Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (*Theobroma cacao* L.) hybrids

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ABSTRACT

The intensive efforts to control the "witches' broom" fungus in Brazil have included research on resistant and more productive cocoa cultivars, but the impact of these crosses on cocoa fermentation is not known. To understand the effects of breeding on cocoa fermentation, this work investigated the microbes and chemical compounds present during the fermentation of three highly productive hybrids (PH 9, PH 15 and PH 16). The microbial species present during the fermentation process were similar in all three hybrids, but the populations of lactic acid, acetic acid, and mesophilic aerobic bacteria and yeast in the PH 9 hybrid fermentations were greater than those in PH 15 and PH 16. *Lactobacillus fermentum*, *Lactobacillus casei* and *Lactobacillus rhamnosus* were the predominant species in all hybrid cocoa fermentations. However, the profile of the bacterial community detected by PCR-DGGE was more complex in the PH 9 fermentation. The yeast *Saccharomyces cerevisiae* was predominant in the all fermentations, followed by *Hanseniaspora uvarum* and the genus *Pichia*. A similar profile of substrate consumption (glucose, sucrose, fructose and citric acid) and kinetics of major metabolites (ethanol, acetic acid and lactic acid) was also observed, but the PH 16 hybrid presented the highest concentrations of sugars, acids and alcohols.

1. Introduction

Theobroma cacao L. belongs to the family *Sterculiaceae* and is economically important due to its valuable seeds. The seeds, commonly known as cocoa beans, are the principal raw material for chocolate production. Cocoa plants are cultivated on plantations in tropical regions throughout the world; Ivory Coast, Ghana, Nigeria, Cameroon, Indonesia and Brazil are the major producers. For many years, cocoa production and commercialization have been the basis of the economy in some Brazilian states and particularly in Bahia. Brazil is the world's sixth largest cocoa producer (ICCO, 2012).

Three main genetic groups of cocoa – *Criollo*, *Forastero* and *Trinitario* – have been established based on their morphological traits and geographical origins (Cheesman, 1944). In the early 1990s, financial crises in the international cacao market and 'witches' broom', a disease caused by the pernicious fungus *Moniliophthora perniciosa* (*Crinipellis perniciosa*), dramatically decreased the production and exports of cocoa beans (Freire, Schwan, & Mororó, 1999; Schwan & Wheals, 2004). These events prompted the search for disease-resistant hybrids, and the Brazilian cocoa producers are currently recovering. The cocoa breeding program aims to increase productivity and disease resistance;

consequently the crosses will affect some characteristics of cocoa pods and seeds, such as size, color, quantity and weight of the seeds, pulp amount, flavor and chemical composition (Camu et al., 2008; Clapperton, Lockwood, Yow, & Lim, 1994; Noor-Soffalina, Jinap, Nazamid, & Nazimah, 2009; Sukha, Butler, Umaharan, & Boult, 2008). Considering the large variability in pulp content and composition, as well as in the cotyledon composition, it should not be a surprise that different hybrids have different fermentation dynamics and requirements. However, the effects of hybrid breeding on cocoa fermentation and chocolate quality are unknown.

The fermentation of raw cocoa beans occurs in two steps: the first stage involves microbial reactions that take place in the pulp and the outer part of the beans; the second phase involves several hydrolytic reactions that occur within the cotyledons (Biehl, Brunner, Passern, Quesnel, & Adomako, 1985; Schwan & Wheals, 2004). The microbial activity in the cocoa pulp is a well-defined temporal succession that is dominated by yeast during the first hours, followed by lactic acid bacteria (LAB), which decline after 48 h of fermentation, and then they are overcome by acetic acid bacteria (AAB). Species of *Bacillus* (e.g. *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus flexus*), other bacteria, and filamentous fungi may also grow throughout fermentation and can affect bean quality and cocoa flavor (Ardhana & Fleet, 2003; Schwan, Rose, & Board, 1995; Schwan & Wheals, 2004). A diversity of yeast species has been reported as the primary colonizers of cocoa fermentations. *Saccharomyces*, *Hanseniaspora* (anamorph *Kloeckera*) and

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Pichia are the predominant genera found. Pereira, Miguel, Ramos, and Schwan (2012) reported that *Saccharomyces cerevisiae* reached maximum populations of 10^7 CFU/g at 24 h during cocoa fermentations conducted in a box, after which it was eclipsed by a range of other species, such as *Pichia kudriavzevii* (anamorph *Candida krusei*, also previously described as *Issatchenkia orientalis*). *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*), *Wickerhamomyces* sp., *Candida ethanolica*, *Candida inconspicua*, *Candida humilis*, *Candida xylopsoci*, *Candida intermedia*, *Debaryomyces etchellsii*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica* were also identified in these fermentations.

The LAB associated with fermentation of cocoa beans have been studied by both cultural and culture independent (PCR-DGGE) methods, as well as molecular methods for species and strain characterization (Camu et al., 2008; Nielsen et al., 2007). Similar profiles were obtained for all fermentations. Both *Lactobacillus plantarum* and *Lactobacillus fermentum* dominated the fermentations, with *L. plantarum* being more prevalent in the earlier stages of the fermentation and *L. fermentum* predominating at the later stages (Pereira, Magalhães, Almeida, Coelho, & Schwan, 2013). The development of yeasts and LAB causes changes in the cocoa mass allowing obligate aerobic acetic acid bacteria (AAB) to replicate and to oxidize ethanol, produced by yeasts, into acetic acid (Schwan & Wheals, 2004). *Acetobacter aceti*, *Acetobacter pasteurianus*, *Acetobacter ghanensis*, *Acetobacter senegalensis*, *Acetobacter lovaniensis*, *Acetobacter fabarum*, *Gluconobacter oxydans*, and *Gluconobacter xylinus* were the main species of acetic acid bacteria isolated during cocoa fermentations in Brazil and in the studies of Schwan and Wheals (2004), Camu et al. (2007), Papalexandratou, Vranckena, De Bruyneb, Vandammeb, and De Vuys (2011) and Pereira et al. (2013).

Different volatile and non-volatile compounds such as alcohols, aldehydes, ketones, esters, carboxylic acids, pyrazines and sugars are produced as a consequence of the microbial activity during cocoa fermentation process (Rodriguez-Campos, Escalona-buendía, Orozco-Avila, Lugo-Cervantes, & Jaramillo-Flores, 2011).

During fermentation, ethanol and acetic acid diffuse into the beans and, combined with the increased temperature, kill the seed embryo; consequently, the internal structure of the cocoa bean breaks down, releasing compounds and pigments that interact biochemically with activated endogenous hydrolases to develop flavor and color precursors (Afoakwa, Paterson, Fowler, & Ryan, 2008; Schwan & Wheals, 2004). This study investigated the microbial communities and the physicochemical changes inside and outside of cocoa beans during the spontaneous fermentation of the three cocoa hybrids cultivated in Brazil.

2. Materials and methods

2.1. Fermentation experiments and sampling

The fermentation experiments were conducted at the Vale do Juliana cocoa farm in Igrapiúna, Bahia, Brazil. The ripe cocoa pods from three different hybrids [Porto Híbrido 9 (PH 9), Porto Híbrido 15 (PH 15) and Porto Híbrido 16 (PH 16)] (Fig. 1) were harvested during the main crop of 2011 (May and June). The cocoa pods were manually opened with a machete, and the beans were immediately transferred to the fermentation house. The fermentation started approximately 4 h after the breaking of the pods and was performed in 0.06 m³ wooden boxes (Schwan & Wheals, 2004). Fermentation was evaluated during 6 days (144 h), and the amount used for each one of the hybrids was 60 kg. The pH value and temperature were evaluated during fermentations.

Bean samples of approximately 100 g each were withdrawn every 12 h during the fermentation process. The samples were taken approximately 40 cm from the surface of the center of the fermenting cocoa mass, placed in sterile plastic pots and transferred to the laboratory. The samples for chemical and culture-independent analyses were stored at −20 °C.

2.1.1. Physical–chemical analysis

2.1.1.1. Sugars, alcohols and organic acids analysis.
The carbohydrates, alcohols and organic acids were extracted as described by Rodriguez-Campos et al. (2011) with minor modifications. Ten grams of fermented cocoa beans from each sample were extracted twice with 10 mL of Milli-Q water with vortexing for 5 min, and each 10 mL homogenate was transferred to another tube. The tubes containing 20 mL of solution were centrifuged (7000 rpm, 10 min, 4 °C), and the supernatant was separated from the precipitate. The precipitate was resuspended in an additional 5 mL of Milli-Q water, and vortexed and centrifuged as described above. The final volume of 25 mL of diluted pulp was centrifuged, and 2 mL of supernatant was filtered through a 0.22 μm membrane (Millipore) for the HPLC analysis of sugars, alcohols and organic acids. After the extraction of compounds (sugars, alcohols and acids) from the cocoa pulp, the testa was separated from the cocoa beans. The cocoa beans were crushed with a pestle and mortar, and extractions were performed as described above.

2.1.1.2. HPLC analyses.
The carbohydrate (glucose, sucrose and fructose), organic acid (acetic acid, lactic acid, malic acid, citric acid and succinic acid) and alcohol (ethanol, glycerol and methanol) analyses were carried out using a liquid chromatography system (Shimadzu, model LC-10A*i*, Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV–Vis detector (SPD 10A*i*) and a refractive index detector (RID-10A*i*). A Shimadzu ion exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was operated at 30 °C for carbohydrates and alcohols and 50 °C for acids. Perchloric acid (100 mM) was used as the eluent at a flow rate of 0.6 mL/min. The acids were detected via UV absorbance (210 nm), while the alcohols and carbohydrates were detected via RID (Miguel, Santos, Duarte, Almeida, & Schwan, 2012). All samples were analyzed in triplicate, and individual compounds were identified based on the retention time of standards injected using the same conditions. The sample concentrations were determined using an external calibration method. Calibration curves were constructed by injecting different concentrations of the standards under the same conditions of sample analysis and the areas obtained were plotted a linear curve whose equation was used to estimate the concentration of the compounds in the sample.

2.2. Microbial count

Fifteen grams of cocoa beans were added to 135 mL of sterile peptone water [0.1% v/w bacteriological peptone (Himedia), pH 7.0 ± 0.2], homogenized for 10 min in a stomacher (Mayo Homogenius HG 400, São Paulo, Brazil) and serially diluted. The LAB were enumerated by pour-plate inoculation in MRS agar (De Man Rogosa Sharpe, Merck, Darmstadt, Germany) containing 0.4% (v/v) nystatin (Merck) to inhibit yeast growth; the AAB were enumerated by spread-plate inoculation on GYC agar [50 g/L glucose (Merck), 10 g/L yeast extract (Merck), 30 g/L calcium carbonate (Merck) and 20 g/L agar (Merck), pH 5.6] supplemented with 0.4% nystatin to inhibit yeast growth. The yeasts were enumerated by spread-plate inoculation on YEPG agar [1% yeast extract, 2% peptone (Himedia), 2% glucose at pH 3.5] containing 100 mg/L chloramphenicol (Sigma) to inhibit bacterial growth. Nutrient agar (Merck) containing 0.4% nystatin was used as a general medium for enumerating viable mesophilic bacteria. The MRS and nutrient agar plates were incubated at 30 °C for 3–4 days; GYC and YEPG agar cultures were incubated at 28 °C for 3–4 days. After the incubation period, the number of colony-forming units (CFU) was recorded. Colonies were picked at random in a number equal to the square root of the total number of colonies present on counted plates, seeking to ensure that all different colony morphologies were recovered in each case (Senguna, Nielsen, Karapinar, & Jakobsen, 2009). All samples of microbial analysis were done in triplicate and the calculation of standard deviation was performed using the program Microsoft Office Excel 2007.

Fig. 1. Fruit, pulp and beans of the three different cocoa hybrids — PH 9 (A), PH 15 (B) and PH 16 (C).

2.3. Culture-independent microbiological analysis

2.3.1. PCR analysis

The total DNA was extracted from samples with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions for DNA purification from tissues. The DNA was stored at −20 °C until further use.

The DNA from the bacterial community was amplified with the primers 338fgc (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) (the GC clamp is underlined) and 518r (5′-ATT ACC GCG GCTGCT GG-3′), which span the V3 region of the 16S rRNA gene (Ovreas, Forney, Daae, & Torsvik, 1997). A fragment of the D1/D2 region of the 26S rRNA gene was amplified with the eukaryotic universal primers NL1GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG-3′) (the GC clamp is underlined) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′), which amplified a fragment of approximately 250 bp (Cocolin, Bisson, & Mills, 2000). All reactions were performed in 25 μL containing 0.625 U Taq DNA polymerase (Promega, Madison, USA), 2.5 μL 10× buffer, 0.1 mM dNTP, 0.2 mM each primer, 1.5 mM MgCl$_2$ and 1 μL of extracted DNA. The amplification was performed as in Ramos et al. (2010). The amplified products (2 μL) were analyzed by electrophoresis on 1% agarose gels before DGGE analysis.

2.3.2. PCR-DGGE analysis

The PCR products were separated on polyacrylamide gels [8% (w/v) acrylamide:bisacrylamide (37.5:1)] in 1× TAE buffer with a DCode System apparatus (BioRad Universal DCode Mutation Detection System, Richmond, CA, USA), according to the procedures previously described by Ramos et al. (2010). Solutions containing 35–70% denaturant [100% denaturant contains 7 M urea and 40% (v/v) formamide] were used for bacteria, and solutions containing 30–60% denaturant were used

for yeast. The gels were run at 60 °C for 6 h at a constant voltage of 120 V. After electrophoresis, the gels were stained with SYBR-Green I solution (Molecular Probes, Eugene, UK) (1:10,000 v/v) for 30 min, and the images were visualized and photographed with a transluminator LPix Image (LTB 20 × 20 HE, LPix®, Brazil).

The DGGE bands were excised from the acrylamide gels. The DNA fragments were purified with a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA, USA) and re-amplified with the primers 338fgc and 518r for bacteria and NL1 and LS2 for yeast. The PCR products were purified and sequenced with an ABI3730 XL automatic DNA sequencer (Macrogen Inc., Seoul, South Korea), and the sequences available in the GenBank database were compared with those in the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

2.4. Statistical analysis

Randomized experimental design with a factorial 3 × 5 (three hybrids × five fermentation times) was analyzed statistically using the processes of analysis of variance according to software SISVAR® version 4.5 (Lavras, Brazil), and Scott-Knott test (5% probability) was used to compare averages.

3. Results

3.1. General aspects of cocoa fruit

The cocoa fruits of different hybrids used in this study are shown in Fig. 1. Each fruit showed a different but characteristic size, peel, seed, pulp and number of beans. The PH 9 hybrid (Fig. 1A) fruit is 16.32 ± 1.27 cm in length and 9.75 ± 0.75 cm in diameter with yellowish color, a rind thickness of 522.35 ± 49.7 g, and 34–47 seeds per fruit, and the seeds weigh 3.19 ± 0.26 g each. The PH 15 hybrid

(Fig. 1B) has a red color, thin and smooth bark (431.40 ± 55.3 g), a rind that is thinner than hybrids PH 9 and PH 16, fruit measuring 14.75 ± 0.93 cm in length and 9.55 ± 0.53 cm in diameter, 42–47 seeds per fruit and seeds weighing 3.39 ± 0.18 g each. The PH 16 hybrid (Fig. 1C) produces red fruits measuring 20.5 ± 0.35 cm and 9.60 ± 0.37 cm in length and diameter, respectively, and has a rind thickness of 665.01 ± 45.5 g, 33–48 seeds per fruit and seeds weighing 4.39 ± 0.49 g.

3.2. Microbial growth

The fermentation of different cocoa hybrids resulted in the typical succession of yeast, LAB and AAB (Fig. 2). Fermentation of the PH 9 cocoa hybrid produced a higher initial yeast population (Fig. 2A). The yeast population of the fermented PH 9 cocoa hybrid increased from 3.39 log CFU/mL at 0 h of fermentation to 4.93 log CFU/mL at 36 h and remained stable up to 96 h (Fig. 2A). The yeast population
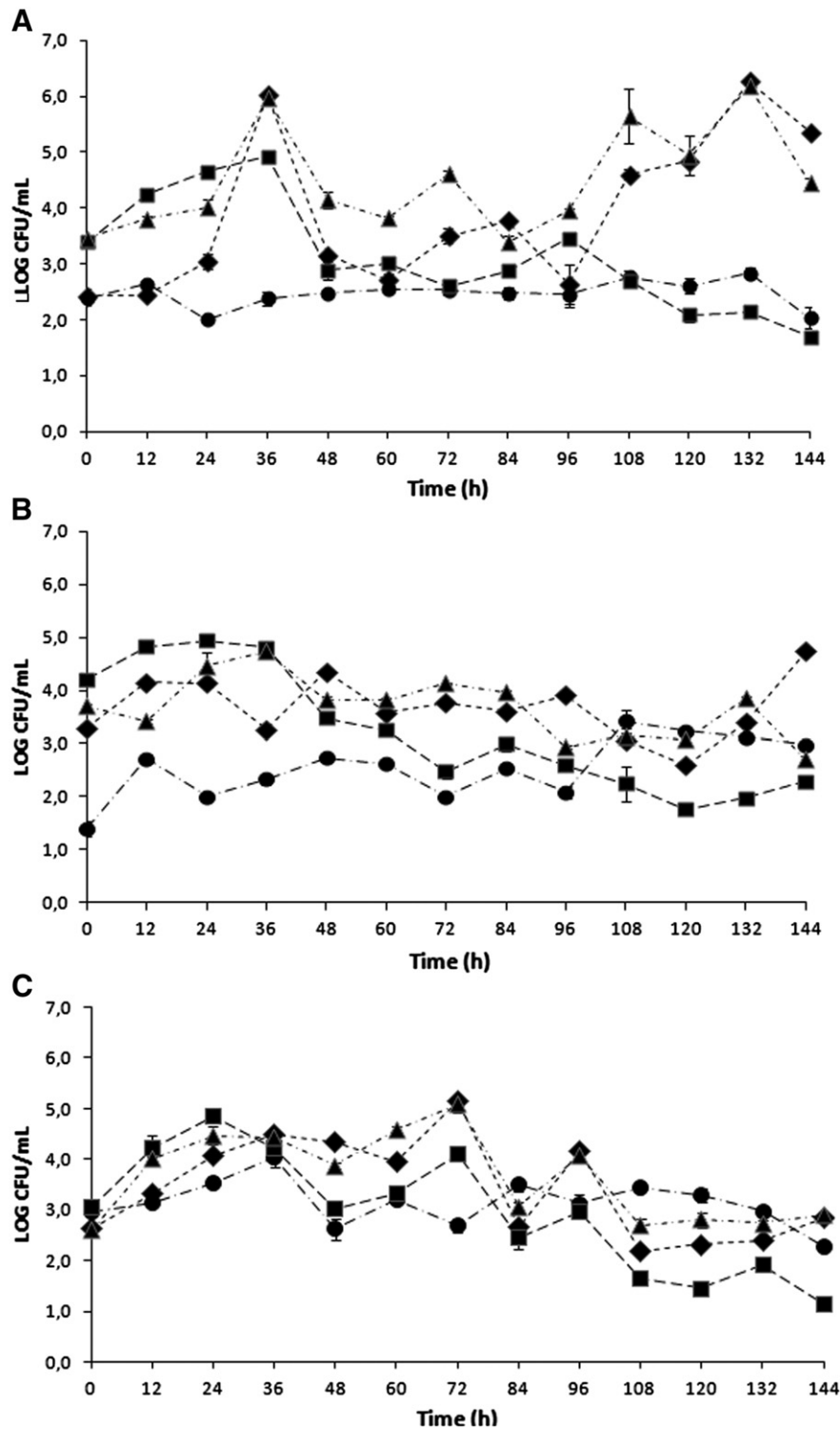

Fig. 2. Microbial succession of yeasts (YEPG counts, ■), LAB (MRS counts, ♦), AAB (GYC counts, ▲), and aerobic mesophilic bacteria (nutrient agar counts, ●) during cocoa bean fermentation using three different hybrids PH 9 (A), PH 15 (B) and PH 16 (C).

in the PH 15 hybrid fermentations increased slightly during the first 36 h of fermentation, and after this period, the populations decreased to 1.69 log CFU/mL (Fig. 2B). The yeast population during the fermentation of the PH 16 hybrid reached its highest values at 24 and 72 h of fermentation (4.84 log CFU/mL and 4.11 log CFU/mL, respectively) (Fig. 2C).

Differences in LAB group populations were observed in all fermentation experiments (Fig. 2). The highest initial count was found for hybrid PH 15 (3.30 log CFU/mL), followed by hybrids PH 16 (2.62 log CFU/mL) and PH 9 (2.43 log CFU/mL). Hybrid PH 15 showed the highest initial population of AAB (3.70 log CFU/mL), followed by hybrids PH 9 (3.46 log CFU/mL) and PH 16 (2.60 log CFU/mL). The population of AAB in the fermentation PH 9 hybrid increased from 3.46 log CFU/mL to 5.97 log CFU/mL at 36 h; afterwards, AAB decreased until the end of fermentation. The AAB during the fermentation of the PH 15 hybrid reached values between 2.70 log CFU/mL and 4.73 log CFU/mL. The AAB population in the PH 16 hybrid started at 2.60 log CFU/mL, increased to 5.08 log CFU/mL at 72 h of fermentation.

The microbial count in nutrient agar medium ranged from 2.04 log CFU/mL to 2.39 log CFU/mL for the PH 9 hybrid. The bacterial counts on nutrient agar for the PH 15 hybrid varied between 1.40 and 2.98 log CFU/mL. The microbes counted on nutrient agar during the fermentation of the PH 16 hybrid varied between 2.96 and 2.28 log CFU/mL.

In the sampling times 0, 48, 84, 120, and 144 h, significant differences (P > 0.05) were observed between the microbial average counts of the hybrids for all groups of microorganisms, except in time of fermentation 84 h, where averages of yeasts populations were statistically equal among hybrids (data not shown).

3.3. Culture-independent analysis with PCR-DGGE

Figs. 3 and 4 show the PCR-DGGE fingerprints for the eukaryotic and prokaryotic communities, respectively, of samples taken during the fermentations of the PH 9, PH 15 and PH 16 cocoa hybrids. The PCR-DGGE fingerprints demonstrate that the PH 9 hybrid had more diverse prokaryotic and eukaryotic communities than the PH 15 and PH 16 hybrids.

Fifteen DNA fragments from eukaryotes were detected by DGGE (Fig. 3 and Table 1). T. cacao was identified because universal eukaryote primers were used. The greatest diversity of yeast was found within 72–96 h of fermentation of the three cocoa hybrids. The eukaryotic DGGE profile detected S. cerevisiae (bands 2, 5 and 7) and H. uvarum (bands 1 and 3) throughout the fermentation of all hybrids. P. kudriavzevii

(band 12) was found only during fermentation of the hybrid PH 15. Particularly, Rhodotorula mucilaginosa (band 4) was detected only in the fermentation of PH 9 and PH 15 hybrids. Issatchenkia terricola (bands 13 and 14) and Aspergillus heteromorphus (band 15) were detected during fermentation of PH 9 and PH 16 hybrids. It is the first report of the presence of these species in the spontaneous fermentation of cocoa in Brazil.

The bacterial DGGE profile indicated that LAB were present throughout the fermentation of all hybrids; L. fermentum (bands 1 and 2), Lactobacillus casei (bands 4 and 5) and Lactobacillus rhamnosus (bands 9 and 10) were found throughout the fermentation period. The PH 9 cocoa hybrid contained a greater diversity of bacteria at 24 and 48 h of fermentation (Fig. 4A). Leuconostoc lactis (band 7), A. pasteurianus (band 12), Gluconobacter liquefaciens (band 11) and one bacteria of the genus Bacillus (band 13) were detected only during the fermentation of PH 9 hybrid. Regarding the prokaryote DGGE profile (Fig. 4B and C), no significant differences were detected during the fermentation of PH 15 and PH 16 hybrids.

3.4. Physical–chemical analysis

The environmental temperature ranged from 18.8 to 26 °C during the fermentation of each hybrid, but the initial temperatures inside the cocoa fermentation boxes were different. The PH 16 hybrid fermentation temperature ranged from 24.3 °C at 24 h to a maximum of 47.4 °C at 144 h. The temperature of the PH 15 hybrid ranged from 23.7 to 49.6 °C at 132 h; PH 9 hybrid reached its maximum temperature (49.3 °C) at 132 h of fermentation. The temperatures at the end of fermentation (144 h) for the PH 9 and PH 15 hybrids were 44 °C and 45.1 °C, respectively. The pH value varied during fermentation for all hybrids. The pH value of hybrid PH 16 ranged from 4 to 4.06, the pH value during hybrid PH 15 fermentation ranged from 4.03 to 4.79 and the pH value of the hybrid PH 9 ranged from 3.84 to 4.3.

3.4.1. Sugars and fermentation products

The consumption of sugars and citric acid, and the formation of metabolites during fermentation of the three hybrids are shown in Fig. 5. Sugars were consumed rapidly during the first hours (0–72 h) (Fig. 5). At the beginning of the fermentation the PH 9 hybrid contained 10.10 g glucose, 5.87 g sucrose, 12.64 g fructose and 1.42 g citric acid per kg of pulp (Fig. 5A). The PH 15 hybrid contained 13.41 g/kg glucose, 5.44 g/kg sucrose, 16.10 g/kg fructose and 1.81 g/kg citric acid (Fig. 5C). The PH 16 hybrid contained the largest initial concentrations of glucose (15.77 g/kg) and fructose (18.61 g/kg), and sucrose and citric acid
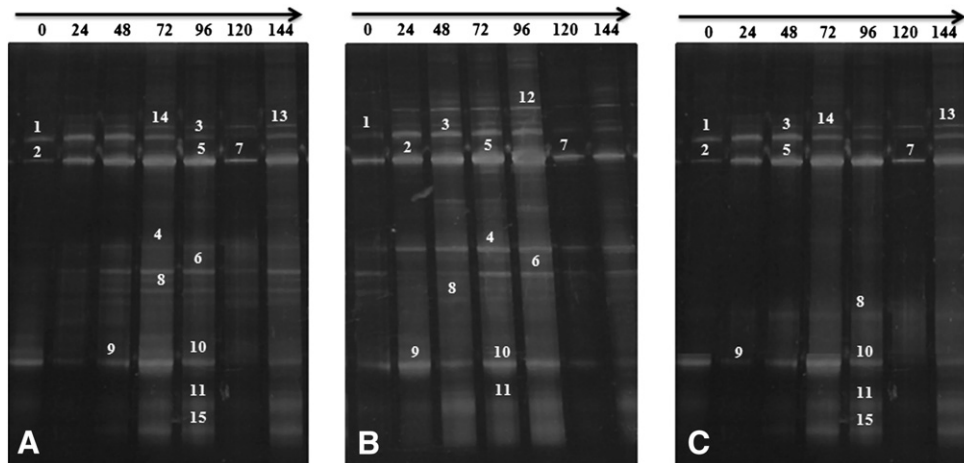

Fig. 3. Changes in eukaryote communities during cocoa bean fermentation using three different hybrids. The fingerprints were generated by PCR-DGGE of the D1/D2 region of the 26S rRNA gene for PH 9 (A), PH 15 (B) and PH 16 (C). The identities of the bands are presented in Table 1, bands indicated by numbers were excised and after reamplification they were subjected to sequencing.
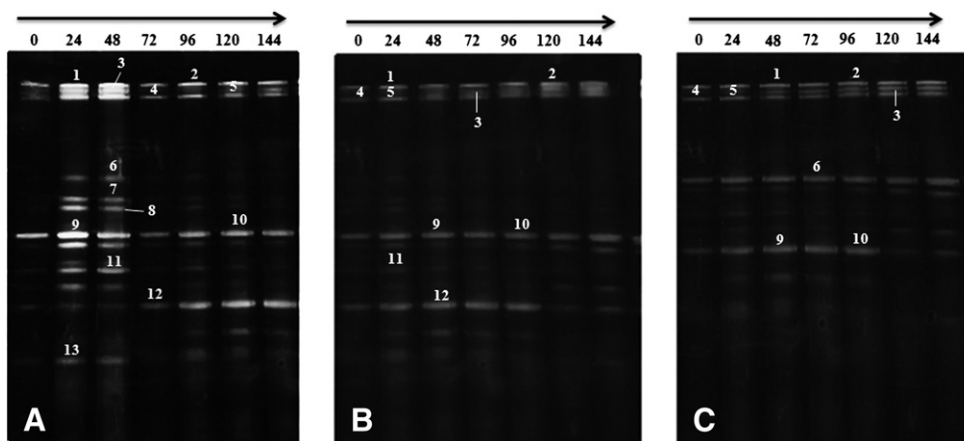
Fig. 4. Changes in prokaryote communities during fermentation of three cocoa bean hybrids. The fingerprints were generated by PCR-DGGE of the V3 region of the 16S rRNA gene for PH 9 (A), PH 15 (B) and PH 16 (C). The identities of the bands are presented in Table 1. Bands marked with numbers were excised, re-amplified and sequenced.

showed values of 5.74 g/kg and 1.54 g/kg, respectively (Fig. 5E). The consumption of these compounds was similar during fermentation of the three hybrids.

Fig. 5 (B, D, and F) showed the maximum values of some compounds (such as ethanol and acetic acid) in the pulp and inside the beans; the measurements were made at the same time. The maximum value of acetic acid in the pulp of the PH 9 hybrid was 1.65 g/kg and inside the beans was 4.54 g/kg at 96 h of fermentation (Fig. 5B). Similar peaks occurred in the PH 15 (120 h) and PH 16 (144 h) (Fig. 5D and F). The final concentration of acetic acid inside the beans was 5.25 g/kg (PH 16), 2.80 g/kg (PH 15) and 2.33 g/kg (PH 9) (Fig. 5B, D and F). The lactic acid concentrations were similar in all fermented PH 15 cocoa beans, both in the pulp and inside the beans. The PH 9 hybrid showed the highest concentration of lactic acid at the end of the fermentation process (Fig. 5B, D and F). The ethanol concentration peak observed in PH 15 hybrid was coincident at 72 h of fermentation in the pulp (3.98 g/kg) and inside the beans (5.40 g/kg) (Fig. 5D). The ethanol found outside (pulp) the cocoa beans reached a maximum concentration

at 72 h and was 7.81 g/kg in PH 16, 5.81 g/kg in PH 9 and 5.40 g/kg in PH 15. The final concentration of ethanol inside the beans was 1.66 g/kg (PH 16), 0.48 g/kg (PH 15) and 0.19 g/kg (PH 9) (Fig. 5B, D and F).

4. Discussion

This study is the first to assess the microbial populations and activities in different cocoa hybrids (PH 9, PH 15 and PH 16). The PH 16 hybrid has larger fruit and more seeds than the other hybrids. Mandarino and Sena Gomes (2009) published a technical report about the productivity of cacao (T. cacao L.) grown in monoclonal blocks in southern Bahia, Brazil. In this report, 9 hybrids were evaluated for their production of dry beans per plant; clones PH 16 and PH 15 were the third and fifth best producers, respectively. Schwan et al. (1995) described the features of Brazilian cocoa; the mature fruits develop directly from the stem of the tree and have pods with a thick outer husk and 30–40 seeds.

During fermentation of the hybrids, significant differences were observed in microbial counts. Variations in microbial counts and species depend on the type/variety of cocoa, country, farm, season, weather conditions, pod ripeness, pod diseases, post-harvest pod storage, pulp/bean ratios, fermentation method and time, batch size and frequency of bean mixing or turning (or lack thereof) (Schwan, 1998). The PH 9 hybrid showed a defined microbial succession, and it harbored the greatest diversity of microbial species, as determined by PCR-DGGE (Figs. 3 and 4).

In the present study, fewer microbes were counted in each of the three hybrids than in Comum cocoa hybrids cultivated in Brazil (Pereira et al., 2013; Schwan, 1998; Schwan & Wheals, 2004). The presence of microorganisms during cocoa bean fermentation reflects the environment (temperature, pH, and oxygen concentration) and the substrates available (which depend on the pulp composition and harvest conditions); consequently, significant amounts of ethanol, lactic acid, and acetic acid can be produced.

The biodiversity, population dynamics, and chemical content (sugars, alcohols and organic acids) of three different cocoa hybrids cultivated in Brazil were evaluated during their spontaneous fermentation. The PH 9 hybrid showed higher concentrations of sugars (glucose and fructose) (data not show) and minor concentrations of acids (lactic and acetic) inside the beans at the end of fermentation (Fig. 5B). A pleasant sensory impression of chocolate flavor can be described as flowery, green, roasted malt, caramel and nuts (Jinap, Dimick, & Hollender, 1995). Highly acidic beans have decreased flavor precursors and result in overly acidified final products (Camu et al., 2008). The yeast and bacterial community profiles of fermented PH 9 were more complex than in the PH 15 and PH 16 hybrids.

Table 1
Identification of the bands based on BLAST in comparison with those in GenBank as obtained by PCR-DGGE using universal primers for yeasts and bacteria.

	Bands[a]	Accession number	Similarity (%)
Eukaryotes			
Hanseniaspora uvarum	1, 3	EU441886.1	99
Saccharomyces cerevisiae	2, 5, 7	EU441887.1	100
Rhodotorula mucilaginosa	4	EU250050.1	99
Hanseniaspora guilliermondii	6	EF550190.1	98
Yarrowia lipolytica	8	EU239379.1	100
Theobroma cocoa	9, 10	JQ228377.1	97
Pichia kluyveri	11	EU293421.1	98
Pichia kudriavzevii	12	JQ419961.1	99
Issatchenkia terricola	13, 14	EU441898.1	97
Aspergillus heteromorphus	15	HQ876769.1	100
Prokaryotes			
Lactobacillus fermentum	1, 2	HQ293047.2	97
Streptomyces bingchenggensis	3	CP002047.1	99
Lactobacillus casei	4, 5	JQ301797.1	100
Uncultured bacterium	6	JQ455591.1	98
Leuconostoc lactis	7	HM058571.1	99
unidentified	8	–	–
Lactobacillus rhamnosus	9, 10	HE661292.1	98
Gluconobacter liquefaciens	11	FJ483515.1	97
Acetobacter pasteurianus	12	AP011170.1	99
Bacillus sp.	13	AY176766	98

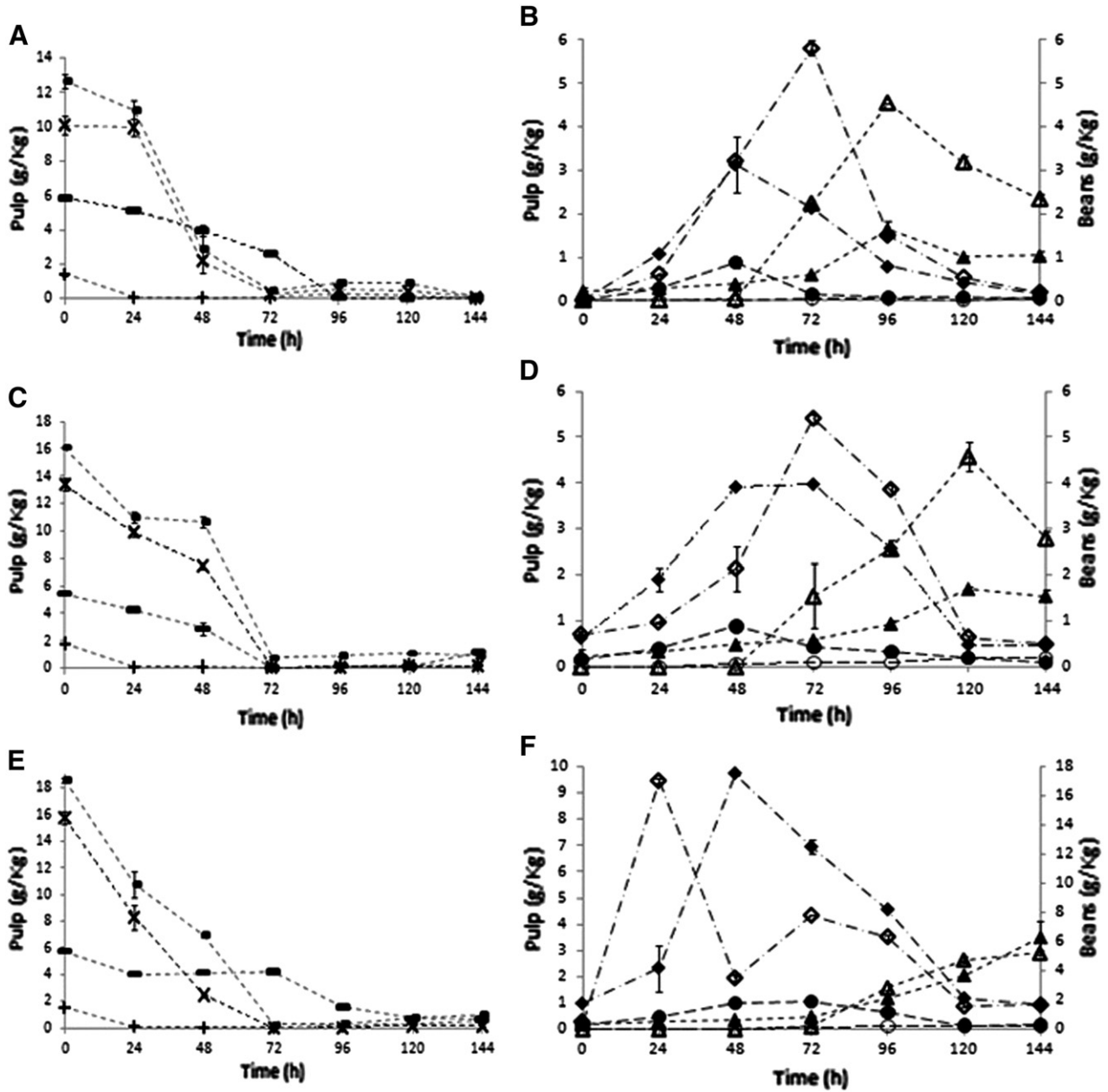[a] Bands are numbered as indicated on the DGGE gel.

Fig. 5. Carbohydrates, acids and alcohols produced in the pulp and inside the beans during fermentation of three different cocoa hybrids PH 9 (A and B), PH 15 (C and D) and PH 16 (E and F). Course of glucose (✕), sucrose (▬), fructose (●) and citric acid (✚) in the pulp cocoa (A, C and E), and course of ethanol (◆), lactic acid (●) and acetic acid (▲) in the pulp and inside the beans (open ss).

PCR-DGGE fingerprinting showed that the most common yeast (*S. cerevisiae*) (Fig. 3) and bacteria (*L. fermentum*, *L. casei* and *L. rhamnosus*) (Fig. 4) were present during the fermentation of each cocoa hybrid. A global investigation of the microbial diversity in cocoa bean fermentations would elucidate this complex process and promote the inclusion of starter cultures in fermentation methods (Schwan, 1998).

This study reports for the first time the presence of *R. mucilaginosa* (detected in hybrids PH 9 and PH 15), *I. terricola* (PH 9 and 16) and *A. heteromorphus* (PH 9 and PH 16) in Brazilian cocoa fermentations (Fig. 3). As previously reported (Ravelomanana, Guiraud, Vincent, & Galzy, 1985; Schwan, Cooper, & Wheals, 1997), yeasts accelerate fermentation by degrading pectin in cocoa pulp. *S. cerevisiae* and *H. uvarum* were the dominant yeast species during fermentation of the PH 9, PH 15 and PH 16 hybrids (Fig. 3). This result confirms the observations of Schwan and Wheals (2004) and Pereira et al. (2012), who suggested that *S. cerevisiae* and *Hanseniaspora* spp. could be used

in cocoa fermentation to enhance the aroma and flavor of chocolate. *P. kudriavzevii* was identified only in the PH 15 hybrid at 24, 48, 72, 96 and 144 h of fermentation. These results agreed with those of Daniel et al. (2009), who studied yeast diversity in heap cocoa fermentation in Ghana. *Hanseniaspora opuntiae* grew primarily during the early phase of fermentation, but *P. kudriavzevii* and *S. cerevisiae* had no specific temporal distribution.

L. fermentum, *L. casei* and *L. rhamnosus* appeared in all three cocoa hybrids. *L. lactis* was identified only in the PH 9 hybrid at 24 and 48 h (Fig. 4). The homo- and heterofermentative species *L. casei* and *L. fermentum*, respectively, were the most prevalent LAB species. These species corresponded with increased concentrations of lactic acid, acetic acid and ethanol. *L. fermentum* and *A. pasteurianus* – identified during PH 9 and PH 15 fermentations – are commonly found during the fermentation of cocoa beans in other countries (Ardhana & Fleet, 2003; Nielsen et al., 2007). *L. fermentum* is strictly heterofermentative. This species

converts citric acid, ferments glucose, oxidizes ethanol, and uses fructose as an alternative external electron acceptor to convert it into mannitol. As it can be seen in Fig. 5A, B, C and D with 72 h of fermentation there was a decrease in glucose and citric acid concentrations and a simultaneous increase in ethanol content.

A. pasteurianus strains are desirable during cocoa bean fermentation because they produce acetic acid from carbohydrates, citric acid and ethanol (Schwan & Wheals, 2004). *A. pasteurianus*, *G. liquefaciens* and *Bacillus* sp. were found during PH 9 fermentation. These species are present in cocoa fermented in Ghana, Indonesia and Brazil (Ardhana & Fleet, 2003; Nielsen et al., 2007; Schwan & Wheals, 2004). *A. pasteurianus* was identified in the PCR-DGGE profiles of hybrids PH 9 and PH 15. The genus *Acetobacter* is composed of acetic acid bacteria that oxidize acetic acid, yielding CO_2 (Nielsen et al., 2007). This information was confirmed by chemical analysis (Fig. 5). The acetic acid concentration first increased but eventually decreased by the end of fermentation of the PH 9 and PH 15 hybrids (Fig. 5B and D).

Bacillus spp. were identified in the PH 9 samples (Fig. 4). The role of *Bacillus* in cocoa fermentation is not well understood. These microorganisms are commonly used in biotechnological industries because they produce large quantities of extracellular enzymes (Schallmey, Singh, & Ward, 2004), short-chained free fatty acids and flavorings such as pyrazines and 2,3-butanediol (Schwan & Wheals, 2004).

Changes in the concentrations of sucrose, glucose and fructose demonstrated that there were metabolic changes during the fermentation of all three hybrids (Fig. 5). In the first 48 h of fermentation, the sugars decreased, which favored the growth of yeasts and LAB (Figs. 2 and 5). Similar results were described in spontaneous fermentation of cocoa using Comum cocoa in Brazil, Australia, Ghana and others (Ardhana & Fleet, 2003; Nielsen et al., 2007; Schwan et al., 1995). The sucrose concentration was low at the beginning of the fermentation because the pods were opened in the field before they were transported to the fermentation house. During this period, sucrose was hydrolyzed into glucose and fructose (Fig. 5). The utilization of sugars and the production of ethanol and acids in the cocoa pulp and cotyledons (Fig. 5) were correlated with yeast and bacteria growth (Fig. 2). The ethanol was evaporated, consumed and/or converted into acetic acid, through the metabolism of AAB (Fig. 2). There is a direct correlation between the development of AAB and the decrease of ethanol concentration in the pulp (Fig. 5). These findings agree with the classic descriptions of these bacteria in the literature (Schwan & Wheals, 2004).

The organic acids detected during cocoa bean fermentation were lactic, acetic and citric acids. In this study, the citric acid concentration decreased and the concentrations of lactic and acetic acids increased in all three hybrid fermentations. *L. fermentum*, *L. casei*, *L. rhamnosus*, *L. lactis*, *Pichia kluyveri* and *P. kudriavzevii* were present in all fermentations. According to Thompson, Miller, and Lopez (2001), species in the genera *Leuconostoc*, *Enterococcus*, *Lactobacillus* and *Pichia* metabolize citric acid into acetic acid, carbon dioxide and lactic acid.

AAB, in particular *A. pasteurianus*, are able to grow on ethanol, mannitol and lactate, and they convert ethanol into acetic acid. This oxidation process is responsible for the temperature increase inside the fermenting cocoa mass (Schwan & Wheals, 2004). In this study, there was an increase in the AAB population (Fig. 2), a decrease in the ethanol concentration and an increase in the acetic acid concentration (Fig. 5), which together increased both the cocoa mass temperature and the penetration of ethanol into the beans of all the hybrids. Furthermore, the acetic acid concentration inside the beans was increased. Brito et al. (2001) reported that part of the acetic acid evaporates and the remainder penetrates into the bean cotyledons. The acetic acid, ethanol and heat kill the cocoa seed embryo, change the subcellular structure of the bean and eventually develop the flavor precursors.

The total amount of alcohols and organic acids present at the end of fermentation differed between the hybrids. Ethanol, lactic acid and acetic acid were present in lower concentrations in the PH 9 hybrid fermentation. These compounds could interfere with the chocolate aroma, which may also vary with the microbial population and the amount of sugar available in the cocoa pulp. Future studies of fermented cocoa hybrids should define the standards for production of high quality chocolates.

5. Conclusions

The efficiency of the integrated management, especially the genetic breeding for the control witches' broom is indisputable, because production data indicated that productivity of Brazilian cocoa is recovering. A better understanding of the microbial communities and the physicochemical changes during the spontaneous fermentation of cocoa hybrids is a prerequisite for developing management procedures and for the production of high quality cocoa.

The hybrids studied (PH 9, PH 15, and PH 16) underwent the same protocol fermentation. PH 9 cocoa hybrid showed a well defined microbial succession, but values of microbial counts were larger compared to the other two hybrids. After fermentation higher concentrations of glucose and fructose (data not show) and minor concentrations of lactic and acetic acids inside the beans of PH 9 hybrids were detected. PCR-DGGE profiles of eukaryote and prokaryote showed higher microbial diversity among the cocoa hybrids studied. Future studies with more cocoa hybrids should investigate the impact of cocoa breeding and define the standards for producing high quality chocolate from hybrid cocoa plants.

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