, 2014.