The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods

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Abstract

Export of cocoa beans is of great economic importance in Ghana and several other tropical countries. Raw cocoa has an astringent unpleasant taste and a spontaneous fermentation is the first step in a process leading to cocoa beans with the characteristic cocoa flavour and taste. The microbiology of Ghanaian cocoa fermentations was investigated using culture-dependent and culture-independent methods. Samples were collected at 12 hour intervals during 96–144 hour tray and traditional heap fermentations. Yeast, Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and Bacillus spp. were enumerated on suitable substrates and identified using phenotypic and molecular methods. The yeast and bacterial micro-populations involved in the cocoa fermentation were further investigated using the culture-independent method Denaturing Gradient Gel Electrophoresis (DGGE).

A microbiological succession was observed during the fermentations. At the onset of fermentation yeasts were the dominating microorganisms. Lactic Acid Bacteria became dominant after 12–24 h of fermentation and remained predominant throughout the fermentations with AAB reaching high counts in the mid phase of fermentation. Bacillus spp. were only detected during heap fermentations where they reached high numbers during the later stages of fermentation. Hanseniaspora guilliermondii was the predominant yeast during the initial phase and Pichia membranifaciens during the later phases of fermentation. A number of other yeast species including three putatively undescribed species were isolated during the fermentations. Lactobacillus fermentum was the dominant LAB in most samples. Several other LAB including Lactobacillus plantarum, Leuconostoc pseudomesenteroides, Leuconostoc pseudofluculineum, Pediococcus acidilactici and a putatively undescribed LAB species were detected during the fermentations. Acetobacter syzygii, Acetobacter pasteurianus and Acetobacter tropicalis were the predominant AAB in all investigated fermentations. During the later stages of heap fermentation Bacillus licheniformis and occasionally other Bacillus spp. were detected in high numbers.

In general the culture-based findings were confirmed using DGGE. However, DGGE indicated that Lc. pseudofluculineum plays a more important role during the fermentation of cocoa than expected from the culture-based findings as it yielded a strong band in most DGGE fingerprints. Cluster analysis of the DGGE fingerprints revealed that the DGGE fingerprints clustered according to fermentation site. Within each fermentation site the profiles clustered according to fermentation time. The DGGE method seems to offer a relatively fast and reliable tool for studying yeast and bacterial dynamics during cocoa fermentations.

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1. Introduction

Ghana is the world’s second largest producer of cocoa beans producing approximately 20% of the world’s cocoa (Anon, 2005). Being the largest export commodity cocoa is of great
economic importance for Ghana as a country and of even bigger socio-economic importance in the cocoa growing farms and villages throughout the country. Cocoa beans are the principal raw material of chocolate and originate as seeds in fruit pods of the tree *Theobroma cacao*. Each fruit pod contains 30–40 beans embedded in a mucilaginous pulp. Raw cocoa has an astringent, unpleasant taste and flavour and has to be fermented, dried and roasted to obtain the characteristic cocoa flavour and taste (Thompson et al., 2001).

Several different fermentation systems are used around the world with heap and box fermentations being most widely used. In Ghana, the heap fermentation system dominates (Baker et al., 1994). Following harvest, the pods are broken open and the beans piled on and covered with plantain leaves. The heaps are variable in size ranging from 20 to 1000 kg (Baker et al., 1994). Aeration of the heaps by turning them every 24–72 h during the fermentation is considered beneficial for the quality of the end product, but this scheme is not followed by all farmers (Baker et al., 1994). To circumvent the laborious process of turning the heaps an experimental tray system claimed to give high-quality product, but this scheme is not followed by all farmers (Baker et al., 1994). Following harvest, the pods are broken open and the beans piled on and covered with plantain leaves. The heaps are variable in size ranging from 20 to 1000 kg (Baker et al., 1994).

In the tray fermentations the raw cocoa beans are placed in 10 cm deep trays and 8–10 trays stacked on top of each other. Air is allowed to circulate between the trays ensuring aeration of the fermenting mass without turning the beans (Allison and Kenten, 1963).

The actual fermentation takes place in the pulp surrounding the beans. The pulp is rich in glucose, fructose and sucrose (total content 10–15%) and the initial pH is relatively low (pH = 3.3–4.0), primarily due to a high concentration of citric acid (1–3%) (Roelofsen and Giesberger, 1947; Thompson et al., 2001; Ardhana and Fleet, 2003; Schwan and Wheals, 2004). A relatively high content of pectin and other polysaccharides (1–2%) makes the pulp viscous limiting diffusion of air (Pettipher, 1986; Schwan et al., 1995; Schwan and Wheals, 2004).

Fermentation of cocoa is a spontaneous microbiological process. The interior of the unopened pods are considered to be sterile or almost sterile but following opening of the pods the cocoa beans are contaminated with a variety of microorganisms originating from e.g. workers hands, containers for transport, knives, pod surfaces, etc. (Roelofsen, 1958; Thompson et al., 2001; Jespersen et al., 2005).

In the initial phases of the fermentation growth of yeasts is favoured due to the high sugar content, low pH and limited oxygen availability in the pulp (Thompson et al., 2001). The primary activity of the yeast is the production of ethanol from carbohydrates and also assimilation of citric acid and degradation of pectin have been reported as important activities (Roelofsen and Giesberger, 1947; Roelofsen, 1958; Schwan et al., 1995; Schwan and Wheals, 2004; Jespersen et al., 2005). Only a limited number of studies have investigated the yeast populations associated with Ghanaian cocoa fermentations. Carr et al. (1979) reported the involvement of *Hansenula, Kloecher*, *Torulopsis, Saccharomyces, Candida, Pichia* and *Schizosaccharomyces* spp. in Ghanaian heap fermentations. Unfortunately the isolates were only identified to genus level (Carr et al., 1979; Carr and Davies, 1980). Outside Ghana the box fermentation system is widely used. Studies of this fermentation system have shown the involvement of various *Candida, Kloecher, Pichia* and *Saccharomyces* spp. in the fermentation (Ostovar and Keeney, 1973; Gauthier et al., 1977; Schwan et al., 1995; Thompson et al., 2001; Ardhana and Fleet, 2003).

Recently, Jespersen et al. (2005) investigated the occurrence and diversity of yeast involved in Ghanaian heap and tray fermentations. This study was the first to use molecular methods for the identification of yeasts involved in the fermentation of cocoa. Jespersen et al. (2005) reported that *Candida krusei, Pichia membranifaciens* and *Hanseniaspora guilliermondii* were the major yeast species involved in the heap fermentations.

In the tray fermentations *Saccharomyces cerevisiae* contributed as well (Jespersen et al., 2005). The heap fermentations studied by Jespersen et al. (2005) were fermented for three days without turning. This is a fermentation scheme followed by some Ghanaian farmers but in general the heaps are fermented 5–6 days and by many farmers turned once or twice during the fermentation (Baker et al., 1994).

The yeast quickly consumes the oxygen present in the fermenting mass creating conditions well suited for the growth of Lactic Acid Bacteria (LAB) also fermenting the sugars producing lactic acid and assimilating citric acid (Schwan et al., 1995; Thompson et al., 2001; Ardhana and Fleet, 2003; Schwan and Wheals, 2004). Besides the mention of the possible involvement of LAB in a few early studies (Knapp, 1937) only the study by Carr et al. (1979) have investigated the role of LAB in heap fermentations. Carr et al. (1979) isolated *Lactobacillus collonides, Lb. fermentum, Lb. mali* and *Lb. plantarum*. However, only a limited number of isolates were identified and no quantitative information about the growth profiles of the different species during fermentation were reported (Carr et al., 1979; Carr and Davies, 1980). To date no studies investigating the involvement of LAB in tray cocoa fermentations have been published.

During box fermentations a number of *Lactobacillus* spp. including *Lb. celllobiosus* (later synonym of *Lb. fermentum* (Dellaglio et al., 2004)) and *Lb. plantarum* have been isolated along with various *Leuconostoc, Lactococcus* and *Pediococcus* species (Ostovar and Keeney, 1973; Carr et al., 1979; Passos et al., 1984; Thompson et al., 2001; Schwan and Wheals, 2004).

The pectinolytic activities of the yeasts causes degradation of the pulp increasing the aeration of the fermenting mass and assimilation of citric acid by yeasts and LAB causes the pH to rise (Thompson et al., 2001). This in turn favours the growth of aerobic Acetic Acid Bacteria (AAB). The AAB metabolise the ethanol initially formed by the yeasts to acetic acid through an exothermal process. Ethanol and acetic acid diffuse into the beans. This in combination with the heat produced by the activities of the AAB leads to bean death, killing the embryo, and induces the biochemical changes leading to well-fermented cocoa beans (Thompson et al., 2001). Besides the study by Carr et al. (1979), data regarding AAB involved in the fermentation of West African heap fermentations is scarce. Carr et al. (1979) reported that AAB forms a significant part of the microorganisms especially in the more aerated parts of the fermenting mass.
and identified a limited number of AAB isolated from fermenting cocoa now classified as *Gluconobacter oxydans*, *Acetobacter aceti* and *Acetobacter pasteurianus* but no quantitative information regarding the growth profiles of the individual species were reported (Carr et al., 1979; Carr and Davies, 1980; Ardhana and Fleet, 2003).

Due to the activities of the AAB, the temperature of the fermenting mass increases to 45–50 °C thereby limiting the growth of many microorganisms. The increased temperature, in combination with increased pH and aeration is associated with growth of aerobic spore-forming *Bacillus* spp. (Thompson et al., 2001). According to Carr and Davies (1980) only *B. subtilis* was isolated from Ghanaian heap fermentations. However, the number of isolates identified to species level was limited. Studies of box fermentations in Brazil, the Caribbean and Indonesia have shown a more varied *Bacillus* community with 5–10 different *Bacillus* spp. being isolated from some fermentations (Ostovar and Keeney, 1973; Schwan et al., 1986; Ardhana and Fleet, 2003). The role of *Bacillus* spp. in the fermentation of cocoa has not been fully clarified. However, due to their high enzymatic activity and production of e.g. short chain fatty acids, pyrazines and 2,3-butanediol the growth and activity of *Bacillus* spp. could cause off-flavours (Schwan et al., 1986; Schwan and Wheals, 2004).

No studies taking advantage of the development within molecular biology-based methods for identifying the bacterial populations associated with the fermentation of the cocoa have been published to date.

Due to the complexity of the process culture-based investigations of the cocoa fermentation are tedious and time consuming. Molecular biology-based finger-printing methods such as Denaturing Gradient Gel Electrophoresis (DGGE), offer a rapid alternative for investigating the process also enabling the detection of organisms difficult to cultivate by conventional methods (Muyzer and Smalla, 1998; Giraffa and Neviani, 2001). DGGE is a semi-quantitative technique based on sequence-specific separation of PCR-derived rRNA gene amplicons in polyacrylamide gels containing a linearly increasing concentration of denaturant (urea and formamide) as described by Muyzer and Smalla (1998). During recent years DGGE has been used to investigate microbiological dynamics in fermented products including wine, fermented cassava, maize dough and coffee beans (Ampe and Miambi, 2000; Cocolin et al., 2000; Masoud et al., 2004; Prakitchaiwattana et al., 2004). Recently we have showed that DGGE is a technique well suited for the study of yeast population dynamics during cocoa fermentations (Nielsen et al., 2005). No studies investigating the bacterial community dynamics during cocoa fermentations using DGGE have been published so far.

The differences in the quality of fermented cocoa beans experienced from country to country, farm to farm and even batch to batch are calling for the development of quality management procedures to ensure cocoa of a consistent high-quality (Lopez and Dimick, 1995). A prerequisite for developing quality management procedures for the fermentation of cocoa is a thorough understanding of the microbiological processes behind. Here we present a detailed microbiological investigation of Ghanaian heap and tray cocoa fermentations for the first time using molecular biology-based methods in combination with phenotypic methods for the microbiological identifications with the aim of reporting detailed quantitative data about the growth and succession of species of yeasts, LAB, AAB and *Bacillus* spp. during the fermentations. In addition bacterial and yeast population dynamics during the fermentations are studied using DGGE in a culture-independent approach.

2. Materials and methods

2.1. Cocoa fermentations

Cocoa pods were harvested by traditional methods during January, August and October 2003 and August 2005 (ambient temperature during the day 28–30 °C) in Mixed Hybrid Cocoa plantations in Ghana, near New Tafo (Eastern Region), Mampong (Eastern Region) and Bompata (Ashanti Region). The cocoa beans were fermented either in tray or traditional heaps at the Cocoa Research Institute of Ghana (CRIG), New Tafo, or as heap fermentations at farms in Mampong and Bompata. The pods were harvested over 3–7 days and opened on the following day with a cutlass. Following opening of the pods the beans were placed on either plantain leaves (heap fermentations) or in wooden trays (tray fermentations). In the heap fermentations approximately 50 kg (small heap) or 500–750 kg (large heap) beans were piled on plantain leaves, covered with plantain leaves and left to ferment. The small heap fermentation was fermented for 4 days with no turning, whereas the large heap fermentations were fermented for 6 days and turned after 48 and 96 h. In the tray fermentations approximately 100 kg beans were placed in each tray [90×120 cm, 10 cm deep, with holes drilled in the base and at the sides to allow aeration and drainage of liquids (sweatings) produced during the fermentations]. 8 trays were stacked on top of each other, the top tray covered with plantain, leaves and left to ferment for 4 days. Following fermentation the beans were sun-dried for 6–10 days.

2.2. Sampling

At 12 hour intervals approximately 100 g beans were sampled with a sterile plastic bag. From the small and large heap fermentations samples were taken approximately 15 cm from the surface. From the large heap fermentation carried out at CRIG samples were also taken from the centre of the fermenting mass. From the tray fermentations samples were taken in the centre of the fermenting mass from the top tray.

2.3. Physical and chemical analysis

The small and large heap fermentations carried out at CRIG and one of the tray fermentations were subjected to various physical and chemical analysis: The temperature was recorded at each sampling site by inserting a thermometer into the fermenting mass. For pH and substrate and metabolite concentration determinations of the pulp the pulp was separated
from the beans basically following the protocol of Ardhana and Fleet (2003). Bean and pulp were physically separated by adding 50 g of distilled H$_2$O (dH$_2$O) to 50 g of sample in a plastic bag and massaging the bag for 5 min. The pulp fraction was recovered by decanting. Twenty g of the pulp fraction was added to 80 g of dH$_2$O, mixed in a Warring blender (Warring Products, New Hartford, USA) and filtered through a 0.45 μm filter (Cameo, Micron Separations Inc., Westboro, USA). One fraction of the filtrate was stored at −20 °C for further analysis. The pH of another fraction of the filtrate was determined using a pH-meter (PHM82, Radiometer A/S, Brønshøj, Denmark). The concentration of sucrose, glucose, fructose, citric acid, acetic acid and ethanol in the filtrate was determined by HPLC (HP series 1100, Hewlett-Packard Company, USA) with an Aminex 87H column (Bio-Rad Laboratories, Hercules, USA) connected to a RI detector (HP1047A, Hewlett-Packard Company). The column was eluted with a degassed mobile phase containing 1 mM H$_2$SO$_4$, pH 2.75, at 30 °C and at a flow rate of 0.5 ml/min (Nissen et al., 2003). The fully fermented sun-dried beans were visually assessed using the cut test (Wood and Lass, 1985; Senenayake et al., 1995).

2.4. Isolation, purification and maintenance of microorganisms

Culture-based microbiological investigations were carried out for the small and large heap fermentations carried out at CRIG and one of the tray fermentations. For culture-based isolations, 20 g of cocoa beans and adhering pulp was added to 180 ml of 1 × Ringers Solution (Merck, Darmstadt, Germany) and mixed for 2 min in a Warring blender at medium speed. From this dilution 10-fold dilutions were prepared in 1 × Ringers. Yeasts were enumerated by surface inoculation on Malt Extract Agar (Merck) containing 100 mg/l chloramphenicol (Sigma, St. Louis, USA) and 50 mg/l chlorotetracline (Sigma) with 3–5 days incubation at 25 °C. Lactic Acid Bacteria were enumerated by surface inoculation on MRS agar (Merck) containing 0.2% sorbic acid (Merck) and 0.1% cycloheximide (Merck) to suppress growth of yeasts, and 0.1% cystein-HCl to obtain anaerobic conditions during incubation (sealed polyethylene bags, 3–4 days, 30 °C). Acetic Acid Bacteria were enumerated by surface inoculation on GYC Agar (glucose 50 g/l (Merck), Yeast extract 10 g/l (Merck), Calcium carbonate 30 g/l (Merck), agar 20 g/l (Merck), pH = 5.6), containing 0.1% cycloheximide to inhibit yeasts and 50 mg/l penicillin (Sigma) to inhibit LAB and incubated for 5–8 days at 25 °C (Drysdale and Fleet, 1988; Ardhana and Fleet, 2003). Aerobic mesophilic bacterial count and Bacillus spp. were enumerated by surface inoculation on Nutrient Agar (Merck) containing 0.1% cycloheximide and incubated for 3 days at 30 °C. Following incubation the number of colony forming units (CFU) was recorded. Subsequently, the morphological characteristics of each colony type were recorded and counts made for each type. For each colony type a number of colonies corresponding to the square root of the number of colonies of that type were streaked and purified (247 yeast isolates, 509 LAB isolates, 255 AAB isolates and 526 isolates from NA were obtained). For long term storage purified isolates were stored at −80 °C. Yeasts were stored in YGP broth (Yeast extract 5 g/l (Merck), glucose 10 g/l, Peptone 10 g/l, pH = 5.6) containing 20% (w/w) glycerol; LAB were stored in 10% skim milk (Merck) containing 20% (w/w) glycerol; AAB were stored in YG broth (Yeast extract 10 g/l; glucose 50 g/l, pH = 5.6) containing 20% glycerol; isolates originating from NA were stored in Nutrient broth (Sigma) containing 20% glycerol (w/w) and 10% skim milk (Merck).

2.5. Identification of yeasts

Initially all yeast isolates were micro- and macro-morphologically characterised as described by Jespersen et al. (2005). Subsequently, the isolates were grouped by rep-PCR and Internal Transcribed Spacer (ITS)-PCR. Initially total cDNA was extracted using the InstaGene Matrix DNA extraction kit following the instructions of the manufacturer (Bio-Rad, Hercules, CA, USA). The rep-PCR reaction was carried out in a 25 μl volume containing 1 U Taq DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA), 2.5 μl 10 × PCR reaction buffer (Amersham Biosciences), 200 μM of each deoxynucleotide triphosphate (Amersham Biosciences), 3.0 mM MgCl$_2$ (Amersham Biosciences), 0.8 μM of primer GTG$_2$ (5′-GTG GTG GTG GTG GTG-3′, DNA Technologies, Aarhus, Denmark), 1% (vol/vol) formamide (Merck), 0.1% (wt/vol) Bovine Serum Albumin (BSA, New England Biolabs, Beverly, USA), 1.5 μl of DNA template, and sterile MilliQ water for adjustment of the volume to 25 μl. The PCR reaction was performed on a Biometra Trio-Thermoblock (Biotron, Göttingen, Germany) under the following thermocycling program: 5 min of initial denaturation at 94 °C, 30 cycles of 95 °C for 30 s, 45 °C for 60 s, 72 °C for 30 s. 15 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by a final elongation step of 60 °C for 16 min (Andrade et al., 2006). The PCR products were separated by 1.5% agarose gel electrophoresis in 1 × TBE (5 h, 140 V) using a Generuler 1 kb DNA ladder as reference (Fermentas, Vilnius, Lithuania). Following electrophoresis gels were stained with ethidium bromide and documented using a Kodak EDAS 290 system (Eastman Kodak, New Haven, USA). The rep-PCR-profiles were normalised and cluster analysis performed using Bionumerics 2.50 (Applied Maths, Sint-Martens-Latem, Belgium). The dendrograms were calculated on the basis of the Dice’s Coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA). ITS-PCR was carried out using primers ITS1 and ITS4 (Esteve-Zarzozo et al., 1999), the PCR mixture described in Nielsen et al. (2005) and the thermocycling conditions lined out in Esteve-Zarzozo et al. (1999). The PCR-products were separated by 1.8% agarose gel electrophoresis using a 100 bp DNA ladder as reference (Fermentas). Gels were ethidium bromide stained, documented and analysed as described above.

Following cluster analysis, representative isolates representing different clusters were identified by sequencing of the D1/D2-region of the 26S rRNA gene, basically following the protocol of Jespersen et al. (2005), and on the basis of carbohydrate assimilation profiles determined using the API ID 32 C kit (BioMerieux, Marcy-L’Etoile, France).
2.6. Identification of Lactic Acid Bacteria

Initial grouping of the LAB isolates were performed on the basis of cell morphology, Gram, catalase and oxidase reactions, growth at 15, 37 and 45 °C determined in MRS broth, gas production from glucose (MRS broth with inverted Durham tubes, determined at 30 °C), and production of NH₃ from arginine (Schillinger and Lücke, 1987). The configuration of the lactic acid enantiomer produced was determined enzymatically (Boehringer Mannheim GmbH, Mannheim, Germany). All homofermentative isolates were tested for the presence of D-meso-diaminopimelic acid (mDAP) in the cell wall (Schillinger and Lücke, 1987).

The carbohydrate fermentation patterns of more than 150 isolates were determined in microtitre plates basically following the protocol of Jayne-Williams (1976). Each isolate was tested for the ability to ferment: amygdaline, arabinose, esculin, cellobiose, fructose, galactose, gluconate, glucose, glycerol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, sucrose, salicin, sorbitol, trehalose and xylose (Jayne-Williams, 1976).

Molecular biology-based grouping of the LAB isolates was performed using rep-PCR (Gevers et al., 2001). DNA was extracted using a previously described protocol (Björkroth and Korkeala, 1996) or using the InstaGene Matrix DNA extraction kit following the instructions of the manufacturer (Bio-Rad). Rep-PCR (GTG₅-primer) was carried out basically as described by Gevers et al. (2001) followed by 1.8% agarose gel electrophoresis at 55 V for 16 h. Following electrophoresis, gels were stained with ethidium bromide, documented, and analysed using BioNumerics 2.50 (Applied Maths) as described above.

Based on the phenotypic and genotypic grouping, representative isolates were selected and the 16S rRNA gene sequenced using a CEQ 2000 Automated Sequencer (Beckmann Coulter, Fullerton, CA, USA), and a CEQ 2000 Dye Terminator Cycle Sequencing Quick Start kit (Beckmann Coulter) or sent to a commercial sequencing facility (DNA-Technology, Aarhus, Denmark). Sequencing was performed using a CEQ 2000 Automated Sequencer (Beckmann Coulter, Fullerton, CA, USA), and a CEQ 2000 Dye Terminator Cycle Sequencing Quick Start kit (Beckmann Coulter) or sent to a commercial sequencing facility (DNA-Technology, Aarhus, Denmark). The primers 7f, 338f, 515r, 968r, 1401r and 1510r (Satokari et al., 2001) were used in the sequencing reactions. Sequences were aligned to 16S rRNA gene sequences in the Genbank database using the BLAST algorithm (Altschul et al., 1997).

Isolates identified as Lb. plantarum/Lb. paraplantarum/Lb. pentosus were differentiated by multiplex PCR with primers targeting the recA gene as described elsewhere (Torriani et al., 2001).

Following purification, it was impossible to revive a limited number of LAB isolates. These isolates were identified directly by grouping them using rep-PCR and sequencing of the 16S rRNA gene as described above.

2.7. Identification of Acetic Acid Bacteria

Gram negative and Gram variable, catalase positive isolates causing clearing of GYC agar due to acid production during growth were considered presumptive AAB. Cell morphology and motility were determined after 3–4 days of growth at 25 °C in YG broth. Production of water-soluble brown pigments was determined after 5 days growth (25 °C) on GYC agar (Drysdale and Fleet, 1988; Gillis et al., 1989). The AAB isolates were identified by Restriction Fragment Length Polymorphism (RFLP) of the 16S rRNA gene and the 16S-ITS-23S region as described elsewhere (González et al., 2006). Results of the PCR-RFLP analysis were confirmed by sequencing of the 16S rRNA gene as described above. Furthermore, the carbohydrate assimilation profile of a few isolates representing all identified species were determined using ABI 50 CHB strips (BioMerieux) incubated at 30 °C and read after 48 and 96 h.

2.8. Identification of Bacillus spp.

Following testing of Gram and catalase reactions, presumptive Bacillus spp. isolates were grown in Nutrient broth added MnCl₂ (50 mg/l) to enhance sporulation, and the micro-morphology examined. DNA was extracted from endospore-forming isolates using the InstaGene Matrix (Bio-Rad), following the instructions of the manufacturer. Following DNA extraction, the 16S-ITS-23S region of the rRNA gene was amplified according to Daffonchio et al. (1998), and the fragments separated by 2% agarose gel electrophoresis (1 × TBE, 100 V, 2 h) using a Generuler 100 bp DNA ladder (Fermentas) as reference. Gels were normalised and cluster analysis performed using BioNumerics 2.50 (Applied Maths) as described above. Grouping on the basis of the 16S-ITS-23S region was confirmed and extended by rep-PCR (GTG₅-primer) carried out as described above. Representative isolates were identified by 16S rRNA gene sequencing as described above and by the use API 50 CHB strips (BioMerieux) incubated at 37 °C, and read after 24 and 48 h.

2.9. Denaturing Gradient Gel Electrophoresis (DGGE)

In addition to the heap and tray fermentations subjected to physical, chemical and culture-based microbiological investigations as lined out above two additional tray fermentations carried out at CRIG and two additional large heap fermentations carried out at farms in Mambong and Bompata, respectively, were included in the DGGE analysis. For the DGGE-investigations, pulp was scraped of the beans, freeze-dried and DNA extracted following the protocol of Nielsen et al. (2005).

Using eukaryotic universal primers primers (NL1GC and LS2), an approximately 250 bp long fragment of the D1/D2-region of the 26S rRNA gene was amplified using PCR as described by Nielsen et al. (2005).

Two sets of universal bacterial primers were tested for the amplification of a fragment of the 16S rRNA gene. The V3 region of the 16S rRNA gene was amplified using the primer set PRBA338fGC/PRUN518r (Övreås et al., 1997) and the V6–V8 region of the 16S rRNA gene amplified using primers 968fGC and 1401r (Zoetendal et al., 1998). The reaction mixture was as described by Nielsen et al. (2005) and the thermocycling conditions as described by Övreås et al. (1997) for the V3-primers, and (except for a higher annealing temperature: 72 °C instead of 68 °C) by Zoetendal et al. (1998) for the V6–V8-primers. All PCR reactions were carried out on a Biometra Trio-
Thermoblock (Biotron). Size and amount of the PCR products were estimated by analysing 10 μl samples by agarose gel (1.5% wt/vol) electrophoresis and ethidium bromide staining.

The DGGE analysis was basically performed as first described by Muyzer et al. (1993) using a DCode System apparatus (Bio-Rad, Hercules, USA). Polyacrylamide gels (8% (wt/vol) acrylamide–bisacrylamide (37.5:1) (Bio-Rad)) in 1×TAE buffer were prepared with a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad) using solutions containing from 35 to 70% denaturant [100% denaturant corresponds to 7 M urea (ICN Biomedicals, Aurora, USA) and 40% (vol/vol) formamide (Merck)] (Muyzer et al., 1993). Gels were run at 60 °C for 16 h at a constant voltage of 70 V. After electrophoresis, gels were stained with SYBR-GOLD (Molecular Probes, Eugene, USA) for 20 min and photographed with UV transillumination (302 nm) using a Kodak EDAS 290 system (Eastman Kodak).

The identity of selected bands in the DGGE profiles was revealed by sequencing. DNA fragments from selected bands

Table 1

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<th>24</th>
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<th>48</th>
<th>60</th>
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<th>84</th>
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<td>4.12</td>
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<td>7.88</td>
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<td>6.25</td>
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<td>100</td>
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<tr>
<td>Bacillus spp. log(CFU Bacillus/g)</td>
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<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>45</td>
<td>N.D.</td>
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<td>% of Bacillus population</td>
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<td>B. megaterium</td>
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</tr>
</tbody>
</table>


a Detected sporadically.

b N.D.: None detected.
were excised from the gels, reamplified, the electrophoretic mobility relative to the fragment from which they were excised, checked and the fragments sequenced following the protocol of Nielsen et al. (2005) and the primers described above. Identification of specific bands in the DGGE gels was further aided by analysing pure cultures isolated during this study using the PCR and DGGE conditions described above.

To facilitate comparison of different DGGE gels a standard consisting of five yeast isolates (all originating from cocoa fermentations carried out in New Tafo, Ghana, and identified

### Table 2
Temperature of the fermenting mass, pulp pH and log(CFU/g) of yeasts, Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and Bacillus spp. (standard deviations in brackets) determined 15 cm from the surface of a large heap cocoa fermentation turned after 48 and 96 h

<table>
<thead>
<tr>
<th>Fermentation time (h)</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
<th>72</th>
<th>84</th>
<th>96</th>
<th>108</th>
<th>120</th>
<th>132</th>
<th>144</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature, °C</td>
<td>28.0</td>
<td>29.5</td>
<td>34.0</td>
<td>36.0</td>
<td>43.0</td>
<td>48.0</td>
<td>48.0</td>
<td>44.0</td>
<td>42.0</td>
<td>44.5</td>
<td>44.0</td>
<td>46.0</td>
<td>44.0</td>
</tr>
<tr>
<td>Yeast, log(CFU&lt;sub&gt;yeast/g&lt;/sub&gt;)</td>
<td>6.97</td>
<td>7.24</td>
<td>6.60</td>
<td>4.90</td>
<td>5.38</td>
<td>5.08</td>
<td>6.87</td>
<td>6.67</td>
<td>7.32</td>
<td>5.40</td>
<td>7.86</td>
<td>5.65</td>
<td>5.55</td>
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<tr>
<td></td>
<td>(0.04)</td>
<td>(0.03)</td>
<td>(0.21)</td>
<td>(0.43)</td>
<td>(0.25)</td>
<td>(0)</td>
<td>(0.02)</td>
<td>(0.16)</td>
<td>(0.34)</td>
<td>(0.02)</td>
<td>(0.12)</td>
<td>(0.14)</td>
<td>(0.14)</td>
</tr>
</tbody>
</table>

% of yeast population

- *H. guilliermondii*: 79, 67, 71
- *S. crataegensis*: 7
- *P. piperi*: 9, 2
- Unidentified Species C: 3
- *I. hanoiensis*: 2
- *C. zemplinina*: 19, 14
- *C. michaelii*: 3
- *C. diversa*: 2
- *P. membranifaciens*: Det.\(^a\) 100 70 50 100 100 90 100 100 100 100 100 100
- *C. lankesteriana*: 15
- *Sc. cerevisiae*: 15 17
- Schiz. pombe: 33
- *I. orientalis*: 10

% of LAB population

- *Lb. plantarum*: 60 21 5 9 9
- *Lb. fermentum*: 40 81 5 5 9 9 8
- Unidentified Species D: 5 5
- *Lc. pseudoficulneum*: 8
- *Lb. hilgardii\(^b\)*: 19
- *Pd. acidilactici*: 5

% of AAB population

- *A. pasteurianus*: 100 55 22 62
- *A. syzygii*: 57 33 72
- *A. tropicalis*: 29 45 100 45
- *A. malorum*: 14
- *G. oxydans*: 14

% of Bacillus spp. log(CFU<sub>Bacillus/g</sub>)

- N.D.: None detected.
- Isolates did not grow upon purification. Identified directly by rep-PCR grouping and 16S rRNA gene sequencing (see text for details).
- Det.: Detected sporadically.
- No data, plates partly overgrown by slimy non-AAB. CFU<sub>AAB</sub> and AAB composition approximately as data for 60 h of fermentation.


*a* Detected sporadically.

*b* Isolates did not grow upon purification. Identified directly by rep-PCR grouping and 16S rRNA gene sequencing (see text for details).

*c* N.D.: None detected.

*d* No data, plates partly overgrown by slimy non-AAB. CFU<sub>AAB</sub> and AAB composition approximately as data for 60 h of fermentation.
as described above as *H. guilliermondii*, *S. cerevisiae*, *Candida stellimalicola*, *Issatchenkia orientalis* and *P. membranifaciens*, respectively) with different empirically determined melting profiles were included in at least 2 lanes in all gels. The DGGE profiles were normalised and cluster analysis performed using Bionumerics 2.50 (Applied Maths). The dendrograms were calculated on the basis of the Dice’s Coefficient of similarity (weighted data) with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

Table 3
Temperature of the fermenting mass, pulp pH and log(CFU/g) of yeasts, Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and *Bacillus* spp. (standard deviations in brackets) determined in the centre of a large heap cocoa fermentation turned after 48 and 96 h

<table>
<thead>
<tr>
<th>Fermentation time (h)</th>
<th>Temperature, °C</th>
<th>pH, pulp</th>
<th>Yeast, log(CFU&lt;sub&gt;yeast/g&lt;/sub&gt;)</th>
<th>LAB, log(CFU&lt;sub&gt;LAB/g&lt;/sub&gt;)</th>
<th>AAB, log(CFU&lt;sub&gt;AAB/g&lt;/sub&gt;)</th>
<th>Bacillus spp. log(CFU&lt;sub&gt;Bacillus/g&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28</td>
<td>3.95</td>
<td>7.23 (0.21)</td>
<td>5.53 (0.04)</td>
<td>N.D. (0)</td>
<td>N.D. (0)</td>
</tr>
<tr>
<td>12</td>
<td>29</td>
<td>4.69</td>
<td>7.47 (0.12)</td>
<td>7.02 (0.03)</td>
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<td>N.D. (0)</td>
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<tr>
<td>24</td>
<td>30.5</td>
<td>4.21</td>
<td>5.73 (0.02)</td>
<td>9.16 (0.13)</td>
<td>N.D. (0)</td>
<td>N.D. (0)</td>
</tr>
<tr>
<td>36</td>
<td>34</td>
<td>3.94</td>
<td>6.34 (0.06)</td>
<td>9.18 (0.04)</td>
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<tr>
<td>48</td>
<td>33.5</td>
<td>3.97</td>
<td>5.19 (0.16)</td>
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<td>N.D. (0)</td>
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<td>60</td>
<td>39</td>
<td>4.04</td>
<td>5.25 (0.07)</td>
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<td>N.D. (0)</td>
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<tr>
<td>72</td>
<td>44</td>
<td>4.12</td>
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<td>9.29 (0.08)</td>
<td>N.D. (0)</td>
<td>N.D. (0)</td>
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<tr>
<td>84</td>
<td>46</td>
<td>4.25</td>
<td>5.82 (0.06)</td>
<td>8.17 (0.08)</td>
<td>N.D. (0)</td>
<td>N.D. (0)</td>
</tr>
<tr>
<td>96</td>
<td>44.5</td>
<td>4.34</td>
<td>4.30 (0)</td>
<td>6.76 (0)</td>
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<td>N.D. (0)</td>
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<tr>
<td>108</td>
<td>43</td>
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<td>4.90 (0)</td>
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<tr>
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<td>N.D. (0)</td>
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<tr>
<td>144</td>
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<td>5.58 (0.04)</td>
<td>N.D. (0)</td>
<td>N.D. (0)</td>
</tr>
</tbody>
</table>

% of yeast population

- **H. guilliermondii**: 78, 53, 21, 25
- **P. kluyveri**: 11
- **C. diversa**: 11
- **Unidentified Species C**: Det.<sup>a</sup> 14
- **S. crataegensis**: 9
- **C. zemplinina**: 33, 57
- **P. membranifaciens**: 22, 91, 13, 13, 9, 11, 31, 50, 100, 100, 67, 100
- **Sc. cerevisiae**: 22, 91
- **I. orientalis**: Det.<sup>a</sup>
- **I. occidentalis**: Det.<sup>a</sup>
- **C. ethanolica**: 13, 3, 50, 33
- **T. delbruecki**: 13
- **Schiz. pombe**: 82

% of LAB population

- **Lc. pseudomesenteroides**: 82
- **Lb. rossii**<sup>b</sup>: 18
- **Lb. fermentum**: 100, 98, 47, 87, 100, 100, 100, 100, 100, 52, 75, 4
- **Le. pseudofaecalis**: 2
- **Lb. plantarum**: 53, 9, 42, 25, 76
- **Unidentified Species D**: 4
- **Pd. acidilactici**: 6
- **Lb. hilgardii**<sup>b</sup>: 20

% of AAB population

- **A. pasteurianus**: 100
- **A. syzygii**: 100
- **A. tropicalis**: 100, 71

% of *Bacillus* population

- **B. licheniformis**: 29, 35, 17, 68, 60, 34
- **B. pumilus**: Det.<sup>a</sup>
- **B. megaterium**: 100, 71
- **B. sphaericus**: 17
- **B. subtilis**: 43


<sup>a</sup> Detected sporadically.

<sup>b</sup> Isolates did not grow upon purification. Identified directly by rep-PCR grouping and 16S rRNA gene sequencing (see text for details).

<sup>c</sup> N.D.: None detected.
2.10. Nucleotide accession numbers

The nucleotide sequences determined in this study have been assigned Genbank Accession Nos. DQ466524–DQ466546 and DQ523483–DQ523503.

3. Results and discussion

3.1. Physical and chemical changes during the fermentation of cocoa beans

The fermentations were all carried out as either traditional heap fermentations as practiced in Ghana for decades (Knapp, 1937; Baker et al., 1994), or as tray fermentations. The temperature of the fermenting mass increased during all investigated fermentations from 28 °C at the onset of fermentation to 46–48 °C reached by mid to late stages in the fermentation (Tables 1–4). Conversion of ethanol into acetic acid by AAB is an exothermal process (Thompson et al., 2001; Schwan and Wheals, 2004). This is confirmed by the finding that in the small heap fermentation, the top of the large heap fermentation and the tray fermentation the increase in temperature corresponds well with an increase in the number of AAB (Tables 1–4). It is noteworthy that only a limited increase in temperature was observed in the centre of the large heap fermentation until the heap was turned for the first time after 48 h (Table 3). Turning aerates the fermenting mass, thereby enhancing growth of aerobic AAB and correspondingly a 10 °C increase in temperature was observed during the next 24 h of fermentation (Table 3).

Table 4: Temperature of the fermenting mass, pulp pH and log(CFU/g) of yeasts, Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (AAB) (standard deviations in brackets) of a tray cocoa fermentation

<table>
<thead>
<tr>
<th>Fermentation time (h)</th>
<th>Temperature, °C</th>
<th>pH, pulp</th>
<th>Yeast, log(CFUyeast/g)</th>
<th>LAB, log(CFU_LAB/g)</th>
<th>AAB, log(CFU_AAB/g)</th>
<th>% of yeast population</th>
<th>% of LAB population</th>
<th>% of AAB population</th>
</tr>
</thead>
<tbody>
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<td>0</td>
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<td>4.12</td>
<td>7.45 (0)</td>
<td>N.D. a</td>
<td>N.D. a</td>
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<td>Lb. plantarum 89</td>
<td>A. syzygii 50</td>
</tr>
<tr>
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<td>33.5</td>
<td>4.28</td>
<td>7.57 (0.10)</td>
<td>7.62 (0.03)</td>
<td>7.05 (0.21)</td>
<td>I. occidentalis 47</td>
<td>Lc. pseudoficiunenum 30</td>
<td>A. matorum 50</td>
</tr>
<tr>
<td>24</td>
<td>34</td>
<td>3.85</td>
<td>7.18 (0.38)</td>
<td>9.71 (0.10)</td>
<td>7.70 (0.14)</td>
<td>I. hanoensis 9</td>
<td>Lb. fermentum 64</td>
<td>A. pasteurianus 50</td>
</tr>
<tr>
<td>36</td>
<td>37.5</td>
<td>3.82</td>
<td>6.33 (0.17)</td>
<td>9.54 (0.34)</td>
<td>7.34 (0.06)</td>
<td>C. zemplinina 11</td>
<td>Lc. pseudomesenteroides 57</td>
<td>A. tropicalis 73</td>
</tr>
<tr>
<td>48</td>
<td>45</td>
<td>3.98</td>
<td>6.49 (0.16)</td>
<td>9.33 (0.04)</td>
<td>7.49 (0.16)</td>
<td>C. sorboxylosa 11</td>
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<tr>
<td>60</td>
<td>46</td>
<td>4.06</td>
<td>4.25 (0.07)</td>
<td>8.27 (0.04)</td>
<td>5.96 (0.03)</td>
<td>C. silve 9</td>
<td>Pd. acidilactici 11</td>
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</tr>
<tr>
<td>72</td>
<td>46</td>
<td>4.20</td>
<td>5.55 (0.07)</td>
<td>9.07 (0.18)</td>
<td>3.99 (0.55)</td>
<td>C. cylindracea 37</td>
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<tr>
<td>84</td>
<td>46</td>
<td>4.29</td>
<td>6.58 (0.03)</td>
<td>8.45 (0.04)</td>
<td>3.99 (0.55)</td>
<td>P. kluyveri 22</td>
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<tr>
<td>96</td>
<td>45.5</td>
<td>4.26</td>
<td>4.29 (0.12)</td>
<td>8.58 (0.19)</td>
<td>5.96 (0.35)</td>
<td>C. diversa 23</td>
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</tr>
</tbody>
</table>


aN.D.: None detected.
The main sugars of the fresh pulp were glucose (5.4–6.6%) and fructose (6.3–7.4%) with only small amounts of sucrose (less than 0.3%). The citric acid content was 0.6–0.7% and no or only low amounts (less than 0.2%) of acetic acid, lactic acid and ethanol were detected in the fresh pulp (data not shown). During the first 24 h, about 80% of the sugars were metabolised in the tray, small heap and top of the large heap fermentation accompanied by an increase in acetic acid, lactic acid and ethanol concentration. The ethanol concentration reached 2% after 24–36 h followed by a decline to non-detectable levels by the end of fermentation (data not shown). The acetic acid concentration peaked at around 2% after 60–72 h of fermentation followed by a decline to 0.7–1% by the end of fermentation, whereas the lactic acid concentration peaked at 1% after 24–48 h of fermentation (data not shown). The development in the centre of the large heap was delayed with a slower metabolisation of sugars and limited production of acetic acid until the first turning after 48 h (data not shown).

During the first 12 h of fermentation the citric acid concentration fell to low or even non-detectable levels (data not shown). This was accompanied by an increase in pH from the starting value of 3.94–4.12 to 4.28–4.69 (Tables 1–4). Subsequently the pH fell again probably due to the production...
of acetic and lactic acid. Towards the end of fermentation, an increase in pH was observed (Tables 1–4), possibly due to evaporation of volatile acids like acetic acid.

The quality of the fully fermented sun-dried beans was visually assessed using the cut test. According to the cut test results, cocoa beans of good quality were produced, as the content of slaty and violet beans were below 1% and 20%, respectively, with the remaining beans being judged as brown (data not shown) (Wood and Lass, 1985).

3.2. Enumeration and identification of yeasts during cocoa fermentation

Yeasts were the dominant microorganisms at the onset of fermentation. From the initial counts of log(CFU/g)=6.07–7.45 the yeast population increased to log(CFU/g)=7.24–8.03 over the next 12 h (Tables 1–4). The first (0 h) sample was the taken after the heaps had been fully built up and the trays completely filled. This takes up to a few hours, leaving time for the yeast to grow, which probably explains the relatively high initial counts. Other studies have reported initial counts of the same magnitude (Carr et al., 1979; Schwan et al., 1995) whereas Jespersen et al. (2005) reported somewhat lower numbers. From 24 h and onwards, yeast counts in the tray and small heap fermentations showed a declining tendency (Tables 1 and 4) whereas the large heap fermentation showed a fluctuating behaviour following yeast turnover after 48 and 96 h: in the outer layers of the large heap fermentation the yeast counts declined after turning followed by renewed yeast growth (Table 2).

The yeasts were identified by a combination of phenotypic and molecular methods. Initially, the micro- and macro-morphological characteristics were recorded, and the rep-PCR-profile and the length of the ITS1-5.8S rRNA gene-ITS2 fragment of each isolate determined. Rep-PCR proved to be a reliable and rapid method for grouping the yeast isolates (Fig. 1). Determination of the length of ITS1-5.8S rRNA gene-ITS2 fragment confirmed the rep-PCR results, as isolates with the same rep-PCR-profile also had the same fragment length (Fig. 1). Representative isolates from each rep-PCR (Fig. 1) group were identified by sequencing of the D1/D2-region of the 26S rRNA gene (Kurtzmann and Robnett, 1998). Most sequenced isolates showed high similarity (99.7–100%) to sequences in the Genbank (data not shown). The identities of these isolates were confirmed by carbohydrate assimilation profiles (API ID 32 C). The assimilation profiles corresponded to the assimilation profiles reported by Kurtzmann and Fell (1998). P. membranifaciens and P. galeiformis are phenotypically difficult to distinguish and have almost identical D1/D2-fragments. However, according to Kurtzmann (1998), no P. galeiformis strains are able to assimilate d-glucosamine, whereas some P. membranifaciens strains have this ability (Kurtzmann, 1998). As all tested isolates assigned to this group assimilated d-glucosamine, they were identified as P. membranifaciens. Three small groups of yeasts all had very low D1/D2 sequence similarity (81.8–92.4%) to sequences deposited in Genbank. Neither was it possible to identify these isolates on the basis of their carbohydrate assimilation profile (Kurtzmann and Fell, 1998). Here these isolates are reported as Unidentified Species A, B and C (Fig. 1 and Tables 1–3). These isolates will be further characterised in future publications.

The yeast community was complex with 5–6 different species detected in each sample during the first 24–36 h of all investigated fermentations, but most detected species quickly became undetectable (Tables 1–4). H. guilliermondii was the predominant yeast in the small and large heap fermentations during the first 24 h. Later in the fermentations, H. guilliermondii was only detected more occasionally (Tables 1–3). Jespersen et al. (2005) and Ardhana and Fleet (2003) have also reported the presence of high numbers of H. guilliermondii (anamorph Kloekera apis) during the first 24 h of cocoa fermentations. The newly described yeast Candida zemplinina (Sipiczki, 2003) was detected in all fermentations, but most notably in the centre of the large heap and in the tray fermentations (Tables 3 and 4). C. zemplinina was detected during cocoa tray fermentations using DGGE by Nielsen et al. (2005) as well, but have, to our knowledge, not been cultivated from other sources than botrytised wine fermentations before. Compared to the heap fermentations H. guilliermondii was less dominant during the initial phases of the tray fermentation. Candida silvae, C. zemplinina and Candida diversa were among the other predominant yeasts during this phase of the tray fermentation possibly reflecting the higher oxygen availability during tray fermentations. After 36–48 h of fermentation a switch in the yeast population was observed in all fermentations with S. cerevisiae and P. membranifaciens becoming the predominant yeasts. Towards the end of fermentation, P. membranifaciens was the predominating yeast in all fermentations constituting up to 100% of the yeast population with occasional appearance of S. cerevisiae, C. krusei and C. ethanolicus (Tables 1–4). In the only other published study investigating the yeast community associated with Ghanaian cocoa fermentations to the species level, Jespersen et al. (2005) also observed a switch towards P. membranifaciens after 72 h of fermentation. However, the fermentations investigated by Jespersen et al. (2005) were only conducted for 3 days (72 h). This fermentation schedule is practiced by some farmers, but generally not recommended (Baker et al., 1994). When the fermentation is allowed to progress for the recommended 4 (tray and small heaps) to 6 days (large heaps), the dominance of P. membranifaciens in the later stages of fermentation seems to be even more pronounced as shown here.

3.3. Enumeration and identification of Lactic Acid Bacteria (LAB) during cocoa fermentation

The number of LAB increased more than 2 log-units during the first 24 h of fermentation, reaching counts as high as log(CFU/LAB)=9.71 in the tray fermentation. With a few exceptions, LAB remained the dominant group of microorganisms throughout the fermentations (Tables 1–4). Carr et al. (1979) also detected LAB in relatively high numbers throughout Ghanaian heap fermentations, but in some samples AAB and late in the fermentation Bacillus spp. reached higher numbers than the LAB. In Indonesian and Brazilian box fermentations,
LAB have also been reported to be the dominant microorganisms after 36–48 h of fermentation, but contrary to the Ghanaian heap fermentations LAB were clearly outnumbered by Bacillus spp. late in the fermentations (Schwan et al., 1995; Ardhana and Fleet, 2003).

The LAB isolates were identified using phenotypic as well as genotypic methods. Initially the isolates (all Gram positive and catalase negative) were characterised on the basis of cell morphology, gas production from glucose and growth at cardinal temperatures. The majority of the isolates (77.8%) were rod-shaped, heterofermentors, often growing at 15 °C (84%) and 45 °C (99.5%). Rod-shaped homofermentors (15.4% of isolates) growing at 15 °C (100%) and in some cases at 45 °C (35.1%), formed the second largest group. The remaining isolates consisted of heterofermentative (4.5%) and homofermentative cocci (2.3%), all growing at 15 °C but not 45 °C.

Following initial grouping, the isolates were genotypically grouped using rep-PCR. Cluster analysis showed a clear separation into clusters (results partly shown in Fig. 2). The majority of heterofermentative isolates clustered together with the _Lb. fermentum_ (DSM20062T) type strain (Fig. 2). Further analysis of the isolates belonging to this group showed that they produced DL-lactate from glucose and NH₃ from arginine. The majority (90% or more) of tested isolates fermented arabinose, fructose, galactose, gluconate, glucose, maltose, melibiose, ribose and sucrose. Less than 3% of the isolates in this group fermented amygdalin, esculin, melezitose and sorbitol. No isolates fermented glycerol and rhamnose. Sequencing of the 16S rRNA gene (5 isolates fully sequenced, 20 partly sequenced) revealed high similarity (99.6–100%) to sequences deposited as _Lb. fermentum_ in the Genbank database.

According to Hammes and Hertel (2003), _Lb. fermentum_ does not grow at 15 °C, whereas 84% of the isolates in this group grew at 15 °C. However, it has previously been reported that _Lb. fermentum_ strains of African origin is capable of growing at temperatures below 15 °C (Abdelgadir et al., 2001). Based on the 16S rRNA gene sequence, phenotypic traits as reported above and the grouping together with the _Lb. fermentum_ type strain in the cluster analysis of the rep-PCR-profiles, these isolates were identified as _Lb. fermentum_ despite the fact that most strains grew at 15 °C.

Two heterofermentative rod-shaped isolates did not cluster together with the _Lb. fermentum_ isolates (represented by isolate LA122 in Fig. 2). These isolates grew at 15 but not 45 °C, produced DL-lactate and fermented arabinose, fructose, galactose, gluconate, glucose, maltose, melibiose, ribose and xylose. Sequencing of the 16S rRNA gene revealed high similarity (99.9%) to _Lb. brevis_ which corresponds well with the phenotypic results (Hammes and Hertel, 2003) and these isolates may thus be identified as _Lb. brevis_.

Rep-PCR showed that the homofermentative rods clustered in three groups (partly shown in Fig. 2). One group clustered together with the type strain of _Lb. plantarum_ (DSM20174T). All isolates belonging to this group produced DL-lactate, had mDAP in the cell wall, grew at 15 °C and 41.9% grew at 45 °C. All tested isolates had a broad carbohydrate fermentation pattern fermenting amygdalin, arabinose, cellobiose, fructose, galactose, gluconate, glucose, lactose, maltose, mannitol, melezitose, melibiose, ribose, sucrose, salicin and trehalose. The presence of mDAP in the cell wall, the lactate isomer produced and the carbohydrate fermentation pattern is a strong indication that these isolates belong to the _Lb. plantarum_ group.

Fig. 2. Dendrogram obtained by cluster analysis of rep-PCR (GTG5) fingerprints from Lactic Acid Bacteria isolates originating from cocoa fermentations. Reference strains of _Lb. plantarum_ (DSM20174T), _Lb. paraplantarum_ (LTH5200), _Lb. pentosus_ (DSM20314T) and _Lb. fermentum_ (DSM20052T) are included for comparison. The dendrogram is based on Dice’s Coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA). Isolates were subsequently identified using pheno-and genotypic methods (see text). Only a limited sub-sample of representative isolates is shown. Abbreviations: _Lb._: Lactobacillus, _Lc._: Leuconostoc, _Pd._: Pediococcus, _Lac._: Lactococcus.
Lb. plantarum/L. pentosus-group. Sequencing of the 16S rRNA gene confirmed that the isolates are closely related to Lb. plantarum showing 99.8–100% similarity. As mentioned above, these isolates clustered together with the type strain of Lb. plantarum in the rep-PCR analysis indicating their identity as Lb. plantarum. However, Lb. plantarum, Lb. paraplanatarum and Lb. pentosus are very closely related, and to obtain an unambiguously identification, the isolates were differentiated using multiplex PCR (Torriani et al., 2001), confirming their identity as Lb. plantarum (results not shown).

A smaller group of homofermentative rods clustered away from the Lb. plantarum isolates in the rep-PCR analysis. These isolates grew at 45 but not 15 °C, produced D-lactate from glucose, contained mDAP in the cell wall and were motile. Sequencing of the 16S rRNA gene revealed that the isolates were closest related to Lb. nagelii (98.0%) and Lb. satsumensis (95.5%), two also motile lactobacilli (Hammes and Hertel, 2003; Endo and Okada, 2005). Given the relatively low 16S rRNA gene similarity between Lb. nagelii and the isolates obtained through this study and the fact that these isolates differ from Lb. nagelii in a number of phenotypic traits, it is probable that these isolates constitute a new species which will be described in a future publication. Throughout this study these isolates will be referred to as Unidentified Species D.

A small group of short rods clustered together in the rep-PCR analysis (represented by LA121 in Fig. 2). These isolates showed no visible gas production, did not produce ammonia from arginine, grew at 15 but not at 45 °C and produced d(-)-lactate from glucose. Leuconostoc spp. normally appear as cocci or ovoid cocci and are characterised by the production of CO₂ from glucose (Björkroth and Holzapfel, 2003), but Leuconostoc strains, appearing as rods and with no visible gas production from glucose, have been isolated from tropical fermented products before (Leisner et al., 2005). 16S rRNA gene sequencing revealed the newly described Leuconostoc pseudoficus (99.7% homology) as the closest relative (Chambel et al., 2006). The description above fits the description of Lc. pseudoficus except that Lc. pseudoficus in the cell wall and were motile. However, given the remaining phenotypic information and the high 16S rRNA gene homology the strains in this group are identified as Lc. pseudoficus.

The heterofermentative cocci all clustered together in the rep-PCR cluster analysis (partly shown in Fig. 2). All isolates produced n(-)-lactate from glucose, grew at 15 and 37 °C but not at 45 °C; no ammonia was produced from arginine and hydrolysis of esculin was not observed. 16S rRNA gene sequencing revealed high similarity to Lb. pseudomesenteroides (100%) and the strains were identified as such on basis of this and the phenotypic observations.

The homofermentative cocci formed two groups in the rep-PCR cluster analysis. One group occurred as pairs and occasionally as tetrads in MRS broth, grew at 15 and 45 °C and produced D-lactate from glucose. 16S rRNA gene sequencing revealed 99.9% similarity to Pediococcus acidilactici. The other group of homofermentative cocci grew at 15 but not 45 °C. 16S rRNA gene sequencing revealed 99.8% similarity to Lactococcus lactis.

It proved impossible to revive a limited number of LAB isolates after purification and storage at −80 °C. These isolates were identified by extracting DNA directly from the plates used for purification and grouping the isolates, using rep-PCR. A few isolates grouped together with Lb. fermentum and Lb. plantarum, respectively. Their identity was confirmed by 16S rRNA gene sequencing. Two groups of isolates formed clusters separate from all other groups in the cluster analysis. 16S rRNA gene sequencing revealed that the isolates of the two groups belong to Lactobacillus rossii and Lactobacillus hilgardii, respectively (Tables 2 and 3).

At the onset of fermentation, Lb. pseudomesenteroides and Lb. plantarum dominated the LAB community but after 12–24 h, Lb. fermentum took over as the dominant LAB (Tables 1–4). Despite differences in size and fermentation method, Lb. fermentum dominated all fermentations investigated, a position only challenged by Lb. plantarum towards the end of the large heap fermentation (Tables 2 and 3). Carr et al. (1979) also isolated Lb. plantarum and Lb. fermentum during Ghanaian heap fermentations but did not state which organisms dominated during the different stages of fermentation (Carr et al., 1979; Carr and Davies, 1980). Ardhana and Fleet (2003) found Lb. fermentum to be the predominating LAB during Indonesian cocoa fermentations, with Lb. plantarum being isolated regularly and Lb. hilgardii more occasionally. Lb. hilgardii was also detected occasionally during this study (Tables 2 and 3). The involvement of Leuconostoc, Pediococcus and Lactococcus spp. in West African cocoa fermentations has not been reported before. However, their involvement in cocoa fermentations has been reported from Brazilian and Belizian fermentations (Passos et al., 1984; Thompson et al., 2001).

Together with previously published studies (Carr et al., 1979; Carr and Davies, 1980; Passos et al., 1984; Schwan et al., 1995; Thompson et al., 2001; Ardhana and Fleet, 2003) the results obtained during this study indicates that Lb. fermentum and Lb. plantarum are indigenous to the fermentation of cocoa around the World.

3.4. Enumeration and identification of Acetic Acid Bacteria (AAB) during cocoa fermentation

Acetic Acid Bacteria were only detected occasionally the first 12 hours of fermentation (Tables 1–4). From 24 hours and onwards the AAB formed a significant part of the micro- population reaching log(CFU_AAB) = 7.70–7.88 after 36–48 h of fermentation in the aerated parts of all fermentations. In the later stages of fermentation the AAB counts showed a declining tendency (Tables 1, 2 and 4). Turning of the fermenting mass clearly influenced the growth of AAB. In the outer layers of the large heap fermentation, the AAB counts decreased 1–2 log-units following turning after 48 and 96 h, whereas the AAB counts in the centre of the fermenting mass increased 2–2.5 log-units following turning (Tables 2 and 3). Carr et al. (1979) did not observe the same correlation between turning and growth of AAB, possibly because they took samples every 24 h and not
every 12 h as in this study. During a Brazilian box fermentation, Schwan et al. (1995) observed maximum AAB counts comparable to those presented here, but the initial growth of AAB was slower not peaking before 72 h of fermentation. By contrast, during Indonesian cocoa fermentations, it has been shown that maximum AAB counts of only $10^5$–$10^6$ CFU/g were reached within only 12 h of fermentation (Ardhana and Fleet, 2003).

The AAB isolates were identified by Restriction Fragment Length Polymorphism analysis of 16S rRNA gene and 16S-ITS-23S rRNA gene fragments (PCR-RFLP) as described by González et al. (2006). Initially, 16S rRNA gene fragments from all isolates were digested with $\text{AluI}$ (Fig. 3). Depending on the RFLP-pattern the analysis was continued using other restriction enzymes (González et al., 2006). The PCR-RFLP results were confirmed by sequencing of the 16S rRNA gene of selected isolates. Sequencing and PCR-RFLP analysis gave identical results in all cases. The identification of $G$. oxydans was further strengthened by the formation of water-soluble pigments on GYC agar and the absence of acid production from D- and L-arabitol (Kersters et al., 2003).

Apparently a succession of different AAB species took place during the fermentations as $A$. syzygii and $A$. pasteurianus were the dominating AAB during the first half of all fermentations. The later stages of fermentation were dominated by $A$. tropicalis. Occasionally, $A$. malorum and $G$. oxydans were detected as well (Tables 1–4). $A$. pasteurianus strains have previously been reported to form a significant part of the AAB community during cocoa fermentations in Ghana (heap), Indonesia (box) and Brazil (box) (Carr et al., 1979; Ardhana and Fleet, 2003; Schwan and Wheals, 2004). The involvement of $A$. syzygii in cocoa fermentations has to our knowledge not been reported before. The finding of previous studies indicating the involvement of $A$. aceti in the fermentation of cocoa could not be confirmed here (Ostovar and Keeney, 1973; Carr et al., 1979; Ardhana and Fleet, 2003; Schwan and Wheals, 2004).

3.5. Enumeration and identification of Bacillus spp. during cocoa fermentation

$Bacillus$ spp. were not detected until after 48–60 h of fermentation in the large heap fermentation peaking at log (CFU$_{Bacillus}$) = 9.10 after 96 h of fermentation (Tables 2 and 3). Turning influenced the distribution of the $Bacillus$ spp. in the fermenting mass strongly as the $Bacillus$ counts fell in the outer parts of the fermenting mass (Table 2) and rose in the centre of the fermenting mass (Table 3) following turning. In the tray fermentation no $Bacillus$ spp. were detected at all and in the small heap fermentation $Bacillus$ spp. never formed a significant part of the microorganisms but were detected in lower numbers from 60 h of fermentation and onwards (Tables 1 and 4). In Indonesian and Brazilian box fermentations $Bacillus$ spp. have been reported to form the majority of the micro-population in the later stages of fermentation outnumbering all other organisms (Schwan et al., 1995; Ardhana and Fleet, 2003). In the fermentations investigated during this study $Bacillus$ spp. formed a significant part of the micro-population, but only outnumbered all other organisms in a few samples (Tables 2 and 3). Carr et al. (1979) also reported that $Bacillus$ spp. formed a significant part of the microorganisms during the later stages of Ghanaian heap fermentations but similarly found that they only outnumbered all other organisms in a few samples. Given these observations it is possible that $Bacillus$ spp. play a bigger role in box fermentations than in heap fermentations, but more fermentations need to be investigated before firm conclusions can be drawn.

Following micro- and macro-morphological examination all endospore-forming isolates were grouped and partly identified on the basis of 16S-ITS-23S rRNA gene polymorphism and rep-PCR (GTG5-primer) (Daffonchio et al., 1998). Results are
partly shown in Fig. 4. Representative isolates were picked from all groups and subjected to 16S rRNA gene sequencing and API 50 CHB carbohydrate assimilation profiling. The different methods yielded similar results, identifying the major group of bacillus isolates as Bacillus licheniformis with B. megaterium and B. pumilus constituting two other significant groups. Furthermore, a smaller number of isolates were identified as B. subtilis, B. cereus and B. sphaericus (Tables 1–3).

In general, B. megaterium was detected in the middle phase (60–96 h), and B. licheniformis and B. pumilus in the mid and late phases of fermentation (Tables 1–3). Contrary to the results reported here Carr and Davies (1980) reported B. subtilis as the only Bacillus spp. involved in Ghanaian heap fermentations (Carr et al., 1979; Carr and Davies, 1980). The involvement of B. licheniformis, B. megaterium, B. pumilus, B. cereus and B. sphaericus in Ghanaian heap fermentations have not been reported before, but their association to Indonesian, Brazilian and Trinidadian box cocoa fermentations have been established previously (Ostovar and Keeney, 1973; Schwan et al., 1986; Ardhana and Fleet, 2003). The role of Bacillus spp. in the fermentation of cocoa has not been fully elucidated, but it has been speculated that they may cause off-flavours due to their high enzymatic activity and production of e.g. short chained free fatty acids (Schwan et al., 1986; Schwan and Wheals, 2004). If this is the case, the results obtained during this study indicates that turning of large heaps are important not only to enhance the desirable activities of AAB but also to minimise the possibly harmful activities of Bacillus spp. in the more aerated parts of the fermentations.

3.6. Investigation of the yeast and bacterial populations associated with Ghanaian cocoa fermentations using Denaturing Gradient Gel Electrophoresis (DGGE)

Given the complexity of the cocoa fermentation traditional culture-based investigations are tedious and time consuming. We have previously showed that Denaturing Gradient Gel Electrophoresis (DGGE) offers an efficient tool for studying yeast population dynamics during cocoa fermentations (Nielsen et al., 2005). Here we extend the method to include the study of bacterial dynamics during the fermentation of cocoa.

In addition to the fermentations investigated using culture-based methods above (Tables 1–4), samples representing additional tray fermentations carried out at the Cocoa Research Institute of Ghana (CRIG) and heap fermentations carried out at farms in Mampong and Bompata, both in Ghana, were included in the DGGE-based investigations as lined out above.

Initially, two different sets of universal bacterial primers targeting the V3 and the V6–V8 region of the 16S RNA gene, respectively, were used for the PCR-based amplification of DNA fragments suitable for DGGE. As seen from Fig. 5, more bands appeared when using the primers targeting the V3-region compared to the V6–V8-primers (on average 10.8±0.5 bands versus 5.5±0.6 bands), indicating that the detection limit is lower using the V3-primers. Furthermore, the V3-primers produced fingerprints with less background smear (Fig. 5). Based on these results, it was decided to use the V3-primers for the remaining samples.

Fig. 6 represents DGGE profiles of 26S rRNA gene fragments of cocoa pulp sampled every 12 h during a 144 h heap fermentation. The identities of selected fragments have been determined by sequencing and by co-migration with yeast isolates originating from cocoa fermentations. The DGGE and culture-based findings correspond well, except for a few discrepancies (Fig. 6 and Table 2). A fragment closely related to H. guilliermondii yielded a strong band until after 84 h of fermentation. According to the culture-based findings H. guilliermondii constituted 67–79% of the yeast population during the first 24 h of fermentation but was not

Fig. 5. DGGE profiles (35–70% denaturant) representing 16S rRNA gene fragments of cocoa pulp sampled after 0, 24, 48 and 72 h of tray fermentation (carried out in January 2003) and amplified using PCR and 2 different primer sets: Lanes 1–4 amplified using primers 968fGC and 1401r (V6–V8-region); Lanes 5–8 amplified using primers PRBA338fGC and PRUN518r (V3-region).

Fig. 6. DGGE profiles (35–65% denaturant) representing 26S rRNA gene fragments (yeast) of cocoa pulp sampled with 12 hour intervals during 144 h of fermentation in a large heap (500 kg) carried out in October 2003 at the Cocoa Research Institute of Ghana. Identity of identified fragments indicated, see arrows. Abbreviations: H.: Hanseniaspora, P.: Pichia, C.: Candida, S.: Saccharomyces, I.: Issatchenkia, S.: Saccharomycopsis.
detected afterwards. The lower limit of detection using DGGE has been estimated to comprise 1% of the yeast population (Prakitchaiwattana et al., 2004). As speculated by Nielsen et al. (2005), it is possible that the presence of a strong band representing H. guilliermondii at sampling times where H. guilliermondii was not detected using culture-based isolations, is due to the fact that H. guilliermondii was present in numbers below the detection limit of the culture-based isolations but above the detection limit of the DGGE method until 84 h of fermentation. Alternatively H. guilliermondii possibly enters an uncultureable state after 36 h of fermentation.

The detection of e.g. C. diversa, C. zemplinina and P. membranifaciens in the DGGE profiles corresponds well with the culture-based findings (Fig. 6 and Table 2).

Similar results were obtained in all other fermentations investigated. Fragments closest related to H. guilliermondii were detected in the early to mid phases of all fermentations. P. membranifaciens was detected from 24 to 36 h of fermentation and onwards and e.g. C. zemplinina, S. cerevisiae and I. orientalis were detected regularly (results not shown). Taken together the DGGE and culture-based results obtained through this study and by Nielsen et al. (2005) and Jespersen et al. (2005) indicate that H. guilliermondii in general is the dominating yeast in the initial phases of Ghanaian cocoa fermentations. Furthermore P. membranifaciens seems to play a prominent role among the yeasts during the later stages of fermentation.

Figs. 7 and 8 represent DGGE profiles of 16S rRNA gene fragments of cocoa pulp sampled every 12 h during a large heap (144 h) and a small heap fermentation, respectively. To a large extent the DGGE findings correspond well with the culture-based findings. In the initial phases LAB dominate the bacterial population with Lb. fermentum yielding a strong signal throughout the fermentation. After 24–36 h of fermentation, bands representing AAB become visible, and during the later stages of fermentation B. licheniformis and occasionally other Bacillus spp. were detected (Figs. 7 and 8 and results not shown). A relatively complex AAB micro-population was observed during the Mampong fermentation as G. oxydans, A. syzygii/A. pasteurianus and A. tropicalis all yielded relatively strong bands during this fermentation (results not shown). Unfortunately A. syzygii and A. pasteurianus migrate to the same position in the DGGE gels and as a consequence it was not possible to discriminate between these two species using DGGE (Figs. 7 and 8).

A band migrating to approximately the middle of the gel in all fermentations yielded a strong band in the initial phases of all fermentations including the fermentations carried out in Mampong and Bompata (indicated with an arrow and “No sequence” in Fig. 8). Unfortunately, it was not possible to obtain a satisfying sequence from this band, and no pure isolates had the same electrophoretic mobility. As a consequence, the band remains unidentified.

Lc. pseudoficusrulneum was occasionally identified during the culture-based part of the study (Tables 1–4). Surprisingly, this species yielded a strong band during all fermentations except the Mampong heap fermentation (Figs. 7 and 8, and results not shown). Given these results, it is indeed possible that Lc. pseudoficusrulneum plays a more important role during the fermentation of cocoa than anticipated from the culture-based findings. A finding underlining that the possibility to detect microorganisms difficult to cultivate is one of the great advantages of the DGGE method (Muyzer and Smalla, 1998; Giraffa and Neviani, 2001).

A weak fragment detected in the initial phase of one of the tray fermentations investigated using bacterial universal primers (V3-region) was surprisingly identified as the yeast H. guilliermondii (results not shown). The unintended amplification of eukaryotic DNA using prokaryotic specific primers has been reported before.
The problem is probably due to amplification of 18S rRNA gene fragments if eukaryotic DNA is present in excess amounts compared to the amounts of prokaryotic DNA. This is likely to be the case in the initial phases of cocoa fermentations where *H. guilliermondii* as seen from Tables 1–4 reaches high counts. However, as the fragment was weak and only detected in the initial phase of one fermentation the problem was considered negligible but underlines that DGGE profiles always should be interpreted with caution.

Cluster analysis of DGGE profiles has previously yielded valuable information about microbial dynamics in various fermented foods and other complex microbial communities (Ampe and Miambi, 2000; Nielsen et al., 2003, 2005). Nielsen et al. (2005) have previously shown that DGGE profiles of the yeast population from cocoa fermentations grouped with fermentation method and site. Furthermore it was shown that within fermentation sites the DGGE profiles clustered according to progress in the fermentation. The same results were obtained during this study, as the profiles grouped with fermentation time and site (results not shown).

Cluster analysis of the DGGE profiles of the bacterial community showed a similar picture. As seen from Fig. 9, the three different fermentation sites form different clusters. Within each fermentation site the profiles clustered according to fermentation time. Denaturing Gradient Gel Electrophoresis seems as speculated by Nielsen et al. (2005), in other words, to offer a possible tool for monitoring the progress of cocoa fermentations within specific production sites.

### 4. Conclusion

A thorough understanding of the microbiological processes leading to well-fermented cocoa is a prerequisite for developing quality management procedures for the production of high-quality cocoa, and perhaps in time to develop suitable starter cultures for the process. Using a combination of conventional culture-dependent microbiological methods aided by molecular methods for identifying the isolates obtained and a molecular biology-based culture-independent method (DGGE) a detailed microbiological investigation of Ghanaian heap and tray fermentations have been carried out. *H. guilliermondii* and *P. membranifaciens* were the dominating yeast during the early and late stages of fermentation, respectively. Isolates representing three putatively undescribed yeast species were isolated during the early stages of fermentation. Based on culture-based isolations *Lb. fermentum* was the dominating LAB in all fermentations. A putatively undescribed motile, homofermentative LAB was occasionally isolated during the fermentations. *A. syzygii*, *A. pasteurianus* and *A. tropicalis* were the predominant AAB during all fermentations. During the later stages of heap fermentation *B. licheniformis* and occasionally other *Bacillus* spp. were detected in high numbers. The culture-based findings were confirmed using DGGE, with a few important exceptions. The primary exception is the possible strong involvement of *Lc. pseudoficulneum* in the fermentations. Furthermore, DGGE proved to be a possible tool for monitoring the progress of cocoa fermentations at specific fermentation sites.

Fig. 9. Dendrogram derived from DGGE analysis of the bacterial populations associated with heap and tray fermentation of cocoa in Ghana. The dendrogram is based on Dice’s coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA). Samples were taken during 0–144 h of fermentation at the Cocoa Research Institute of Ghana and at farms in Mampong and Bompata, respectively. Abbreviations: LHT: Large Heap Top fermentation (i.e. pulp sampled 15 cm from the surface of the heap); LHC: Large Heap Centre fermentation (i.e. pulp sampled in the centre of the heap); SH: Small Heap fermentation; Tray: Tray fermentation; CRIG: Cocoa Research Institute of Ghana.
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