Short communication

Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic

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Abstract

Cocoa fermentation was monitored at the IDIAF (Instituto Dominicano de Investigaciones Agropecuarias y Forestales) "Mata Larga" experimental station, in San Francisco de Macoris, Dominican Republic. The maximum average fermentation temperature reached 51 °C after 48 h and the pH reached 4.5 after 144 h of fermentation. A significant decrease in glucose, fructose and citric acid was seen in the pulp over the first 48 h. There was a delay of 24 h between maximum microbial growth and maximum concentrations of the respective metabolites, which occurred after 48 h for ethanol and after 72 h for acetic acid. A maximum concentration in lactic acid was found after around 120 h of fermentation. The aerobic mesophilic flora increased from 6.1×10^6 to a maximum of 4.2×10^7 CFU g^{-1} of dry matter after 48 h of fermentation. Yeasts displayed maximum development after 24 h (6.1×10^7 CFU g^{-1} of dry matter), whilst for lactic and acetic acid bacteria it occurred after 48 h (7.3×10^7 and 1.5×10^8 CFU g^{-1} of dry matter respectively). The yeasts isolated belonged to the genera *Hanseniaspora* and *Candida*, the lactic acid bacteria to the genus *Lactobacillus*, and the acetic acid bacteria to the genus *Acetobacter*. The differences compared to other fermentation trials concerned the micropopulation from a qualitative point of view.
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1. Introduction

In 2002 world production of cocoa exceeded the 3 million tonne mark. Although Latin America was the origin of cocoa growing, it no longer produces more than 15% of world volume, primarily divided between Brazil (6%), Ecuador (3%), the Dominican Republic (1.5%), Colombia (1%) and Mexico (1%) according to market reports (Anonymous, 2002, 2003).

The Dominican Republic is currently one of the main organic cocoa exporting countries. Organic cocoa production currently stands at around 6000 tonnes out of the total 35,000 tonnes of cocoa produced by the country (Anonymous, 2003). Even with the increase in production, cocoa fermentation is still carried out traditionally (Roche et al., 1988).

Cocoa "fermentation" is one of the stages in post-harvest processing that mostly governs ultimate product quality. Fermentation remains empirical and does not give rise to beans of consistent quality, which obliges processors continuously to make changes of their formulations (Lehrian and Patterson, 1984; Lanaud et al., 1999). Fermentation helps to break down the mucilaginous pulp surrounding beans and causes cotyledon death (Sanchez et al., 1985; Sanchez, 1989; Gotsch, 1997). It also helps to trigger biochemical changes inside the beans that contribute to reducing bitterness and astringency, and to the development of flavour precursors (Barel, 1997).

During the first phase of fermentation, yeasts possess an intense metabolism favoured by the acidity of the environment, the richness in fermentable carbohydrates and the low oxygen content of the mass (Lehrian and Patterson, 1984). The yeast fermentation metabolism very quickly leads to consumption of all the simple sugars to give ethanol and carbon

dioxide. Alcoholic fermentation is a moderately exothermic reaction (93.3 kJ by molecule of glucose consumed). It leads to a moderate increase in the temperature of the mass, which reaches 35 to 40 °C (Ravelomanana et al., 1984; Barel, 1998; Jespersen et al., 2005). At the same time, polysaccharides in the cells of the mucilaginous tissue are broken down by the pectinolytic action of yeasts (Sanchez et al., 1985).

Greater aeration of the mass due to the disappearance of the mucilage enables acetic acid bacteria to develop and intervene (Barel, 1997). By oxidation, they convert the ethanol produced during alcoholic fermentation into acetic acid (Jinap, 1994). Oxidation is a highly exothermic reaction (496 kJ by molecule of ethanol converted into acetic acid), which raises the temperature to 50 °C (Lagunes-Gálvez, 2002). Regular stirring is necessary to promote aeration, so as to achieve quick and uniform fermentation, leading to a rapid increase in temperature (Passos et al., 1984; Vander-Horst, 1984; Schwan et al., 1990).

Despite work published on the microflora of cocoa, there is not enough information on fermentation monitoring. The purpose of our work was to study the parameters of cocoa fermentation in the Dominican Republic, from a quantitative and qualitative viewpoint, so as to more effectively determine the role of microorganisms and how they function.

2. Materials and methods

2.1. Fermentation monitoring and sampling

Cocoa pods, mostly from Trinitario or Trinitario hybrid trees, were harvested at the "Mata Larga" experimental station belonging to IDIAF (Instituto De Investigacion Agropecuaria y Forestal) at San Francisco de Macoris in the Dominican Republic in December 2002.

Three successive fermentation operations were carried out with 100 kg of fresh cocoa per trial, in wooden boxes measuring $60 \times 60 \times 60$ cm. Fermentation was carried out over 6 days, stirring after 24, 48 and 96 h. The three fermentations were monitored by taking random bean samples from different zones in the mass of cocoa, so as to obtain 100-g samples for microbiological analyses, or 800-g samples for biochemical analyses. The samples were taken every 6 to 12 h for the first 48 h of fermentation, then every 24 h up to the sixth day. Samples intended for biochemical analyses were frozen in liquid nitrogen and stored at −20 °C.

2.2. Physico-chemical analyses: pH, temperature and water content

A sample of 20 g of cocoa beans was immersed in 100 mL of distilled water and the pH was measured in the supernatant (Lopez et al., 1989).

The ambient temperature and the temperature of the fermenting cocoa mass were monitored continually with a portable meter (temperature and relative humidity) Zephyr II+ Solomat® type 2300B1241 and W14981274576 (GrayWolf Sensing Solutions, Clare, Ireland).

Water content was determined in compliance with international standard ISO 2291–1972 (drying at 103 °C for 16 h) on cocoa shell and mucilage at different stages of fermentation.

2.3. Microbiological analyses

Samples of 20 g from 100-g initial sample were immersed in 200 mL of physiological solution (0.8% NaCl w/v) and stirred by a magnetic bar for 10 min. After decimal dilutions in the same medium, counts were carried out in duplicate on different culture media. The aerobic mesophilic flora was counted on PCA medium (plate count agar, Merck® Darmstadt, Germany), lactic acid bacteria on MRS agar (De Man Rogosa Sharpe, Biokar® Diagnostics, Beauvais, France), yeasts on a Sabouraud agar medium with chloramphenicol (Biorad® Marnes la Coquette, France) and acetic acid bacteria on Carr medium and on agar medium adapted to acetic acid bacteria (Guiraud, 1998). For all the media, the incubation period was two days at 28 °C. The results of the counts expressed as CFU per g of cocoa dry matter (DM) were obtained in compliance with standard ISO 4833.

For each counting agar plate, every types of the different colonies (colonial and microscopic type) were sampled, as a single colony, or for the numerous colonies, three colonies at random. The selected colonies were purified by isolation on the same media, frozen in liquid nitrogen and stored at −20 °C in peptone water (casein peptone, 10 g L^{-1}; sodium chloride 5 g L^{-1}; Biorad® Marnes la Coquette, France) supplemented with 50% glycerol (w/v).

The frozen strains were revived in peptone water (Biorad® Marnes la Coquette, France) at 28 °C for 24 h and spread onto the different isolation media depending on the species.

The microbial strains were observed under the microscope in their fresh state and after Gram staining (Bio-Merieux® reagents, Marcy l'Etoile, France). Catalase and oxidase tests were performed on slides with Bio-Merieux® reagents (Marcy l'Etoile, France). The pectin depolymerase activity of yeasts was quantified after the strains had been cultured for 7 days without stirring, at 28 °C on medium containing pectin (composition: glucose 10 g L^{-1}; Unipectin 325 NH 95, Biorad® Marnes la Coquette, France, 15 g L^{-1}; yeast extract 10 g L^{-1}). The media were clarified by centrifugation ($5000 \times g$, 10 min), then passed through an OSTWALD NFT 51-032 viscometer in borosilicate glass (ERTCO, Canadawide Scientific Ltd, Otawa, Canada) with a flow time for water of 10 s. The control consisted of non-inoculated culture medium (Sanchez, 1983).

Yeasts were tentatively identified according to the morphological, physiological, fermentation and assimilation properties (Kreger-van Rij, 1987; Kurtzman and Fell, 1998) and using API 20C AUX strips (Bio-Merieux®, Marcy l'Etoile, France). Lactic acid bacteria were identified according to the gas production from glucose (Gibson and Abdel-Malek test), growth at pH 3.0–5.5, growth at 15–47 °C (Guiraud, 1998; Passos et al., 1984) and using API 50CH strips (Bio-Merieux®, Marcy l'Etoile, France). The API tests were exploited with APILAB plus bacterial identification software (Bio-Merieux®, Marcy l'Etoile, France). Acetic acid bacteria were identified using the media and techniques proposed by Frateur (Guiraud, 1998;

Ardhana and Fleet, 2003): catalase, growth on ethanol and acetate, oxidation of ethanol and calcium lactate, ketogenesis of glycerol, production of 5-keto-gluconic acid from glucose and formation of water-soluble brown pigments. The Bergey's Outline Manual (2004) was used for the bacterial nomenclature.

2.4. Biochemical analyses

According to Van Der Stegen and Van Dujin (1987), these were carried out on combined pulp and bean shell, starting from the hypothesis that shell did not undergo any major transformations during fermentation. For organic acids and sugars, extraction was carried out at ambient temperature by placing 10 g of sample in 100 mL of ultra-pure water and stirring for 10 min with a magnetic bar. The extract was passed through a Dowex 1×4 anion exchange column (Fluka, Busch, Switzerland) activated by a water/methanol mixture (3/1, v/v) to fix organic acids. The sugars were in the eluate recovered on leaving the column. The acids were salted out of the column after passage of a 5 mM H_2SO_4 solution, then purified on an Amberlite XAD-4 column (Fluka, Busch, Switzerland).

After filtration through membranes with a porosity of 0.45 μm (cellulose acetate, Millex Millipore, Ireland) the eluates were injected into an HPLC chain (Shimadzu LC-6A apparatus, Kyoto, Japan) on a PL Hi-Plex H column (300×7.7 mm; particle size, 8 μm; Polymer Laboratories, Marseille, France) with a 5 mM H_2SO_4 mobile phase and a flow rate of 0.6 mL min^{-1} (Van Der Stegen and Van Dujin, 1987). Organic acids were detected by a UV detector (Shimadzu SPD-6A spectrophotometric detector, Kyoto, Japan) at 210 nm, and the sugars were detected by a refractometer (Shimadzu RID-6A refractometric detector, Kyoto, Japan).

Ethanol and acetic acid were quantified by gas chromatography (Shimatzu type GC 8A chromatograph, Kyoto, Japan), connected to a Shimadzu CR6A Chromatopac integrator (Kyoto, Japan), on a Porapak Q isothermal column at 170 °C (1 m × 2.2 mm; 80–100 mesh, Alltech, Lexington, Kentucky USA). The quantified solution was obtained by aqueous extraction at ambient temperature by placing 20 g of sample in 100 mL of ultra-pure water stirred for 10 min with a magnetic bar. 2-isopropanol was used as the internal standard. The vector gas was nitrogen. The temperature of the injector and the detector was 210 °C. For both products, a standard range was performed with solutions of between 0 and 200 mg L^{-1}.

2.5. Expression of results

As the external conditions and the data obtained during the 3 fermentations were very similar, we show the mean values and standard deviations for all the measurements.

3. Results and discussion

3.1. Changes in physical parameters

At the beginning of fermentation (i.e. after pod opening), the average moisture content of the shell-mucilage combination

was 76.6%±0.8 and the pH value was 4.00±0.10 (which was slightly higher than what is usually reported: 3.6 on average). Mucilage acidity is known to depend primarily on the presence of citric acid (Lopez and Passos, 1984). During our fermentation operations, after 144 h, the pH value rose to 4.48±0.02 and the water content fell to 69.3%±0.9. Lehrian and Patterson (1984) and Schwan and Whelas (2004) showed that after citric acid consumption, and despite the production of acetic acid, the pH value of the mass of cocoa could increase to 5 or even 6.

The temperature changes and effects of stirring were described by Lopez and Passos (1984), Passos et al. (1984) and Schwan et al. (1990). During our fermentation monitoring, the rise in temperature reached a maximum value of 51 °C after 48 h. That value corresponded to what has generally been described (Carr, 1982; Lehrian and Patterson, 1984; Barel, 1998; Schwan and Whelas, 2004). Barel (1997) considered as satisfactory a rise in temperature to around 45 °C in 48 h maximum, and linked it to good ultimate cocoa quality. The variations in the ambient temperature followed the daytime and night-time cycles, with variations of around 10 °C, without greatly influencing the temperature of the fermenting mass (Fig. 1). There was no more sweating after 24 to 36 h of fermentation, suggesting that there was no longer any pectinolytic activity.

3.2. Biochemical parameters

Analyses were carried out on the shell-mucilage combination. For the substances considered as possible fermentation substrates at the beginning of the process, the values after pod opening were 56.6±8.3 mg g^{-1} dry matter (DM) for glucose and 88.8±6.9 mg g^{-1} DM for fructose. The sugar concentration we obtained was lower than the values usually found, which often amount to 100 mg g^{-1} (Forsyth and Quesnel, 1963; Carr, 1982). We did not detect any sucrose, which partly contributed to the low total sugar content. That was indicative of advanced fruit ripening (Jinap et al., 1993). Another substrate found was 5.9±3.8 mg g^{-1} (WM) of citric acid. We found an initial
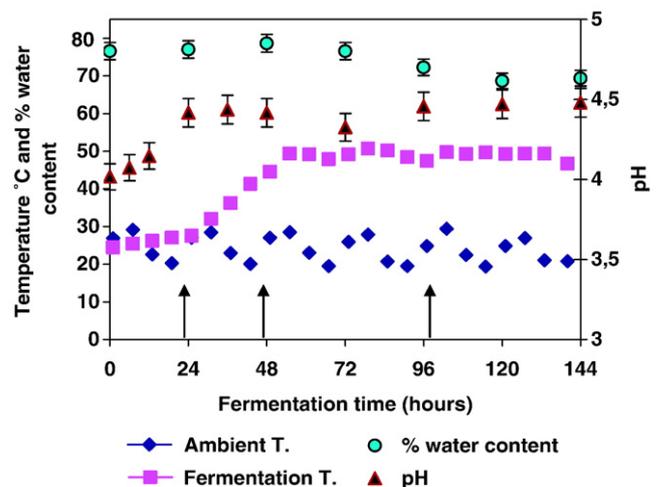

Fig. 1. Changes in physical parameters (ambient temperature, fermentation temperature, water content and pH). Stirring: →.

content that was much lower than that usually reported, as opposed to 20 to 25 mg g^{-1} (WM) (Biehl et al., 1989; Lanaud et al., 1999; Bucheli et al., 2001). That explained the initial pH value of the mucilage that we measured.

A significant drop in sugar and citric acid concentrations was seen in the first 48 h, and continued throughout fermentation (Fig. 2).

The variation in microbial metabolites produced during fermentation is shown in Fig. 3. The ethanol content increased rapidly, reaching a value of 9.6±0.1 mg g^{-1} DM after 48 h; this evolution is currently described (Rohan, 1958; Carr, 1982; Lehrian and Patterson, 1984). The ethanol concentration then fell rapidly, becoming nil after 96 h. It was found that the maximum ethanol content obtained was lower than expected when sugar consumption was considered, particularly as pectin breakdown was an additional source of fermentable sugars. This showed that ethanol was rapidly converted or eliminated. The main cause of ethanol disappearance was its oxidation into acetic acid by acetic acid bacteria (Lehrian and Patterson, 1984; Schwan, 1998; Schwan and Whelas, 2004). However, there were also other reasons for ethanol disappearance: in aerobiosis, some yeast are able to oxidize it and there may have been loss through sweating and evaporation.

Oxidation of alcohol into acetic acid is considered fundamental in cocoa fermentation. It causes a rise in temperature (in our case up to 51 °C) which kills the bean. The maximum acetic acid content in the pulp reached a maximum after 72 h (22.5± 2.1 mg g^{-1} DM) i.e. 24 h after the maximum temperature; a rise in its concentration was found at the end of fermentation. For acetic acid, the maximum values were checked by HPLC analysis.

The existence of lactic fermentation, which is not favourable to cocoa quality (Barel, 1997), is not an exceptional phenomenon. Lactic acid production is therefore often found (Passos et al., 1984). During our trials, we found maximum concentrations of lactic acid of around 0.70±0.86 mg g^{-1} of wet matter
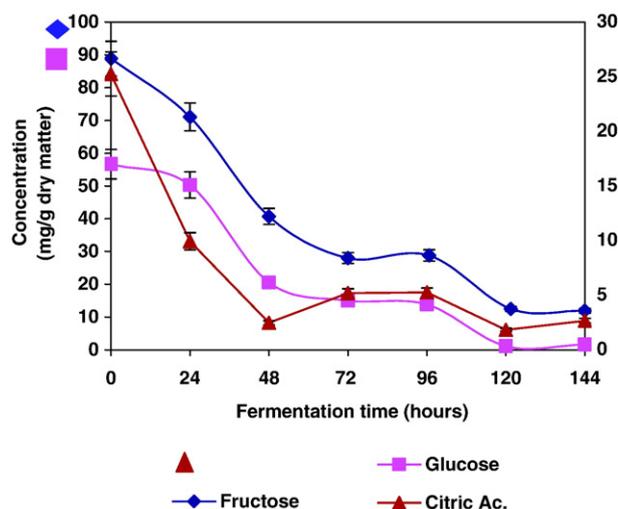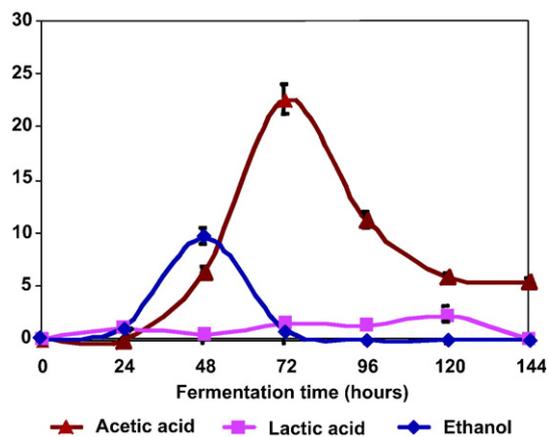

Fig. 3. Variation in metabolites produced during fermentations.

after 120 h of fermentation, i.e. values similar to those observed by Carr et al. (1979) in Ghana and Malaysia. Such values are not indicative of poor fermentation.

3.3. Changes in the micropopulation

At the beginning of fermentation, the aerobic mesophilic flora reached 6.1±5×10^6 CFU g^{-1} dry matter. Yeasts and acetic acid bacteria were present and reached 1.5±1×10^6 CFU g^{-1} DM and 9.7±5×10^5 CFU g^{-1} DM respectively. Lactic acid bacteria were only quantifiable after 12 h (with 2.7±4× 10^4 CFU g^{-1} DM). There did not seem to be any latency phase for the aerobic flora, yeasts and acetic acid bacteria (Fig. 4). Maximum development occurred after 24 h for yeasts (6.1± 3×10^7 CFU g^{-1} DM) and after 48 h for the aerobic mesophilic flora (2±1×10^8 CFU g^{-1} DM), acetic acid bacteria (1.5± 1×10^8 CFU g^{-1} DM) and lactic acid bacteria (7.3± 9×10^7 CFU g^{-1} DM), i.e. a population level similar to that observed by Ostovar and Keeney (1973) in Trinidad and by Ardhana and Fleet (2003) in Indonesia.

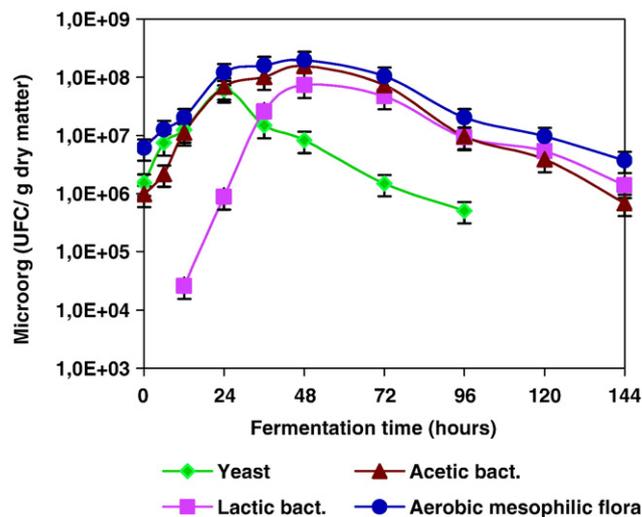Fig. 2. Change in glucose, fructose and citric acid concentration during fermentations.

Fig. 4. Average count for the micropopulation present during fermentations.

Table 1
Sampling times for the main isolates of yeasts, lactic and acetic acid bacteria

	Fermentations hours						
	0	24	48	72	96	120	144
Yeasts							
Candida inconspicua		+	+	+	+	+	+	+
Hanseniaspora guillermondii	+	+	+	−	−	−	−
Pichia fermentans	+	−	+	+	−	−	−
Yarrowia lipolytica	−	+	+	+	−	−	−
Candida zeylanoides	−	+	+	−	−	−	−
Lactic acid bacteria							
Lactobacillus plantarum or *Lactobacillus paraplantarum*	−	+	+	+	+	+	+
Lactobacillus paracasei subsp. *paracasei*	−	−	+	+	+	+	+
Lactobacillus pentosus	−	+	+	+	+	−	−
Lactobacillus brevis	−	−	+	+	+	−	−
Acetic acid bacteria							
Acetobacter lovaniensis	−	−	−	+	+	−	−

3.4. Identification of microbial strains present during fermentation

3.4.1. Yeasts

According API and complementary biochemical tests, the 43 yeast strains isolated were found tentatively to belong to the genera *Candida*, *Hanseniaspora*, *Pichia* and *Yarrowia* (Table 1). Table 2 shows the biochemical characteristics of the species isolated that are important for the fermentation of cocoa. Among the yeast species isolated, many had low fermenting power. The species *Candida insconspicua* proved to be the most abundant and persisted throughout fermentation. It did not produce ethanol and did not display any pectinolytic activity; however, it was capable of assimilating lactic acid. The species *Hanseniaspora guillermondii* fermented sugars to give ethanol. It displayed pectinolytic activity. It was present during the first 36 h of fermentation. Apart from yeasts of the genus *Hanseniaspora*, all the other species isolated were able to assimilate ethanol. That helped to explain the low maximum ethanol concentration found. The species *Pichia fermentans* var *fermentans*, *Yarrowia lipolytica* and *Candida*

zeylanoides var *zeylanoides* were not present throughout fermentation. They are all capable of oxidizing citric acid and only the species *C. zeylanoides* is not able to produce ethanol. The species (i) *Candida glabrata*, (ii) *Candida krusei* and *Hanseniaspora valbyensis* were isolated on separate occasions respectively after (i) 48 and (ii) 72 h of fermentation. These three fermentative species produced ethanol and, of them, the species *C. glabrata* was incapable of oxidizing citric and lactic acids. None of the species of yeasts isolated was able to metabolize acetic acid in a significant way.

Mucilage breakdown is a major fermentation phenomenon since it modifies aeration conditions and produces fermentable carbohydrate substrates. Pectinolytic enzymes produced by yeast strains are responsible for such hydrolysis (Carr, 1982; Cascante et al., 1994; Schwan, 1997). Three yeast species isolated, *Candida inconspicua*, *C. zeylanoides* and *H. guillermondii* had a pectin depolymerization activity.

Most of the yeast species isolated during our trials had already been observed during cocoa fermentation by Ostovar and Kenney (1973) in Trinidad, by Carr et al. (1979) and Ardhana and Fleet (2003) in Malaysia, by Ravelomanana (1985) in Madagascar and Ivory Coast, by Cascante et al. (1994) in Ecuador, by Schwan and Whelas (2004) in Brazil, by Jespersen et al. (2005) and Nielsen et al. (2005) in Ghana.

3.4.2. Lactic acid bacteria

The presence of lactic acid bacteria is usual (Carr et al., 1979; Carr, 1982). In our trials, lactic acid bacteria were quantifiable from 12 h onwards (with 10^3 CFU g^{-1} WM). According API and complementary biochemical tests, we tentatively identified four species of heterofermenting lactic acid bacteria from the 44 strains isolated: *Lactobacillus pentosus* (6 isolates), *Lactobacillus plantarum* or *Lactobacillus paraplantarum* (24 isolates), *Lactobacillus paracasei* subsp. *paracasei* (10 isolates) and *Lactobacillus brevis* (4 isolates). They were therefore capable of producing lactic acid, acetic acid, ethanol and CO_2. The heterofermenting properties were verified according the Gibson and Abdel–Malek test (Passos et al., 1984). On one occasion after 48 h of fermentation we observed the existence of strains belonging to the species *L. brevis*.

Table 2
Characteristics of the yeast species isolated during cocoa fermentation

Species	Number of isolates	Ethanol fermentation	Ethanol assimilation	Acetic acid assimilation	Citric acid assimilation	Lactic acid assimilation	Pectinolytic activity
Candida inconspicua	18	−	+	−	−	+	16 (−) 2 (+ weak)
Hanseniaspora guillermondii	8	+	−	−	−	−	7 (−) 1 (+ slow)
Yarrowia lipolytica	6	−	+	−	+	−	−
Candida zeylanoides	4	3 (+) 1 (−)	+	−	+	−	3 (+) 1 (−)
Pichia fermentans	3	+	+	−	+	+	−
Candida krusei	2	+	+	−	+	+	−
Candida glabrata	1	+	+	−	−	−	−
Hanseniaspora valbyensis	1	+	−	−	+	−	−

L. plantarum was found at other production sites in America, but only after 72 h of fermentation. The species *L. pentosus* had never been isolated before in America, but given its genetic resemblance to *L. plantarum*/*L. paraplantarum*, it may have been described as the latter (Roissart and Luquet, 1994).

3.4.3. Acetic acid bacteria

According to the Frateur tests, the strains isolated (5) all were found tentatively to belong to the species *Acetobacter lovaniensis*. This isolates produces acetic acid from ethanol and can utilise that acid to CO_2 and water. The level of the acetic acid bacterial population mentioned in the literature is similar, from 10^3–10^4 cells g^{-1} (WM), just after pod opening, rising to 10^6–10^7 cells g^{-1} during fermentation (Lopez, 1972; Samah et al., 1993; Schwan, 1998). During our fermentation monitoring, the acetic acid bacterial population was high from the outset with 10^5 CFU g^{-1} (WM). There was a delay of 24 h between the maximum acetic acid bacterial population and the maximum acetic acid content. Only one species was identified, *A. lovaniensis*. That observation might not reflect the richness and diversity of this population as it is difficult to count acetic acid bacteria and preserve viable pure strains for identification (Schwan and Whelas, 2004). The only species isolated was able to oxidize acetic acid. The species *A. lovaniensis* has only been reported in Malaysia (Schwan and Whelas, 2004).

It would be interesting to use biomolecular techniques to fine-tune the identification of microbial species and study the biodiversity of microorganisms present during fermentation (Jespersen et al., 2005; Nielsen et al., 2005), and learn about their ecophysiology. Such studies could focus more particularly on acetic acid bacteria, which play a fundamental role in the development of chocolate flavour precursors.

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