Diversity of Yeasts Involved in Cocoa Fermentation of Six Major Cocoa-Producing Regions in Ivory Coast

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Abstract

Cocoa beans (*Theobroma cacao* L.) are the raw material for chocolate production. Fermentation of cocoa pulp is crucial for developing chocolate flavor precursors. This fermentation is led by a succession of complex microbial communities where yeasts play key roles during the first stages of the process. In this study, we identified and analyzed the growth dynamics of yeasts involved in cocoa bean fermentation of six major cocoa-producing regions in Ivory Coast. A total of 743 yeasts were isolated, and were identified by sequencing of D1/D2 regions of 26S rDNA gene. These isolates included 11 species with a predominance of *Pichia kudriavzevii* (44,81 %), *Pichia kluyveri* (20,99 %) and *Saccharomyces cerevisiae* (18,97 %) respectively. In addition, the length polymorphism of the genetic marker ITS1-5.8S-ITS2 and PCR-RFLP analysis revealed an intraspecific diversity within the three-main species involved in cocoa fermentation of six major local regions in Ivory Coast. This intraspecific diversity could be exploited for selecting appropriate starter cultures.

Keywords: Cocoa, fermentation, yeast, diversity, PCR-RFLP

Introduction

Cocoa fermentation is a crucial step of post-harvest processing of cocoa. It's a spontaneous process in while the different flavor and aroma precursors of cocoa beans desired in chocolate industry are produced (Schwan & Wheals, The microbiology of cocoa fermentation and its role in chocolate quality, 2004. De Vuyst, Lefeber, Papalexandratou, & Camu, 2010). Cocoa fermentation efficiency requires the activity of successive indigenous microorganisms' consortia, including yeasts, lactic acid and

acetic acid bacteria, and *Bacillus* bacteria (Ouattara, et al., 2008. Ho V. , Zhao, Srzednicki, & Fleet, 2013). Among these microorganisms, yeasts are essentials because considered as initiators of this process (Soumahoro, et al., 2015. Ho, Zhao, & Fleet, 2014). Indeed, without yeasts in cocoa fermentation, the chocolate obtained after manufacturing is without savour nor flavor and depreciated by (Ho, Zhao, & Fleet, 2014). Yeasts involving in cocoa fermentation play crucial roles in the cocoa pulp fermentation process, including the production of ethanol and organic acids, which are believed to arrest germination of the cocoa seeds and contribute to essential chemical conversions inside the cocoa beans (Hansen, Del Olmo, & C., 1998) In fact, most yeasts initiate an alcoholic fermentation of the pulp sugars, generating ethanol which, along with acetic acid, enters the bean to kill the embryo and trigger endogenous biochemical reactions that produce the chocolate flavor precursors (De Vuyst, Lefeber, Papalexandratou, & Camu, 2010). Apart from alcoholic fermentation, some yeasts such *Pichia kudriavzevii* and *Saccharomyces cerevisiae* are able to produce volatile aroma compounds that contribute as precursors of chocolate flavor (Verstrepen, et al., 2003. Kostinek, et al., 2008). Others yeasts mainly *Pichia kluyveri* and *Kluyveromyces marxianus* produce pectinolytic enzymes which are believed to play a central role in the degradation of the viscous pectin-rich pulp (Schwan, Rose, & Board, 1995. Lopez & Dimick, 1995). The action of yeast pectinolytic enzyme is essential for the growth of other microorganisms involving in cocoa fermentation (Schwan, Rose, & Board, 1995).

This importance of yeasts in cocoa fermentation explain why these microorganisms were mostly studied in order to assess their diversity and identify different key species which could be exploited for selecting appropriate starter cultures in cocoa fermentation.

Thus, in Ghana (near New Tafo and Old Tafo), *Saccharomyces cerevisiae, Pichia kudriavzevii*, and *Hanseniaspora opuntiae* were founded as dominant yeast species (Daniel, Vrancken, Takrama, Camu, & De Vuyst, 2009), while *Candida tropicalis* was yeast species dominant in Indonesia (precisely in East Java)**,** (Ardhana & Fleet, 2003). At San Francisco de Macoris, in Dominican Republic, (Lagunes-Galvez, Loiseau, Paredes, Barel, & Guiraud, 2007) reported that *Candida inconspicua* was the most abundant and persistent yeast during cacao fermentation. From Itajuípe, Bahia State in Brazil, *S. cerevisiae* and *Hanseniaspora sp.* were dominant yeasts (Pereira, Miguel, Ramos, & Schwan, 2012) while *P. kudriavzevii*, *S. cerevisiae*, *Saccharomycopsis crataegensis* and *Hanseniaspora guilliermondii* were the predominant yeasts isolated from fermented cocoa bean mass in the Mexican state of Tabasco (Arana-Sánchez, et al., 2015)**.** All researches indicate that yeast populations involved in cocoa fermentations vary from country to country, from local region to local region, around the world **(**(Nielsen,

Hønholt, Tano-Debrah, & Jespersen, 2005. Daniel, Vrancken, Takrama, Camu, & De Vuyst, 2009. Romero-Cortes, Robles-Olvera, Rodriguez-Jimenes, & Ramírez-Lepe, 2012). This variability of yeast species from area to area could be impact the final cocoa beans quality in fine chocolate. Indeed, the spontaneous nature of cocoa bean fermentations may be the source of variable cocoa beans broad quality (Maura, et al., 2016). This situation causes huge economic losses for cocoa-producing countries estimated among 200 billion FCFA/year in Ivory Coast (ANADER, 2014). That why, many investigations suggest the use of microbial starter culture to improve fermentation process. In this context, the yeast species as *Pichia kudriavzevii, Saccharomyces cerevisiae, Pichia klyuveri* and Candida sp., were used as starter to control cocoa fermentation (Crafack, et al., 2013. Mahazar, et al., 2015. Meersman, et al., 2016. Pereira, et al., 2017). These different species improved cocoa fermentation process and the chocolate quality, but results obtained are still insufficient for cocoa fermentation process standardization. Therefore, the research to select appropriate yeast starter must be continuous and a perfect knowledge of the diversity of yeast strains involved in cocoa fermentation is necessary to reach this goal. Thus, considering the variability of yeasts involving in cocoa fermentation of country to country and local region to local region within the same country, investigations must be carry out in the different cocoa-producing/exporting areas with a view to a perfect knowledge fermented cocoa yeasts.

From Ivory Coast, first cocoa producer/exporter, on 17 cocoa-producing regions, only yeast involving in cocoa fermentation of two local regions, including Me and Agneby-Tiassa was studied in order to assess molecular diversity and to select appropriate yeast starter (Koné, et al., 2016. Samagaci, Ouattara, Niamké, & Lemaire, 2016). None information exists concerning yeast species involving in cocoa fermentation from the other local regions of Ivory Coast.

The aim of this study is to assess molecular diversity within yeast populations involved in cocoa fermentation from six major local regions of Ivory Coast in order to investigate the keys species for selecting appropriate yeasts starters for Ivorian cocoa fermentation.

Materials and Methods

1. Fermentations, isolations and growth conditions of yeasts

The cocoa pods were harvested in October 2015 from six (6) major cocoa-producing regions of Ivory Coast: Gôh (6° 07’ 60’’ N, 5° 55’ 60’’ W), San Pedro (4° 44’ 41’’ N, 6° 38’ 23’’ W), Gbôklè (4° 57’ 04’’ N, 6° 05’ 19 W), Haut-Sassandra (6° 53’ N, 6° 27’ W), Cavally (6° 32’ 37’’ N, 7° 29’ 37’’ W), Tonkpi (7° 24’ N, 7° 33’ W). One hundred (100) Kg of cocoa beans, constituted of mixed genotypes (Forastero, Trinitario, Criollo), were

fermenting using traditional heap method with banana leaves, during 6 days. One hundred (100) g samples were regularly taken each 12 h to fermentation time. The numeration and isolation of yeasts were carried out using the decimal dilution method (Pereira, Miguel, Ramos, & Schwan, 2012). After approximate decimal dilution in sterile saline solution, the fresh fermented samples were plated onto MYGP agar (3 g/L yeast extract; 3 g/L malt extract, 5 g/L bactopeptone; glucose 10 g/L and agar 15 g/L) containing 100 mg/L chloramphenicol (Sigma). Yeasts strains were identified morphologically after 3 days incubation at 30 °C, and then maintained in glycerol (20 %) at −20 °C for further studies.

2. Physicochemical analysis

2.1. Temperature and pH determination

The temperature was recorded directly on mass fermenting with a precision thermoter (*ALLA, France*). The pH determination of cocoa beans followed the protocol of Senanayake, Jansz, & Buckle, (1997) For pulp pH, 10 g cocoa beans were shaken in 100 mL of MiliQ water for 15 min. The beans were separated by decanting and the pH of the supernatant was measured using a digital pH meter (*EUTECH, Germany*).

2.2. Titratable acidity determination

Titratable acidity was determined according to the method AOAC, (1990). Cocoa beans were mixed in MilliQ water and filtrate. Titration was carried out by pouring drop by drop a solution of NaOH (0.1. N) (V_1) in 10 mL of filtrate in the presence of phenolphthalein (3 drops). Titratable acidity was expressed milliequivalents (meq) for 1 g of fresh matter by the following relation:

Titrable acidity (meq/g) = (N xV_1 x10^2) / (m xV_0)

V_0: volume (mL) of the taken filtrate, V_1: volume (mL) of poured NaOH, N: normality of NaOH, m: mass sample.

2.3. Reducing sugars determination

The quantity of reducing sugars was determined by using the method of Bernfeld, (1955). Before to quantify reducing sugars, the extraction of water-soluble sugars of pulp was carried out according to the method of Agbo, Uebersax, & Hosfieldn, (1985). Thus, 5 g of cocoa beans were homogenized in 50 mL distilled water at 60 °C until cooling at ambient temperature. The mixture was then filtered on filter paper (Whatman, Ø 185 mm) and the filtrate was supplemented with 100 mL of distilled water. To 100 µL of water-soluble sugar extracts were added 200 µL of DNS (3,5-dinitrosalycilic acid). Then, the solution was heated during 5 min. After cooling, 2 mL of distilled water were added and the optical density was

obtained with a spectrophotometer (*PIOWAY, Singapore*) at 540 nm against a blank. A standard curve was established with glucose solution (1 mg/mL) and the quantity of reducing sugars of each sample was obtained from the regression equation established using the standard curve.

3. Identification of yeast species

3.1. Yeast DNA extraction and PCR amplification

Yeasts were grown at 30 °C to the mid-log phase in YPD medium before harvesting. Yeast genomic DNA was extracted using the classic phenol/chloroform method described by Hoffman, (2001) and used as matrix for the PCR reactions. All PCR reactions were performed using a thermocycler (Mycycler, Bio Rad, USA) in a final volume of 50 µL containing 1 µL of DNA extract, 1 U Taq DNA polymerase **(**New England BioLabs**)**, 5 µL 10X magnesium-free buffer, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µM dNTP (Euromedex, France).

The ITS1/ITS2 region of the 5.8S rDNA was PCR amplified as described by Zarzoso, Belloch, Uruburul, & Quero, (1999) using the eukaryotic universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3').

The ITS1/ITS2 PCR program started by an initial denaturation at 95 °C for 5 min, followed by 35 cycles (94 °C for 1 min, 55.5 °C for 1 min, 72 °C for 2 min) and ended by a final extension at 72 °C for 10 min. The amplified products were used then for digestion with restriction enzymes during RFLP. The D1/D2 region of 26S rDNA was PCR amplified as described by Hamdouche, et al. (2015) using the eukaryotic universal primer gc-NL1 (5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCCATATCAATAAG CGGAGGAAAAG 3') and the reverse primer LS2 (5' ATTCCCAAACAACTCGACTC 3').

The D1/D2 PCR program consisted to one cycle at 94 °C for 3 min, followed by 30 cycles (95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min), and a final extension at 72 °C for 10 min. The amplified products were submitted directly for sequencing in order identify different yeast species involving in Ivorian cocoa fermentation.

3.2. PCR-RFLP analysis and yeast molecular identification

For RFLP analysis, PCR-amplified ITS1/ITS2 regions of 5.8S rDNA were digested with *Hha*I, *Hae*III or *Hinf*I (New England BioLabs) restriction enzymes. Reactions, composed of 10 µL of ITS1/ITS2 PCR, 2 µL of supplied buffer, 7.6 µL of water and 0.4 µL of each restriction enzyme, were incubated at 37 °C for 15 min and further analysed by electrophoresis in 1.3 % (w/v) agarose gel in 0.5 X TAE buffer.

For molecular identification of isolated yeasts, PCR-amplified D1/D2 regions of 26S rDNA were sequenced at BIOFIDAL (Lyon, France) using the Pseq D1/D2 primer (5' GGGCCATATCAATAAGC 3'). 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.

Results

1. Fermentation conditions and yeasts growth

1.1. Fermentation conditions

The temperature of cocoa mass increased rapidly during 24 h to 72 h of fermentation time and then decreased progressively after this time for all local regions. However, the higher peak of temperature (47 °C) was obtained in Cavally after 48 h of fermentation; while the lower peak of temperature was recorded in San Pedro after 24 h of fermentation (Figure 1). Moreover, it also observed that Gbôklè and San Pedro reached maximal temperature after 24 h while Haut-Sassandra and Cavally reached this value at 48 h, and Gôh and Tonkpi had a peak of temperature after 72 h of fermentation (Figure 1).

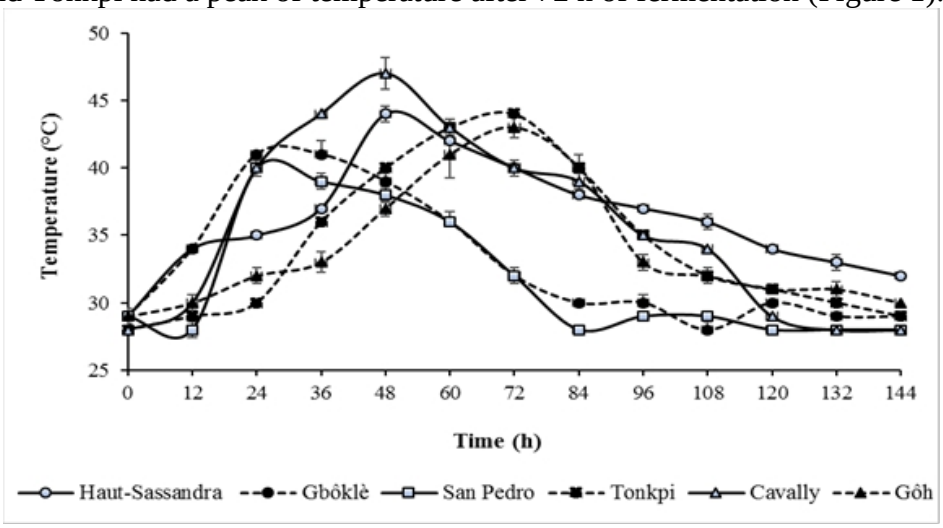

Figure 1. Evolution of temperature during cocoa heap fermentation.

Concerning the pH, it raised progressively during all fermentation process, to 3.4 at the beginning of the process up to 7.7 at the end of process (Figure 2). However, during the first 60 h, pH of cocoa pulp remain acid with values ranging between 3.8 – 4.9.

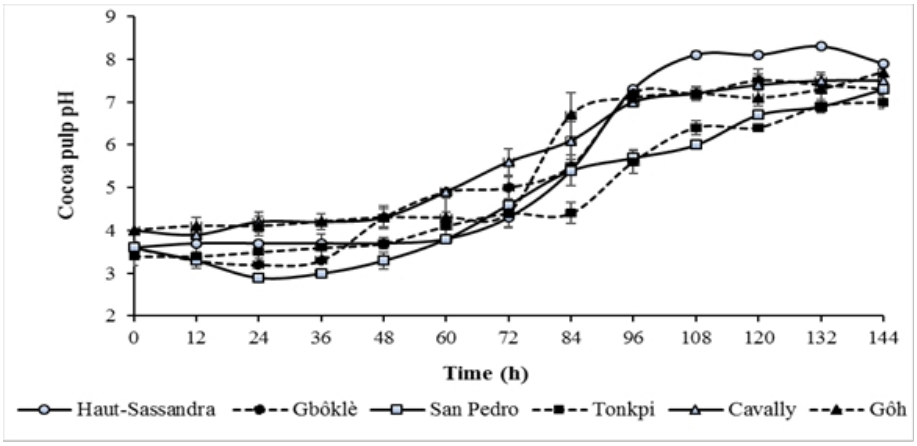

Figure 2. Evolution of cocoa pulp pH during cocoa heap fermentation.

The titratable acidity variation of cocoa pulp was similar for the six regions. However, the acidity value for the regions of Gbôklè, San Pedro, Haut-Sassandra, Cavally and Tonkpi increased until to reach a maximum value at 24 h of fermentation time. Only the region of Gôh recorded its higher titratable acidity at 12 h of fermentation time (Figure 3). The higher acidity value (4.43 meq/g of cocoa beans) was obtained in San Pedro while the lower acidity value (1.96 meq/g of cocoa beans) was recorded in Cavally. Regions of Gôh and Tonkpi were recorded the same maximum acidity value (2.06 meq/g of cocoa beans) but at different times of fermentation around 12 h and 24 h, respectively (Figure 3).

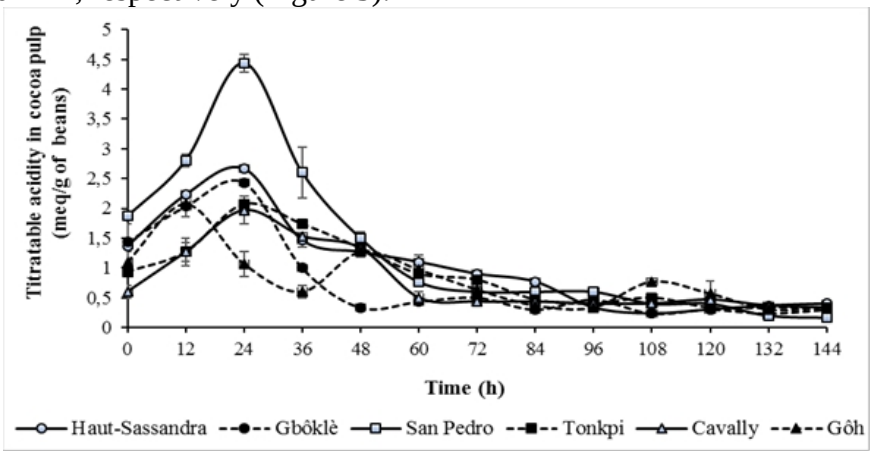

Figure 3. Evolution of titratable acidity of cocoa pulp during cocoa heap fermentation.

Concerning the quantity of pulp reducing sugars during cocoa fermentation in the six regions, the figure 4 showed a rapid decrease of reducing sugars quantity during 72 h of fermentation time. Indeed, it observed 77.35 to 87.32 % of reducing sugars consumed in cocoa pulp for the six fermentations.

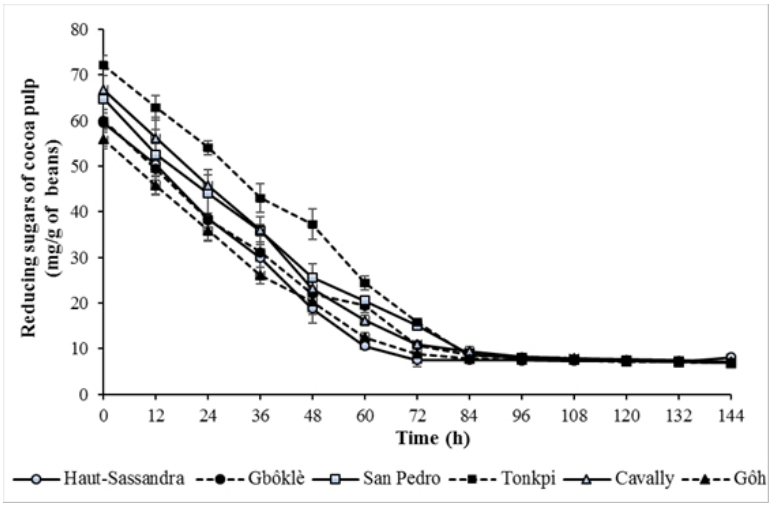

Figure 4. Evolution of reducing sugars of cocoa pulp during cocoa heap fermentation.

1.2. Yeasts growth

The yeasts growth kinetic (Figure 5) showed that the yeasts start the cocoa fermentation with 10^4-10^5 even 10^6 CFU/g until 10^7 and 10^8 CFU/g at 12–24 h. The occurrence time of yeasts during these six cocoa fermentations is 12 h – 60 h. The kinetic of yeasts growth concerning Cavally, Tonkpi, Gôh and Gbôklè regions is the same. Indeed, for these regions, it observed an increase of yeasts population during the first 24 h and decrease progressively after 24 h of fermentation. On the other hand, San Pedro and in Haut-Sassandra showed different kinetics of growth. The yeasts growth kinetic from San Pedro region showed a slight increasing of yeasts populations during the first 24 h before to reach a peak at 36 h of fermentation time. As for yeasts growth kinetic from Haut-Sassandra region, it increases rapidly during the first 12 h and decreased rapidly until 48 h (Figure 5).

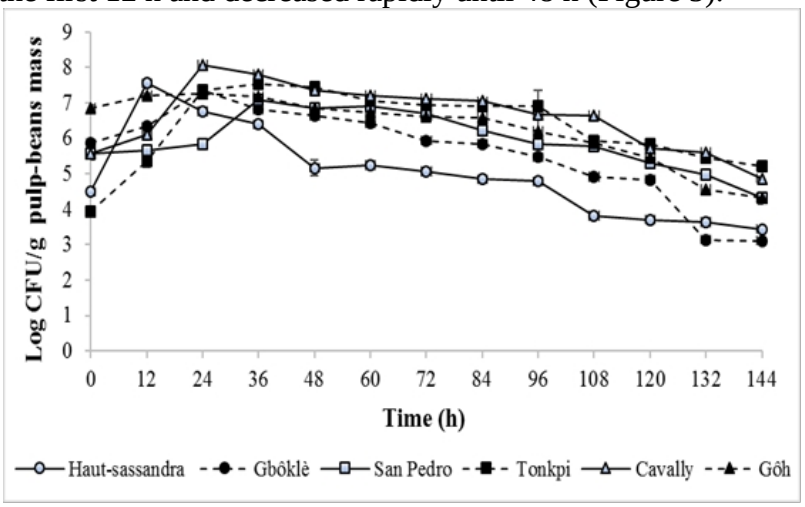

Figure 5. Yeasts growth dynamic during cocoa heap fermentation.

2. Analysis of yeasts diversity during cocoa fermentation

During the cocoa fermentation process, 743 yeast strains were isolated for all six regions. The ITS1/ITS2 regions of 5,8S gene of these isolates were amplified, and cut with restriction enzyme.

Table 1. Length and RFLP analysis of ITS1/ITS2 region of 5.8S rDNA of yeast isolates involved in cocoa heap fermentation of the six major regions.

Region	Group	Marker length ITS1-5.8S-ITS2 (bp)	Restriction profile			Number of isolates
			*Hae*III (bp)	*Xha*I (bp)	*Hinf*I (bp)	
GBÔKLE	I_{Gb}	400 - 500	350+100	250+150	400-500	**11**
	II_{Gb}	500	500	500	500	**84**
	III_{Gb}	600	450+100	350+250	200+200	**7**
	IV_{Gb}	800	350+250+200	500+300	500+100	**11**
HAUT-SASSANDRA	I_{HS}	400	400	400	400	**2**
	II_{HS}	500	500	300+200	150+200	**8**
	III_{HS}	800	800	500+300	500+100	**109**
SAN PEDRO	I_{SP}	400-500	400-500	200+150	400-500	**61**
	II_{SP}	500	500	300+200	150+200	**21**
	III_{SP}	600	400+200	300	500+100	**17**
	IV_{SP}	800	400+200	400+200	500+100	**21**
TONKPI	I_{T}	500	400+100	500	250	**106**
	II_{T}	600	500+100	600	300	**17**
	III_{T}	800	800	800	400+200	**2**
GÔH	I_{G}	500	400+100	200+100	400+100	**18**
	II_{G}	600	500+100	200+100	300+150	**67**
	III_{G}	800	800	800	400+200	**46**
CAVALLY	I_{C}	500	400+100	300+150	200+250	**85**
	II_{C}	600	450+150	600	500+100	**4**
	III_{C}	800	400+200	800	400+200	**46**

bp: base pairs; *Hae*III, *Xha*I and *HinfI*: restriction enzymes.

The results of different restrictions profiles generated are presented in table 1. Gbôklè and San Pedro regions presented four (4) different restriction profiles while Haut-Sassandra, Cavally, Gôh and Tonkpi regions presented three (3) restriction profiles (Table 1). Almost all restrictions profiles obtained were different between them and different of one region and another.

Analysis of sequences of D1/D2 domain of 26S gene of yeast strains has allowed to identify yeasts species involved in cocoa fermentation of the studied regions. Eleven (11) species were identified in the six local regions, including *P. kudriavzevii*, *P. kluyveri*, *S. cerevisiae*, *P. manshurica*, *H. uvarum*, *D. hansenii*, *K. ohmeri*, *Debaryomyces sp.*, *C. humilis / K. exigua*, *H. uvarum / H. opuntiae* and *C. tropicalis.* The distribution of these species for each local region is presented in table 2. Results indicated that *P.*

kudriavzevii, *Saccharomyces cerevisiae* and *P. kluyveri* were mainly species involved in cocoa fermentation for all local regions (Table 2). Moreover, a weakest diversity was observed in Cavally region with only three species while a highest diversity was obtained from Tonkpi region with six species (Table 2).

The RLFP analysis has allowed to reveal an intraspecific diversity for identified species. Indeed, the results showed that some identified species presented several restrictions profiles while other presented only one (Table 3). The species as *Pichia kudriavzevii*, *Pichia kluyveri*, *Pichia manshurica*, *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* revealed several restrictions profiles for the same species. *Pichia kudriavzevii* and *Saccharomyces cerevisiae* showed a large intraspecific diversity with 7 different restriction profiles. *Pichia kluyveri* revealed 3 different restriction profiles. *Pichia manshurica* and *Hanseniaspora uvarum* revealed 2 restriction profiles (Table 3).

Table 2. Distribution of yeast strains involved in cocoa heap fermentation at different local major regions of Ivory Coast.

Region	**Haut-Sassandra**	**Gbôklè**	**San-Pedro**	**Tonkpi**	**Cavally**	**Gôh**
Species	*P. kluyveri* **(72)**	*P. kudriavzevii* **(62)**	*P. kluyveri* **(61)**	*P. kudriavzevii* **(98)**	*P. kudriavzevii* **(85)**	*P. kudriavzevii* **(67)**
	H. uvarum **(37)**	*S. cerevisiae* **(40)**	*S. cerevisiae* **(21)**	*S. cerevisiae* **(9)**	*S. cerevisiae* **(46)**	*H. uvarum* **(24)**
	S. cerevisiae **(8)**	*P. manshurica* **(11)**	*P. kudriavzevii* **(21)**	*P. manshurica* **(8)**	*D. hansenii* **(4)**	*S. cerevisiae* **(22)**
	K. ohmeri **(2)**	*P. kluyveri* **(5)**	*Debaryomyces sp*. **(17)**	*C. humilis / K. exigua* **(6)**		*P. kluyveri* **(18)**
				H. uvarum/H. opuntiae **(2)**		
				C. tropicalis **(2)**		

The values in bracket represent the number of the species founded in the region. *P. kluyveri: Pichia kluyveri; H. uvarum: Hansenisapora uvarum; S. cerevisiae: Saccharomyces cerevisiae; K ohmeri: Kodamaea ohmeri; P. kudriavzevii: Pichia kudriavzevii; P. manshurica: Pichia manshurica; C. humilis / K. exigua: Candida humilis / Kazachstania exigua; H. uvarum/H. opuntiae: Hanseniaspora uvarum/ Hanseniaspora opuntiae; C. tropicalis: Candida tropicalis; D. hansenii: Debaryomyces hansenii*.

Table 3. Restriction profiles of yeast strains involved in cocoa heap fermentation.

Strains	**Restriction profiles**	**Number of strains**
Pichia kudriavzevii	II_{Gb} ; III_{Gb} ; IV_{Gb} ; II_{SP} ; I_T ; II_G ; I_C	333
Pichia kluyveri	III_{HS} ; I_{SP} ; I_G ; IV_{Gb}	156
Saccharomyces cerevisiae	II_{Gb} ; IV_{Gb} ; II_{HS} ; IV_{SP} ; II_T ; III_G ; III_C	141
Hanseniaspora uvarum	III_{HS} ; III_G	61
Pichia manshurica	I_{Gb} ; I_T	19
Debaryomyces sp	III_{SP}	17
Candida humilis / Kazachstania exigua	II_T	6
Debaromyces hansenii	II_C	4
Kodamaea ohmeri	I_{HS}	2
Hanseniaspora uvarum / Hanseniaspora opuntiae	II_T	2
Candida tropicalis	III_T	2

Discussion

Cocoa fermentation is a spontaneous process necessary in post-harvest process in cocoa. The spontaneous nature of cocoa bean fermentations may be the source of microbial variation, and hence of variable end-product quality (Maura, et al., 2016). In this study, the diversity of yeasts, initiators of process, was studied in order to explain the variability of cocoa beans quality from Ivory Coast and identify keys species for selecting appropriate yeasts starters for Ivorian cocoa fermentation. At first sight, physicochemical parameters are determined to appreciate the activity of cocoa microflora particularly yeasts involved in the fermentative process.

Our study recorded temperatures values between 40 - 47 °C at different times of fermentation. Although, some studies reported values reach never above 42 °C (Kouamé, 2017. Visintin, Alessandria, Valente, Paola Dolci, & Cocolin, 2016), most studies report values increasing up to 50 °C during cocoa beans fermentation (Ardhana & Fleet, 2003. Lagunes-Galvez, Loiseau, Paredes, Barel, & Guiraud, 2007). In generally, increase of temperature during cocoa fermentation process is induce by the conversion of the sugars to ethanol by yeasts, then ethanol to acetic acid by acetic acid bacteria which are exothermic reactions (Schwan & Wheals, The microbiology of cocoa fermentation and its role in chocolate quality, 2004). Consequently, intensity of peak of temperature is linked to the intensity of these reactions and therefore to the capacity of yeasts and acetic acid bacteria to produce hight amount of ethanol and acetic acid respectively. Since ethanol and acetic acid are necessary for obtaining quality cocoa, the Cavally region in which the higher temperature peak (47 °C) was recorded could allow production of quality cocoa beans and selection of yeasts strains with high ethanol production capacity for the standardization of the cocoa fermentation process.

During the cocoa fermentation, the pulp pH change acid to basic. The same result was reported by several studies (Guehi, Dabone, Ban-Koffi, Kra, & Zahouli, 2010. Mahazar, et al., 2015. Ouattara, et al., 2016). The increasing of pulp pH could be due to the catabolism of citric acid by yeasts and lactic acid bacteria which offer favorable conditions for the other bacteria growth notably *Bacillus* and acetic acid bacteria (Leal, Gomes, Efraim, Tavares, & Figueira, 2008. Crafack, et al., 2013) which are also necessary to fermentation process (Soumahoro, et al., 2015. Yao, Goualié, Ouattara, & Niamké, 2017). Moreover, the citrate metabolism is known to have a benefic effect on fermentation process because it leads to produce some aroma molecules such as acetoin which are desirable flavor in fermentation process (Illeghems, Weckx, & De Vuyst, 2015).

The decreasing of reducing sugars observed in the six regions, during the first 72 h of fermentation time, indicates the effectiveness of the

fermentative activity of cocoa microflora notably yeasts which convert these sugars to ethanol (Ho V. , Zhao, Srzednicki, & Fleet, 2013). A similar rapid decreasing trend was observed by Afoakwa, Kongor, Takrama, & Budu, (2013) in Ghana**.** However, it also reported that, during the first hours of fermentation time, some yeasts, lactic acid bacteria and acetic acid bacteria convert sugars in acids (Swiegers, Bartowsky, Henschke, & Pretorius, 2005. Papalexandratou, Camu, Falony, & De Vuyst, 2011. De Vuyst & Weckx, The cocoa bean fermentation process: from ecosystem analysis to starter culture development, 2016), which explain the increasing titratable acidity during the first 24 h. These acids produced in the pulp diffuse into the beans and subsequently induce important biochemical reactions leading to well fermented cocoa beans (Afoakwa, Kongor, Takrama, & Budu, 2013). This diffusion into cotyledons gives rise to a decrease of acids concentrations in pulp, which explain the decrease of titratable acidity after 24 h of fermentation.

Isolation of yeasts was carried out to identify the different species involved into each local region. Thus, the yeasts growth dynamics during cocoa fermentation showed that yeasts are present during fermentation process. No lag phase was observed during these fermentative processes as reported by (Samagaci, G., Goualié, & Niamke, 2014). This is probably due to suitable growth conditions for yeasts prevailing in the initial phase of cocoa fermentation (Nielsen, et al., 2007). Indeed, high reducing sugars concentrations in cocoa pulp (55.91-72.09 mg/g) at the beginning of fermentation were recorded for all regions. Moreover, the pH (3.8-4.9) of each local region, is quite remaining acid during the first 60 h of fermentation time. This could be explained the optimal occurrence of yeasts during 12-60 h of fermentation time. These results corroborate those of Ho, Zhao, & Fleet, (2014) which have observed yeasts occurrence during 48 h-72 h of cocoa fermentation. According to these authors, yeasts are cocoa fermentation process initiators and consider as necessary at this process (Ho, Zhao, & Fleet, 2014). Indeed, the primary activity of yeasts is the conversion of reducing sugars to ethanol and CO_2 (Schwan & Wheals, The microbiology of cocoa fermentation and its role in chocolate quality, 2004. Lagunes-Galvez, Loiseau, Paredes, Barel, & Guiraud, 2007). This activity is very crucial to cocoa fermentation because ethanol produced by yeasts is then oxidized by acetic acid bacteria to acetic acid which, by its diffusion into cotyledons, contribute to the formation of aroma precursors of chocolate (Thompson, Miller, Lopez, & Camu, 2013).

During the fermentation process, 743 yeasts were isolated from the six regions. The analysis of RLFP and sequencing of domain D1/D2 of 26S gene of these isolates showed 18 restriction profiles and 11 yeasts species respectively. This indicates existence of a diversity in yeasts population

involved in Ivorian cocoa fermentation. In addition, our results indicated that the fermentation process was dominated by *Pichia kudriavzevii*, *Pichia kluyveri* and *Saccharomyces cerevisiae* at rate prevailing of 84,78 %. The high prevailing of these species could be due to ability of these species to withstand the stressful conditions from cocoa fermentation (Daniel, Vrancken, Takrama, Camu, & De Vuyst, 2009. De Vuyst, Lefeber, Papalexandratou, & Camu, 2010. Pereira, Miguel, Ramos, & Schwan, 2012. Samagaci, Ouattara, Niamké, & Lemaire, 2016. Also, the dominance of these species is an interesting result because most studies, concerning the control of cocoa fermentation by yeasts, reported the use of these three species as appropriate starter cultures (Crafack, et al., 2013. Meersman, et al., 2016. Pereira, et al., 2017).

Indeed, some *Pichia kudriavzevii* used as starter, successfully carried out the fermentation process, with efficient sugar metabolism and ethanol formation. Their metabolic activity during the fermentation process influenced the final volatile fraction of fermented cocoa beans (Pereira, et al., 2017). Thus, the dominance of *Pichia kudriavzevii* in Gbôklè, Tonkpi, Cavally and Gôh regions will allow to obtain cocoa beans fermented from these regions with excellent aromas.

Moreover, according to Crafack, et al. (2013), *Pichia kluyveri* influences positively the flavor profile of cocoa beans and presents great pectinolytic activity to degrade pectin of the cocoa pulp. Thus Haut-Sassandra and San Pedro, the two regions dominated by Pichia kluyveri, could be susceptible to give cocoa beans fermented with good flavor profile. Besides, Haut-Sassandra and San Pedro are neighbor regions and this proximity could explain the dominance of the same species, *Pichia klyuveri* in these regions.

Concerning *Saccharomyces cerevisiae*, Meersman, et al. (2016) showed that thermotolerant hybrid strains of *Saccharomyces cerevisiae* would be able to increase acetate ester production necessary to aroma formation of chocolate. Thus, *Saccharomyces cerevisiae* strains which prevail in all regions can be exploited in order to identify appropriate starters cultures to cocoa fermentation or others food fermentations.

Besides the predominant species, the occurrence of some species at low prevalence notably *Candida tropicalis* is to underline because known to influence quality and safety cocoa. Indeed, the addition of *Candida* sp. on cocoa beans fermentation inhibit the growth of pathogenic microorganisms. *Candida* sp.-fermentation ensured both the quality and safety of the end product (Mahazar, et al., 2015). Thus, the prevalence of *Candida tropicalis* from Tonkpi region could contribute to guaranty both the cocoa quality and cocoa safety.

Eleven (11) species were involved in cocoa heap fermentations of the six Ivorian major local cocoa producing regions studied. This heterogeneity of yeasts population implicated into cocoa bean fermentation was also shown in studies around the world (Nielsen, Hønholt, Tano-Debrah, & Jespersen, 2005. Daniel, Vrancken, Takrama, Camu, & De Vuyst, 2009. Arana-Sánchez, et al., 2015). However, some yeast species isolated from our investigations were differents from those previously isolated in Ivory Coast. Indeed, *Pichia galeiformis, Galactomyces geotrichum* and *Wickerhamomyces anomalus* were isolated in cocoa fermentation from Me region (Ivory Coast) (Koné, et al., 2016). *Candida intermedia, Candida nacodendra,* and *Hanseniaspora guilliermondii* were isolated in cocoa fermentation from Agneby-Tiassa region (Ivory Coast) (Samagaci, Ouattara, Niamké, & Lemaire, 2016). In addition, *Torulaspora delbrueckii, Candida ethanolica* and *Schizosaccharomyces pombe* are also be detected recently in Ivorian cocoa fermentation by Visintin, Alessandria, Valente, Paola Dolci, & Cocolin, (2016). All these results indicate the occurrence of a diversity and a variability of yeast microflora involved in Ivorian cocoa fermentation to one local region to another. This yeast species diversity and variability would be responsible to the variability of cocoa beans quality observed in Ivory Coast. Because, yeast species involved into each producing region could display a specific role that could impact cocoa beans quality in fine chocolate produced as reported above.

Analysis of RLFP profiles of species identified allowed to class yeast species in two groups. Firstly, yeast species which present one restriction profile as reported by Zarzoso, Belloch, Uruburul, & Quero, (1999) and (Schwan, Almeida, Souza-Dias, & Jespersen, 2007). Indeed, theses authors demonstrated that one restriction profile is specific of one species. However, some yeast species notably *Pichia kudriavzevii, Saccharomyces cerevisiae, Pichia klyuveri, Pichia manshurica* and *Hanseniaspora uvarum* presented several restriction profiles constituting the second group. Similar results were reported by Samagaci, Ouattara, Niamké, & Lemaire, (2016) who showed 2 restrictions profiles for *Candida tropicalis, Candida nacondendra* and *Hanseniaspora uvarum*. Also, some authors reported great intraspecies variation among populations of *Pichia kluyveri Saccharomyces cerevisiae* and *Pichia kudriavzevii* respectively, isolated to cocoa fermentations (Jespersen, Nielsen, Hønholt, & Jakobsen, 2005. Meersman, et al., 2013. Pereira, et al., 2017). Taken together, our results indicate occurrence an intraspecific diversity within yeasts population. This intraspecific diversity could be exploited for the choosing appropriate starter cultures to resolve the variability problem of cocoa quality, through the standardization of cocoa fermentation. Indeed, within group strains of a same species exist subdivisions which distinguish them by their genotypic and/or phenotypic

features. The differentiation occurs when the species undergo significant mutations generated by natural processes, such as sexual reproduction, changes in ploidy, transposons, genetic recombination or horizontal gene transfer (Steensels, et al., 2014). **(**These natural mutations can form strains with important fermentative characteristics, which can be exploited for selecting appropriate starter cultures. Thus, the strains of *Pichia kudriavzevii*, *Pichia kluyveri* and *Saccharomyces cerevisiae*, which present a great intraspecific diversity and more abundant in this study, constitute some potential ways concerning starters research for the control of fermentative cocoa process still spontaneous in Ivory Coast.

Conclusion

The results of this study indicate that it exists a molecular diversity of yeasts involved in the fermentation of Ivorian cocoa, with a predominance of *Pichia kudriavzevii*, *Saccharomyces cerevisiae* and *Pichia kluyveri*. This diversity of yeast species, varying one local region to another, can explain the variability of cocoa beans quality in Ivory Coast. Moreover, the great intraspecific diversity of these predominant yeast species indicate that some strains of the same species can present different characteristics which can be used for selecting appropriate starter cultures in industry. Thus, considering the crucial role of yeasts in cocoa fermentation, it would arrange to research yeast starters to control the fermentative cocoa process as realized already in beer, wine and bread industries. This would contribute to resolve the problem of the variability of cocoa beans quality which cause huge economic loss in Ivory Coast.

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