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Dynamics and Biodiversity of Populations of Lactic Acid Bacteria and Acetic Acid Bacteria Involved in Spontaneous Heap Fermentation of Cocoa Beans in Ghana▽

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The Ghanaian cocoa bean heap fermentation process was studied through a multiphasic approach, encompassing both microbiological and metabolite target analyses. A culture-dependent (plating and incubation, followed by repetitive-sequence-based PCR analyses of picked-up colonies) and culture-independent (denaturing gradient gel electrophoresis [DGGE] of 16S rRNA gene amplicons, PCR-DGGE) approach revealed a limited biodiversity and targeted population dynamics of both lactic acid bacteria (LAB) and acetic acid bacteria (AAB) during fermentation. Four main clusters were identified among the LAB isolated: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Leuconostoc pseudomesenteroides*, and *Enterococcus casseliflavus*. Other taxa encompassed, for instance, *Weissella*. Only four clusters were found among the AAB identified: *Acetobacter pasteurianus*, *Acetobacter syzygii*-like bacteria, and two small clusters of *Acetobacter tropicalis*-like bacteria. Particular strains of *L. plantarum*, *L. fermentum*, and *A. pasteurianus*, originating from the environment, were well adapted to the environmental conditions prevailing during Ghanaian cocoa bean heap fermentation and apparently played a significant role in the cocoa bean fermentation process. Yeasts produced ethanol from sugars, and LAB produced lactic acid, acetic acid, ethanol, and mannitol from sugars and/or citrate. Whereas *L. plantarum* strains were abundant in the beginning of the fermentation, *L. fermentum* strains converted fructose into mannitol upon prolonged fermentation. *A. pasteurianus* grew on ethanol, mannitol, and lactate and converted ethanol into acetic acid. A newly proposed *Weissella* sp., referred to as "*Weissella ghanaensis*," was detected through PCR-DGGE analysis in some of the fermentations and was only occasionally picked up through culture-based isolation. Two new species of *Acetobacter* were found as well, namely, the species tentatively named "*Acetobacter senegalensis*" (*A. tropicalis*-like) and "*Acetobacter ghanaensis*" (*A. syzygii*-like).

Cocoa beans are the principal raw material for chocolate production (39, 43, 73, 81). These seeds are derived from the fruit pods of the cocoa tree (*Theobroma cacao* L.), which is cultivated in plantations in the equatorial zone, with the Ivory Coast, Brazil, and Ghana as the major producers (2). The cocoa beans are embedded in a mucilaginous pulp inside the pods. Raw cocoa beans have an astringent, unpleasant taste and flavor and have to be fermented, dried, and roasted to obtain the desired characteristic cocoa flavor and taste (26, 73). The final chocolate flavor is influenced by the origin and cultivar of the cocoa beans, the on-the-farm fermentation and drying process, and the roasting and further processing performed by the cocoa and chocolate manufacturer (4, 10, 32, 33, 57, 75).

After removal of the beans from the pods, the first step in cocoa processing is a spontaneous 3- to 10-day fermentation of beans and pulp in heaps, boxes, baskets, or trays, of which spontaneous heap fermentation is the most widely used method in Ghana (4, 39). A microbial succession of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) takes place during fermentation (3, 67, 73). The yeasts liquefy the pulp through depectinization, which reduces pulp viscosity, and produce ethanol from sugars (and citric acid) under anaerobic conditions in an acid (pH < 4.0), carbohydrate-rich environment. As pulp is drained away, ethanol formation proceeds and both temperature and pH increase, which creates ideal conditions for the growth of LAB and AAB. LAB convert sugars and organic acids mainly into lactic acid. As more air comes in, AAB start to grow that oxidize the ethanol, initially produced by the yeasts, to acetic acid. Ethanol and acetic acid diffuse into the beans, and this, in combination with the heat produced by this exothermic bioconversion, causes the death of the seed embryo as well as the end of fermentation. Also, this initiates biochemical changes in the beans, leading to the formation of precursor molecules for the development of a characteristic flavor and color of the beans (26, 73). These properties are further developed during drying, roasting, and final processing of well-fermented cocoa beans (10, 73). Besides the actions of yeasts, the activities of both LAB and AAB are essential for the production of high-quality cocoa. *Bacillus* spp. may develop at the end of fermentation (in-

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creased temperature and less acidic and more-aerobic conditions), but their role in the process is not clear (3, 51, 67).

Several studies have dealt with yeasts involved in cocoa bean fermentation (3, 30, 49, 63, 66). Few studies have focused on the population dynamics and biodiversity of LAB and AAB during cocoa bean fermentation in relation to the metabolomics of this ecosystem. This may be due to difficulties in accessing and sampling such fermentations and in isolating and growing AAB and to the limited biodiversity that has been seen among LAB and AAB from fermented cocoa beans (67). In general, AAB belonging to the genus *Acetobacter* have been found more frequently than those of *Gluconobacter* (8, 51, 53, 67, 72). In the case of LAB, lactobacilli dominate compared with *Leuconostoc*, *Pediococcus*, and *Lactococcus* (8, 54, 55, 72). As most of the microbiological studies on cocoa date many years back, classical culture-dependent methods were used and no molecular identification methods were included. Moreover, the taxonomy of both LAB and AAB has undergone several changes during the last decade (18). This requests an updated isolation, identification, and biodiversity study of LAB and AAB from fermenting cocoa beans, including both molecular identification and culture-independent methods.

Culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) of PCR-derived gene amplicons have been developed to assist in the study of the microbial biodiversity and population dynamics of complex ecosystems over space and/or time, including food (19, 22, 47). Compared to traditional culturing, these methods, generally based on nucleic acids such as the 16S rRNA gene, aim at obtaining both a qualitative and a semiquantitative picture of a microbial community without the need to isolate and culture its single components. However, it has previously been demonstrated that while these techniques are valuable, there is a need to use a combined system to overcome the bias of the "culture-(in)-dependent-only" approach. Therefore, culture-independent and cultivation methods should be applied in parallel for population dynamics and biodiversity studies. Recently, such an approach was applied to study the yeast microbiota during cocoa bean fermentation in Ghana (49). Concerning AAB, this technique has been applied to determine their diversity during wine and vinegar fermentations (16, 44). With respect to LAB, DGGE studies encompass several fermented foods, such as cheese, wine, sourdough, and fermented sausages (19, 58, 61).

The aim of the present study was to assess the population dynamics of LAB and AAB and the evolution of important fermentation parameters (temperature, pH, sugars, and metabolites) during spontaneous heap fermentations of cocoa beans in Ghana. Both culture-dependent and -independent methods were applied to monitor and identify LAB and AAB. Cluster analyses of the repetitive-sequence-based PCR (rep-PCR) and bacterial DGGE profiles were performed to reveal possible differences between fermentation processes. Through metabolite target analysis, a link was made between the substrates and metabolites found in pulp and beans and the microbes identified.

MATERIALS AND METHODS

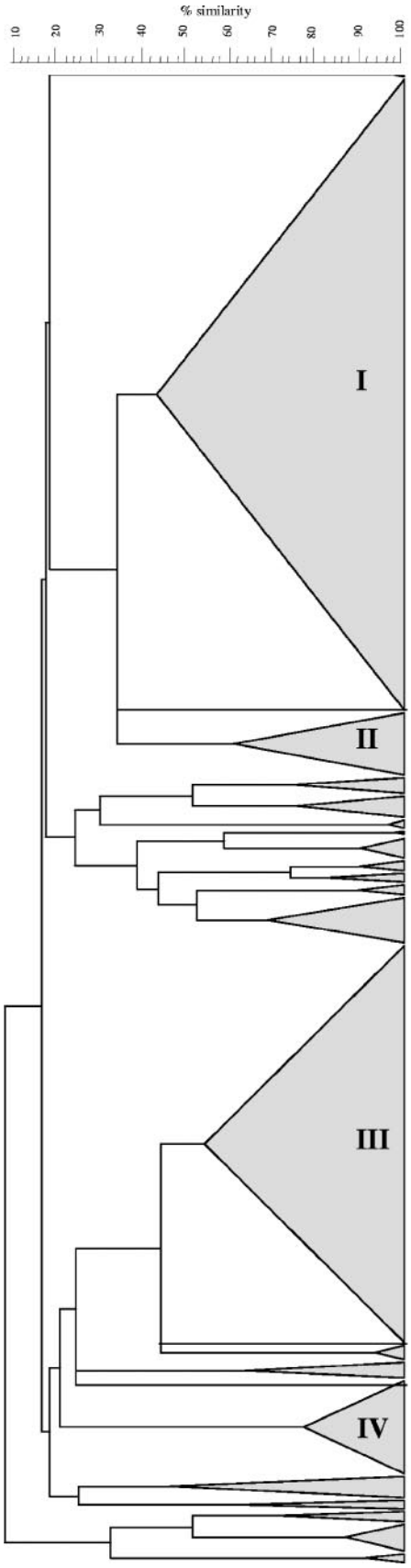
Cocoa bean fermentation. Two field experiments were set up in Ghana to sample spontaneous cocoa bean fermentations (heap method), one during the mid-crop (June to July 2004; heaps 1, 2, and 3) and one during the main crop (October to November 2004; heaps 4, 5, 6, and 7), representing the two major harvest seasons. As traditional cocoa bean fermentation processes are supposed to be region and site specific, two small farms (A and B), located about 15 km from each other near New Tafo and Old Tafo, respectively, were chosen. During the mid-crop, fermentations at farm A were followed twice (heaps 1 and 3) and those at farm B once (heap 2), while during the main-crop, fermentations at both farms A (heaps 4 and 6) and B (heaps 5 and 7) were followed twice.

Cocoa pods from mixed hybrid cocoa tree plantations (Criollo and Forastero) were harvested by traditional methods (such as manual harvest and transport in unwashed baskets) and used for fermentation within 2 to 3 days. Only matured pods were used for fermentation. Plantation workers cut the pods with unwashed machetes, beans plus surrounding pulp were scooped out manually, the placenta was not removed, and the husks were left to rot in the surroundings. At each farm, approximately 250 to 1,000 kg of wet beans and pulp was placed on banana and plantain leaves on the ground, resulting in heaps of 95- to 180-cm diameter and 40- to 64-cm height which were then covered with extra banana and plantain leaves and left to ferment. The beans were not mixed during fermentation. The entire fermentation lasted 6 days at both farms. The drainage of liquids produced during fermentation (sweatings) was allowed to penetrate into the ground. Drying of the fermented cocoa beans took around 10 to 14 days, depending on the weather, and resulted in amounts of 33 to 200 kg of dried beans. During fermentation, there was an on-line follow-up of temperature (outside and inside the heaps), pH (inside the heaps), and rainfall (pluviometer). Temperature and pH were measured by inserting a digital pH 340i sensor (WTW GmbH, Weilheim, Germany) in the middle of the fermenting cocoa bean mass.

Sampling. Samples of the seven heaps were taken according to a fixed time schedule, namely, at the start of the fermentation (time zero, fresh cocoa beans) and after 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 84, 96, 120, and 144 h of fermentation. Sampling was always done at the same depth of the bean mass (approximately 30 cm from the upper surface) but in different points of the heap. Each sample consisted of 600 g of beans that was aseptically removed and transferred into sterile plastic bags. Besides samples from the mucilaginous pulp of the opened pods, swab samples corresponding to a surface of 25 cm^2 were taken from the environment (surfaces of cocoa pods, banana leaves, baskets, machetes, and farmers' hands). For culture-independent and metabolite analyses, 128 cocoa bean samples were cooled, frozen, and transported on dry ice to Belgium.

Plating, enumeration, isolation, and maintenance. In Ghana, the culture-dependent approach was performed immediately after sampling (fresh samples were transiently stored on ice and treated in the laboratory within 1 h). Therefore, 180 ml of 0.1% (wt/vol) peptone water (Oxoid, Basingstoke, United Kingdom) was added to 20 g of pulp and beans in a sterile stomacher bag that was vigorously shaken for 3 min in a Stomacher 400 (Seward, Worthington, United Kingdom) to obtain a uniform homogenate. Samples (1.0 ml) of the homogenate were serially diluted 10-fold in 0.1% (wt/vol) peptone water, from which aliquots (0.1 ml) were plated on different selective agar media that were incubated at different temperatures for 1 to 4 days in a standard incubator (Jouan, St. Herblain, France) for the monitoring, isolation, and enumeration (by recording the number of CFU) of specific groups of microorganisms responsible for fermentation: plate count agar (PCA; Oxoid) for the total aerobic bacterial count (37°C), malt extract agar (MEA; Oxoid) plus 100 mg $liter^{-1}$ of oxytetracycline for yeasts (37°C), deoxycholate-mannitol-sorbitol (DMS) agar (25) plus 400 mg $liter^{-1}$ of cycloheximide for AAB (42°C), de Man-Rogosa-Sharpe (MRS [15]) and medium 17 (M17) agar (Oxoid) of Terzaghi and Sandine (71) plus 400 mg $liter^{-1}$ of cycloheximide for LAB (37°C), and kanamycin esculin azide (KAA, Oxoid) agar plus 400 mg $liter^{-1}$ of cycloheximide for enterococci (37°C). Swabs were transferred to 10 ml of 0.1% (wt/vol) peptone water and vortexed for 2 min; 0.1-ml aliquots were spread on the agar media and incubated as described above. Morphologically different colonies were picked up from a suitable dilution of each sample on MRS, M17, KAA, and DMS agar media, grown in test tubes with the appropriate medium, purified through subculturing and plating, and stored at −80°C in the same medium supplemented with 25% (vol/vol) glycerol as a cryoprotectant. This culture-dependent approach yielded 790 bacterial isolates (498 LAB isolates from MRS and M17, 40 LAB isolates from KAA, and 252 AAB isolates) for identification in Belgium. The numbering of the identified bacterial strains is listed in Fig. 1 and 2. It turned out that approximately 15% of the LAB and AAB isolates could not be revived after transport from Ghana to Belgium.

Identification and cluster analysis. In Belgium, all bacteria were checked for purity through successive transfers in and plating on the appropriate media and subsequently identified through a polyphasic taxonomic approach, which made use of both phenotypic (colony and cell morphology, mobility, Gram staining,



244	*Weissella paramesenteroides*
LMG 6907^{T}	*Lactobacillus plantarum*
276, 178†, 172, 171, 181, 185, 192BB, 194A, 190, 197, 177, 180, 188, 192B, 189, 187†, 167B, 191, 173, 195, 176, 75, 272, 450, 454, 451, 370, 289, 363, 327A, 226, 202, 353, 239, 76, 148, 146, 85, 87, 294, 233, 314, 19, 30, 59, 64, 3, 6, 17, 21, 303, 369†, 99, 100, 230, 273, 291, 243, 10, 216, 300, 336, 212, 262, 254, 255†, 250, 265, 142, 297, 199, 218, 229, 66, 71, 35, 56, 65†, 57, 346, 318A, 248, 249, 245A, 267, 270, 261, 147, 196, 186, 449, 258A, 414†, 74, 285, 282, 80, 83, 313†, 38, 287, 309†, 357	*Lactobacillus plantarum*
373, 24B	*Enterococcus* sp.
183†, 192A, 184†, 217†, 319A, 344A, 169, 193†, 95, 69B, 70	*Enterococcus casseliflavus*
LMG 18480, LMG 9297^{T}, 200†	*Weissella confusa*
LMG 7939, LMG 6893^{T}, 20B†	*Leuconostoc mesenteroides* subsp. *mesenteroides*
252†, 257	*Weissella sp.*
283†	*Lactobacillus brevis*
295†, 316, 32B, 3B	*Lactobacillus plantarum*
79, 145†	*Lactobacillus plantarum*
48†, 49B	*Lactobacillus fermentum*
91, 93†	*Enterococcus columbae*
26, 27†, 12, 31, 2, 25, 69, 29	*Lactobacillus fermentum*
LMG 6902^{T}	*Lactobacillus fermentum*
13, 231†, 219A, 283B1, 198, 219B, 452A, 241B2, 241B3, 247B, 238, 342A, 298, 293A, 293B, 241B1, 242†, 247A, 241A, 258B, 5, 7, 39, 46, 279, 372, 259, 33, 34, 55, 14, 32, 20, 24, 28, 4, 23, 92, 143, 47, 53, 52†, 51, 443, 448, 296†, 220, 222, 236, 339, 37, 40, 9, 18, 81, 253A, 84, 86, 201, 61, 62, 60, 203, 204, 211†, 72,	*Lactobacillus fermentum*
88, 63	*Enterococcus* sp.
166†‡, 167A, 354	*Enterococcus faecium*
215, 194B†‡, 225	*'Weissella ghanaensis'*
286	*Leuconostoc sp.*
361, 253B, 274, 78, 240†, 292, 251, 264, 284, 54, 67, 1, 280, 328, 263, 268	*Leuconostoc pseudomesenteroides*
362, 175†, 174	*Weissella cibaria*
311	*Weissella kimchii*
LMG 6899, 305	*Lactobacillus mali*
LMG 6890, 221	*Lactococcus lactis* subsp. *lactis*
333A, 344C, 351, 452B†, 333B	*Lactobacillus plantarum*
271†, 245B	*Lactobacillus mali*

FIG. 1. Dendrogram based on the numerical analysis of generated, digitized $(GTG)_5$ PCR fingerprints from fermented cocoa bean isolates identified as lactic acid bacteria. Banding patterns were clustered together with the reference strains by using UPGMA, with correlation levels expressed as percentage values of the Pearson correlation coefficient. Species validation of representative strains of each cluster included SDS-polyacrylamide gel electrophoresis (indicated by †) and/or 16S rRNA gene sequence analysis (indicated by ‡).

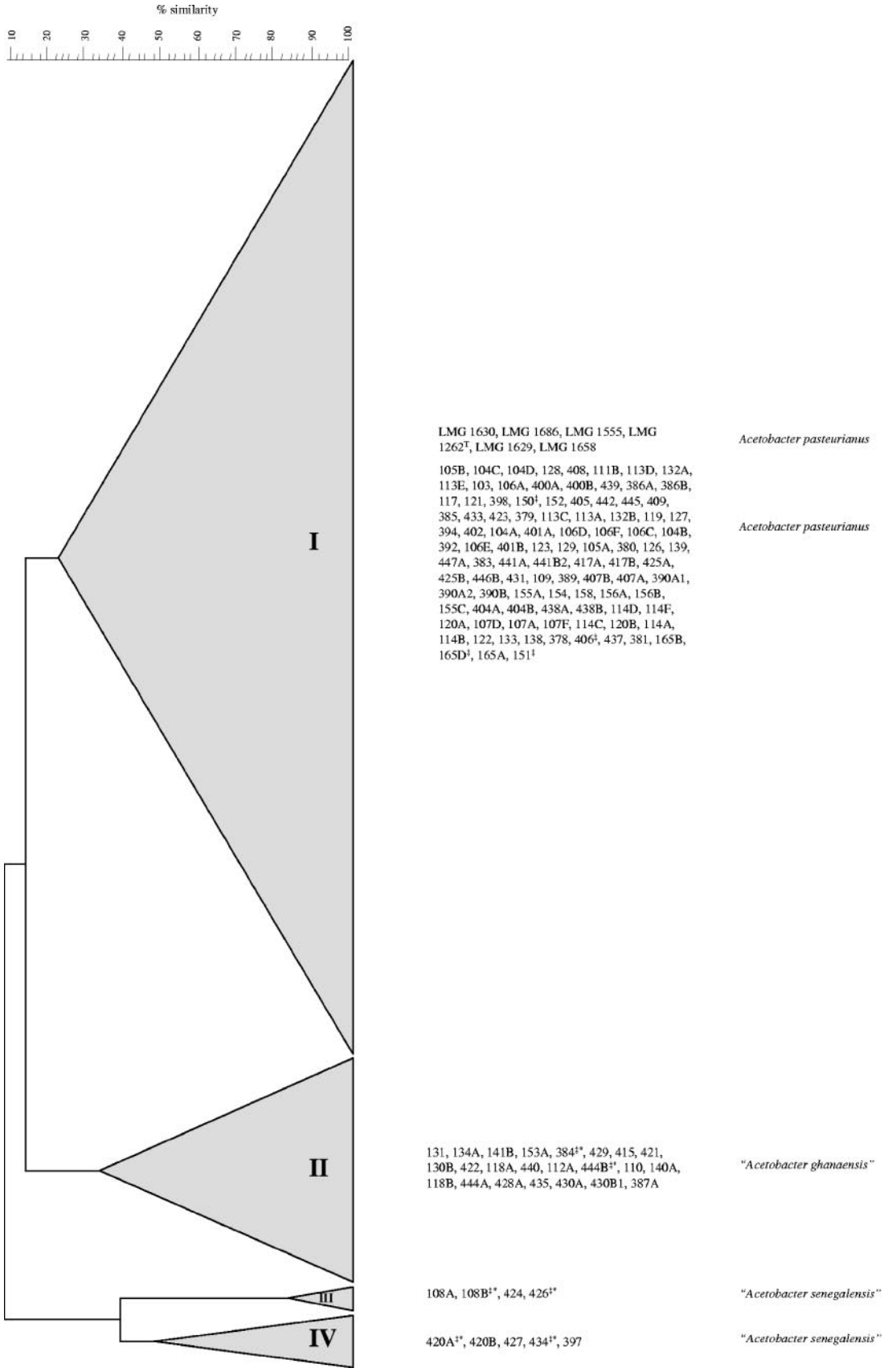


FIG. 2. Dendrogram based on the numerical analysis of generated, digitized $(GTG)_5$ PCR fingerprints from fermented cocoa bean isolates identified as acetic acid bacteria. Banding patterns were clustered together with the reference strains by using the UPGMA, with correlation levels expressed as percentage values of the Pearson correlation coefficient. Species validation of representative strains of each cluster included 16S rRNA gene sequence analysis (indicated by ‡) and/or DNA/DNA hybridizations (indicated by ∗).

catalase activity, and oxidase activity; organic acid production was determined through high-pressure liquid chromatography as described by Van der Meulen et al. [76]) and genotypic analyses. Finally, potential LAB (382) and AAB (170) isolates were grown in MRS and mannitol-yeast extract-peptone medium (2.5% [wt/vol] D-mannitol, 0.5% [wt/vol] yeast extract, and 0.3% [wt/vol] bacteriological peptone [Oxoid]), respectively. Their classification and identification were performed with an optimized PCR of repetitive DNA elements (rep-PCR), based on the $(GTG)_5$ primer, for both LAB (21) and AAB (L. De Vuyst, N. Camu, T. De Winter, K. Vandemeulebroecke, V. Van de Perre, M. Vancanneyt, P. De Vos, and I. Cleenwerck, unpublished results) in combination with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of whole-cell proteins for LAB (56), 16S rRNA gene sequence analysis of representatives of the different clusters obtained after numerical analysis of the rep-PCR profiles for both LAB and AAB (21), and/or DNA/DNA hybridizations for AAB (9). For $(GTG)_5$ PCR, total DNA was extracted from cells obtained through microcentrifugation (13,000 rpm for 20 min) of 10-ml overnight cultures of LAB in MRS medium and AAB in mannitol-yeast extract-peptone medium, as described by Gevers et al. (21), except that for AAB, proteinase K (VWR International, Darmstadt, Germany) was used in an amount of 2.5 mg ml^{-1} TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) instead of mutanolysin. For DNA/DNA hybridizations, total DNA was prepared from approximately 0.5 g (wet mass) by using the protocol of Wilson (79) with minor modifications (9). For more-detailed identifications of AAB, DNA base composition and supplementary phenotypic tests were performed as previously described (9, 46). Finally, cluster analysis of the rep-PCR profiles was performed according to season and farm. Numerical cluster analyses were performed with BioNumerics version 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were obtained by means of the unweighted-pair group method with arithmetic averages (UPGMA) clustering algorithm, with correlation levels expressed as percentage values of the Pearson correlation coefficient.

Direct extraction of DNA from fermented cocoa bean samples. Twenty grams of frozen beans plus pulp samples was homogenized twice in a Stomacher 400 for 5 min, with 70 ml saline added each time. The combined fluid (±120 ml) was removed by decanting and subsequently centrifuged at 170 × *g* at 4°C for 5 min to remove large particles. The supernatant was filtered through a 20-μm-pore-size filter (Whatman, Brentford, United Kingdom). The filtrate was centrifuged at 8,000 × *g* at 4°C for 20 min to pellet the cells, which were subsequently frozen at −20°C for at least 1 h. The thawed pellet was washed in 1 ml TES buffer (6.7%[wt/vol] sucrose, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspended in 300 μl STET buffer (8% [wt/vol] sucrose, 5% [wt/vol] Triton X-100, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA). Seventy-five microliters of lysis buffer (TES containing 1,330 U ml^{-1} mutanolysin and 100 mg ml^{-1} lysozyme; Sigma-Aldrich, St. Louis, MO) and 100 μl proteinase K (TE containing 2.5 mg ml^{-1}) were added, and the suspension was incubated at 37°C for 1 h. After the addition of 40 μl preheated (37°C) 20% (wt/vol) SDS in TE buffer and a pinch of glass beads with a diameter of 150 to 212 μm (Sigma-Aldrich), cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by a 10-min incubation at 65°C. One-hundred microliters of TE buffer were added, and the lysate was extracted with 1 volume of phenol-chloroform-isoamyl alcohol (49:49:1) (Sigma-Aldrich) for 30 s. Phases were separated by microcentrifugation (13,000 rpm for 5 min at 4°C) using Phase Lock Gel tubes (Eppendorf AG, Hamburg, Germany). The aqueous phase was further purified by using a NucleoSpin column according to the manufacturer's instructions (Macherey Nagel GmbH, Düren, Germany). This was done primarily to remove potentially PCR-inhibiting compounds, as cocoa pulp contains polysaccharides, proteins, enzymes, and polyphenols. Finally, the eluted phase was carefully mixed with 70 μl 5 M NaCl and 1 ml isopropanol, and the DNA was precipitated on ice for at least 15 min. The DNA was collected by microcentrifugation (13,000 rpm for 30 min at 4°C), and the pellet was washed in ice-cold 70% (vol/vol) ethanol. The DNA was dried in a vacuum excicator and resuspended in 100 μl TE. Three microliters of DNase-free RNase (10 mg ml^{-1}; Sigma-Aldrich) was added, and the whole suspension was incubated at 37°C for 10 min. The final samples were stored at −20°C until further use.

PCR. The primers used in this study were a primer pair that amplifies DNA from species of LAB (LAC1-LAC2 [78]) and a primer pair that amplifies DNA from species of AAB and LAB (WBAC1-WBAC2 [44]). LAC1 (5′-AGCAGTA GGAATCTTCCA-3′) and LAC2 (5′-ATTTCACCGCTACACATG-3′) target the V3-V4 region of the 16S rRNA gene, and WBAC1 (5′-GTCGTCAGCTCG TGTCGTGAGA-3′) and WBAC2 (5′-CCCGGGAACGTATTCACCGCG-3′) target the V7-V8 region of the 16S rRNA gene. To facilitate DGGE separation, a GC-rich sequence (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCC CCCGCCCC-3′) was attached to one of the primers in each primer pair. PCR amplifications were performed using a DNA T3 thermocycler (Biometra, Westburg, The Netherlands) in a final volume of 50 μl, containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, 1.25 IU of *Taq* DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), and 3 μl of extracted DNA (approximately 500 ng). One single PCR core program was used for all primer pairs: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 20 s, annealing at a primer-specific temperature (LAC, 61°C; WBAC, 67°C) for 45 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min, followed by cooling to 4°C. PCR amplification products were stored at −20°C. Amplicons (10 μl) were run in 1.5× TAE (40 mM Tris-acetate, 2 mM Na_2EDTA, pH 8.5) agarose (0.8%, wt/vol) gels at 100 V for 30 min, flanked by the EZ Load 100-bp molecular ruler (Bio-Rad, Hercules, CA).

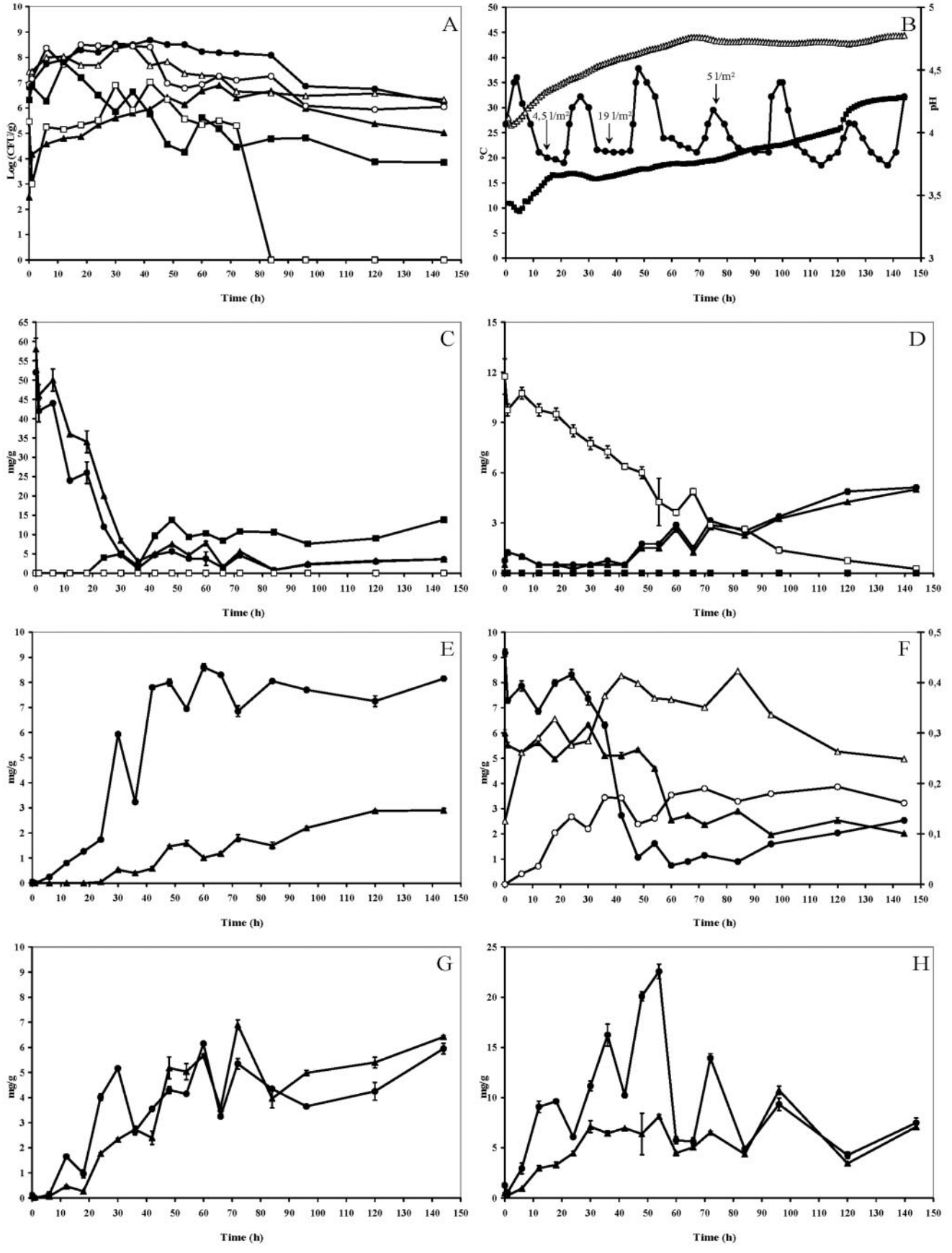
DGGE analysis. (i) Cocoa bean samples. PCR products were analyzed on DGGE polyacrylamide gels by using a protocol based on that of Muyzer et al. (47). The gels (160 by 160 by 1 mm) consisted of 8% (vol/vol) polyacrylamide (National Diagnostics, Atlanta, GA) in 1× TAE buffer, using a 35 to 60% and a 50 to 70% denaturant gradient (100% denaturing polyacrylamide solution corresponded with 7 M urea [National Diagnostics] and 40% [vol/vol] formamide [Sigma]) for PCR products obtained with the LAC1-LAC2 and WBAC1-WBAC2 primers, respectively. Electrophoresis of PCR samples was carried out in 1.0× TAE running buffer at 70 V for 16 h at a constant temperature of 60°C, using the DCode system apparatus (Bio-Rad). After electrophoresis, all gels were stained with ethidium bromide (50 μl of ethidium bromide in 500 ml of 1.0× TAE buffer) for 10 min, followed by visualization of the DGGE band profiles under UV light. Digital capturing of images was performed with the Gel Doc EQ system (Bio-Rad). The resulting fingerprint pictures were analyzed using BioNumerics version 4.0 software (Applied Maths). DGGE analyses were performed twice. Normalization of the gels was performed by using band ladders of known bacterial DNA in three lanes in all gels. Therefore, DNA originating from pure cultures of *Lactobacillus plantarum* LMG 6907^T, *Lactobacillus fermentum* LMG 6902^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893^T, *Lactobacillus casei* LMG 6904^T, *Pediococcus acidilactici* LMG 11384^T, and *Lactobacillus acidophilus* LMG 9433^T and of *L. plantarum* LMG 6907^T, *Enterococcus faecalis* LMG 7937^T, *Acetobacter pasteurianus* LMG 1262^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893^T, and *Acetobacter syzygii* LMG 21419^T was mixed in equal volumes of the same concentration and used as a reference ladder for LAB and AAB, respectively, after the corresponding PCR amplicons were positioned in a DGGE gel with the appropriate gradient. For cluster analysis of DGGE profiles, the calculation of similarities in the profiles of bands was based on the Dice coefficient to provide a qualitative discrimination among the patterns.

(ii) Colonies from agar plates. For PCR-DGGE analyses of bulk cells, colonies were washed off from an appropriate dilution on MRS agar plates of the corresponding heap samples with saline (0.85% [wt/vol] NaCl) in Ghana; this cell suspension was frozen and transported on dry ice to Belgium. Total DNA from the cells was extracted as described above for $(GTG)_5$ PCR analysis. PCR-DGGE was performed as described above for LAB.

(iii) DNA sequencing. For the sequencing of DGGE bands, bands of interest were excised from the gels with a sterile blade, mixed with 50 μl of sterile water, and incubated overnight at 4°C to allow the DNA of the bands to diffuse out of the polyacrylamide gel blocks. Two microliters of this aqueous solution was used to reamplify the PCR products with the same primers, including the GC clamp. The amplicons were checked for purity by another DGGE run under the conditions described above with amplified DNA of the original sample as a control. Only reamplified PCR products migrating as a single band and at the same position with respect to the control were amplified with the primer without the GC clamp and sequenced in a commercial facility using capillary sequencing technology (VIB, Brussels, Belgium). Searches in the GenBank database were performed with the BLAST program (1) to determine the closest known relatives of the partial 16S rRNA gene sequences obtained.

Metabolite target analysis. (i) Sample preparation. Frozen samples of beans plus pulp were used to prepare aqueous extracts for metabolite analysis. Beans were physically separated from pulp by manual peeling. Samples (20 g) of separate fractions of pulp and beans were mixed with 80 ml of ultrapure water (MilliQ; Waters Corp., Milford, MA) with an Omnimixer (Phillips, Brussels, Belgium) for 5 min. The homogenate was centrifuged at 17,000 × *g* at 4°C for 15 min, and the supernatant was retained. The sediment was washed with 20 ml of ultrapure water and centrifuged, and the washing supernatants were combined with the first ones to provide aqueous extracts for further analyses. These extracts were clarified by filtration through 0.45-μm-pore-size filters (Whatman) before further use.

(ii) Gas chromatography-mass spectrometry. Short-chain fatty acids, branched short-chain fatty acids, and other volatile compounds, as well as


A
Log (CFU/g)
Time (h)
B
°C
pH
4,5 l/m²
19 l/m²
5 l/m²
Time (h)
C
mg/g
Time (h)
D
mg/g
Time (h)
E
mg/g
Time (h)
F
mg/g
Time (h)
G
mg/g
Time (h)
H
mg/g
Time (h)

ethanol, methanol, acetaldehyde, diacetyl, and acetoin, in aqueous extracts were determined with gas chromatography-mass spectrometry according to the methods described by Van der Meulen et al. (76), except that 2,6-dimethyl phenol was used as an internal standard in all cases.

(iii) HPAEC with pulsed amperometric detection. The amounts of glucose, fructose, sucrose, mannitol, and erythritol of aqueous extracts were determined by high-pressure anion exchange chromatography (HPAEC) with pulsed amperometric detection as described by Van der Meulen et al. (76), except that extracts (700 μl) were treated with acetonitrile (700 μl) to remove proteins.

(iv) HPAEC and conductivity with ion suppression. Organic acids (citric acid, acetic acid, lactic acid, gluconic acid, ketogluconic acids, formic acid, oxalic acid, malic acid, and fumaric acid) in aqueous extracts were determined by HPAEC and conductivity with ion suppression, using an AS-19 column (Dionex, Sunnyvale, CA). The mobile phase, at a flow rate of 1.0 ml min^{-1}, consisted of ultrapure water (0.015 μS cm^{-1}; eluent A) and 100 mM KOH (eluent B). The following gradient was applied: 0 min, 96% eluent A and 4% eluent B; 20 min, 96% eluent A and 4% eluent B; and 60 min, 0% eluent A and 100% eluent B. The aqueous extracts were treated with acetonitrile as described above, appropriately diluted, filtered (0.2 μm, Minisart RC 4; Sartorius, Darmstadt, Germany) prior to injection, and run together with the appropriate external standards.

(v) Liquid chromatography-mass spectrometry. Amino acids, amino acid metabolites, and succinic acid in aqueous extracts were quantified through liquid chromatography-mass spectrometry on a Waters 2695 chromatograph coupled to a Quattro Micro mass spectrometer (Micromass; Waters Corp.). Succinic acid and amino acid metabolites were determined according to the method of Van der Meulen et al. (76). In the case of amino acids, a symmetry column (Waters Corp.) was used. The mobile phase, at a flow rate of 0.2 ml min^{-1} and linearly increasing to 0.5 ml min^{-1} over a period of 45 min with a flow rate of 0.2 ml min^{-1} afterwards, was composed of 0.1% (vol/vol) formic acid in ultrapure water (eluent A) and 90% (vol/vol) acetonitrile in ultrapure water (eluent B). The following gradient was used (vol/vol): 0 min, 90% eluent A and 10% eluent B; 45 min, 10% eluent A and 90% eluent B; 46 min, 90% eluent A and 10% eluent B; and 60 min, 90% eluent A and 10% eluent B. One hundred microliters of internal standard (0.002% [wt/vol] 2-aminobutyric acid in ultrapure water) was added to 500 μl of aqueous extract. The amino acids were derivatized using an AccQ Fluor reagent kit according to the manufacturer's instructions (Waters Corp.). The derivatized samples as well as the appropriate external standards were injected.

All sample preparations and analyses were performed in triplicate, and the mean values ± standard deviations are represented as milligrams per gram of pulp or beans.

RESULTS

Population dynamics of Ghanaian cocoa bean heap fermentations: culture-dependent approach. Figure 3 shows the course of the different microbial groups selected (Fig. 3A) and the temperature and pH (Fig. 3B) for heap 5, which is representative of the seven spontaneous cocoa bean heap fermentations performed. For comparison, the lower and higher values of the seven fermentations performed are included below. In general, simultaneous growth of yeasts, LAB, and AAB took place; no other major groups of microorganisms were involved, as reflected by the PCA counts (Fig. 3A). Yeast counts of 4.3 to 7.0 log CFU g^{-1} of pulp and beans were present in the heaps at the beginning of the fermentation. The size of the yeast population increased during the first 12 to 18 h and grew to a maximum population of 6.6 to 7.5 log CFU g^{-1}. Upon prolonged fermentation, the yeast population declined to 3.0 to 4.7 log CFU g^{-1}, and in heap 1, no yeasts were retrieved after 144 h of fermentation. All fermentations were characterized by a high level of LAB from the start (4.4 to 8.0 log CFU g^{-1}), as reflected by both MRS and M17 counts. LAB grew during fermentation to maximum populations of 7.5 to 8.9 log CFU g^{-1} after 30 to 48 h. Afterwards, there was a slight decrease of the LAB population, sometimes stabilizing upon prolonged fermentation (5.0 to 6.5 log CFU g^{-1}). KAA counts (3.6 to 5.7 log CFU g^{-1}) were always lower than those on MRS and M17. However, KAA counts always decreased upon prolonged fermentation, even reaching zero levels after 72, 84, and 120 h in heaps 4, 5, and 6, respectively. AAB, initially present in densities of 2.0 to 4.6 log CFU g^{-1}, grew slower and reached lower maximum population densities (6.4 to 7.5 log CFU g^{-1}) after about 66 h of fermentation. Compared to LAB, the AAB population slightly decreased upon prolonged fermentation and sometimes stabilized (4.9 to 6.2 log CFU g^{-1}).

All fermentations were characterized by an initial pH of approximately 3.5 that increased during the first 18 h, in some fermentations preceded by a slight and short decrease (data not shown) and always followed by a constant, almost linear increase to approximately pH 4.0 to 4.3 after 120 to 144 h of fermentation (Fig. 3B).

The ambient temperature during the day and night was 24 to 39°C and 19 to 24°C, respectively, with maxima being slightly lower in the case of rainfall (Fig. 3B). The temperature inside the heaps went from on average 26.3°C (26.0 to 30.0°C) at the start of the fermentations to a maximum temperature of on average 43.5°C (42.2 to 47.7°C). Rainfall slightly influenced the temperature course of the heap, with ups and downs of 1 to 4°C (Fig. 3B).

In general, no pronounced differences were observed between the repetitions and the seasons for microbial counts, pH, and temperature during cocoa bean heap fermentation at both farms, indicating the validity of the sampling, isolation, and measurement procedures.

Identification of the isolates. Phenotypic analyses indicated that isolates from MEA were yeasts (large colonies and bigger cells than those of LAB and AAB) and that 240 out of 382 isolates from MRS, M17, and KAA belonged to the LAB group (gram positive; rods or cocci; nonmotile; catalase negative; oxidase negative; production of lactic acid, acetic acid, and/or ethanol), while 132 out of 170 isolates from DMS belonged to the AAB group (gram negative; rods; motile or nonmotile; catalase positive; oxidase negative; production of acetic acid, gluconic acid, and 2-keto-gluconic acid). Noteworthy was the selective isolation of AAB on DMS and of *L. plantarum* on KAA under the conditions used, representing

FIG. 3. Course of a spontaneous Ghanaian cocoa bean heap fermentation (heap 5) representative of the seven fermentations performed. (A) Microbial succession of yeasts (MEA, ■), LAB (MRS, ●; M17, ○; KAA, □), AAB (DMS, ▲), and total aerobic bacteria (PCA, △). (B) Temperature inside (△) and outside (●) the heap and pH (■) inside the heap. Measurements of rainfall are indicated by arrows. (C) Course of residual glucose (●), fructose (▲), and sucrose (□) and of mannitol produced (■) in the pulp. (D) Course of residual glucose (●), fructose (▲), and sucrose (□) and of mannitol produced (■) in the beans. (E) Course of lactic acid produced in the pulp (●) and in the beans (▲). (F) Course of residual citric acid (full symbols, left axis) and of succinic acid produced (open symbols, right axis) in the pulp (● and ○) and in the beans (▲ and △). (G) Course of acetic acid produced in the pulp (●) and in the beans (▲). (H) Course of ethanol produced in the pulp (●) and in the beans (▲).

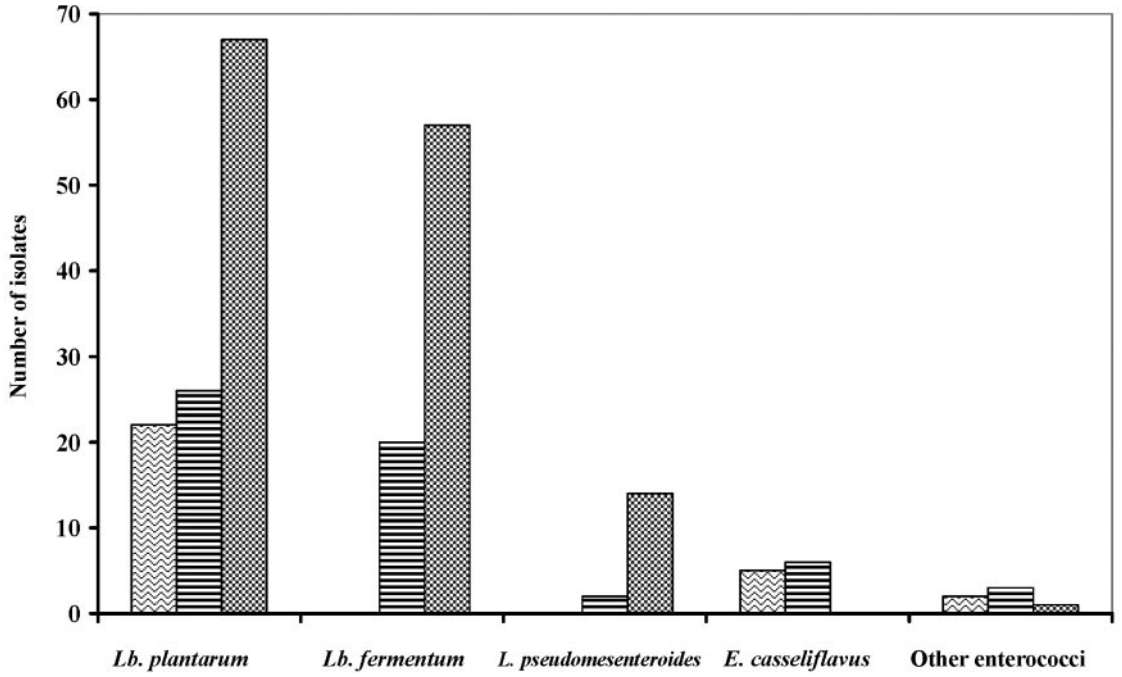


FIG. 4. Comparison of different media for isolation of lactic acid bacteria from fermented cocoa bean samples. The media are as described in Materials and Methods: ≋, KAA; ≡, M17; and ▩, MRS. The dilutions from which the different species were picked are not shown. *Lb.*, *Lactobacillus*.

78% and 72% of the isolates, respectively. Most *L. plantarum* strains were isolated from MRS; almost no enterococci were found neither on KAA (15%) nor on the other media (Fig. 4).

LAB classification and identification (Fig. 1) revealed four main clusters among the isolates: *L. plantarum* (cluster I, 114 strains), *L. fermentum* (cluster III, 76 strains), *Leuconostoc pseudomesenteroides* (cluster IV, 16 strains), and *Enterococcus casseliflavus* (cluster II, 11 strains). For all heaps, *L. plantarum* (4 to 7 log CFU g^{-1}) was the most abundant species in the first 42 h of fermentation, while *L. fermentum* (4 to 7 log CFU g^{-1}) was still isolated upon a longer fermentation time (Table 1). *Leuconostoc pseudomesenteroides* was isolated only in the beginning of almost all heap fermentations (5 to 7 log CFU g^{-1}), except for heaps 3 and 6, where it was absent (Table 1). Also, *E. casseliflavus* was isolated only at the beginning of the fermentations (2 to 6 log CFU g^{-1}), except for heaps 1 and 2, where it was absent (Table 1). The remaining 23 LAB isolates were distributed among different taxa (Fig. 1; Table 1). For instance, different *Weissella* species were picked up in the beginning of the fermentations (Table 1). Interestingly, isolates 194B, 215, and 225 represented a novel species most closely related to the genus *Weissella*, as based on its 16S rRNA gene sequence divergence from known species; this novel species is further referred to as "*Weissella ghanaensis*" (K. De Bruyne, N. Camu, P. Vandamme, L. De Vuyst, and M. Vancanneyt, unpublished results) and was picked up from heap 7 only. Also, isolates 252 (confirmed by 16S rRNA gene sequence analysis) and 257 belong to a new *Weissella* sp. Occasionally, isolates belonging to *Enterococcus faecium*, *Lactobacillus brevis*, *Lactobacillus mali*, and *Leuconostoc mesenteroides* were found, but only in the beginning of the fermentations (Table 1). *Leuconostoc pseudomesenteroides* (six isolates) and *E. casseliflavus* (three isolates) as well as other enterococci (five isolates), *Weissella* spp. (five isolates), *Leuconostoc durionis* (one isolate), *L. mali* (one isolate), and *Lactococcus lactis* subsp. *lactis* (one isolate) were found on farmers' hands, baskets, machetes, and pods (Table 1; Fig. 1). Baskets and leaves were contaminated with yeasts, *L. plantarum* (24 isolates), and *L. fermentum* (8 isolates), too (Table 1).

AAB classification and identification (Fig. 2) revealed only four clusters among the isolates: *A. pasteurianus* (cluster I, 100 strains), *Acetobacter syzygii*-like (cluster II, 23 strains), *Acetobacter tropicalis*-like (cluster III, 4 strains), and *A. tropicalis*-like (cluster IV, 5 strains). The most dominant isolates from the beginning of the cocoa fermentation belonged to *A. pasteurianus* and *A. tropicalis*-like (Table 1), both in amounts of 3 to 6 log CFU g^{-1}. Later on, isolates of *A. tropicalis*-like were replaced by those of *A. syzygii*-like, the latter occurring in amounts of 4 to 6 log CFU g^{-1}. Both species finally disappeared, while *A. pasteurianus* survived longer throughout the fermentations (Table 1). All AAB species were picked up from all heaps, except for *A. syzygii*-like and *A. tropicalis*-like, which were not picked up from heap 3 and heaps 1, 3, and 5, respectively.

DNA/DNA hybridizations between AAB isolate 444B and isolates 430A and 415, all from cluster II, revealed high DNA binding values of 99% and 88%, respectively, and intermediate DNA binding values with *A. syzygii* LMG 21419^T (46%) and *Acetobacter lovaniensis* LMG 1617^T (47%), the phylogenetically closest *Acetobacter* species, which are below species level (69). This novel species is further referred to as "*Acetobacter ghanaensis*" (I. Cleenwerck, N. Camu, K. Engelbeen, T. De

TABLE 1. Sources of the different species of LAB and AAB identified, listed according to the number of isolates[a]

Taxon (no. of isolates)	Heap	Sample(s)
LAB		
Lactobacillus plantarum (114)	1	S0, S1, S2, S3, S4, S6, S8, S9, leaves
	2	S4, S9
	3	S0, S1, S2, S3, S5, machete, hand, pod
	4	S0, S1, S2, S3, S4, S6, S7
	5	S0, S1, S2, S3, S4, S5, S8, hand, basket
	6	S0, S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, basket, hand
	7	S0, S2, S3, S5, S6, S7, S8, S13 basket, hand
Lactobacillus fermentum (76)	1	S0, S1, S2, S4, S6, S9, leaf
	2	S0, S1, S2, S3, S6, S9, S11, S13
	3	S1, S3, S4, S5, machete, pod
	4	S1, S3, S4, S5
	5	S3, S4, S5, S8, machete
	6	S3, S5, S7, S9, S10, S11, S12, S14, hand, leaf
	7	S0, S8, S12
Leuconostoc pseudomesenteroides (16)	1	S4, S6
	2	S4
	3	Machete, hand
	4	S0, S1
	5	S0, S1, S2, machete, basket, hand
	6	Pod
	7	S6
Enterococcus casseliflavus (11)	4	S0
	5	Hand
	6	S0, leaf, basket
	7	S0, S2
Enterococcus spp. (5)	1	Leaf
	3	S2, S3
	4	S3
Enterococcus faecium (3)	4	S0
Lactobacillus mali (3)	4	S4
	5	pod
Weissella cibaria (3)	5	S1, hand
"*Weissella ghanaensis*" (3)	7	S2
Enterococcus columbae (2)	3	Machete
Weissella sp. (2)	6	S1, S3
Lactococcus lactis (1)	6	Pod
Lactobacillus brevis (1)	5	S4
Leuconostoc durionis (1)	5	Hand
Leuconostoc mesenteroides (1)	1	S6
Weissella confusa (1)	6	Hand
Weissella paramesenteroides (1)	6	Machete
Weissella kimchii (1)	5	S1
AAB		
Acetobacter pasteurianus (100)	1	S4, S5, S9, S10, S11, S12, S13, S14
	2	S2, S9, S10, S11, S12, S13
	3	S1, S2, S3, S7
	4	S5, S6, S8, S9, S10
	5	S5, S6, S8, S9, S10, S11, S12
	6	S1, S2, S3, S7, S10, S12
	7	S1, S2, S5, S6, S7, S12
"*Acetobacter ghanaensis*" (23)	1	S3, S5, S11, S12
	2	S10, S11, S12
	4	S6
	5	S1
	6	S5, S6, S9, S12
	7	S3, S4, S7, S8, S10
"*Acetobacter senegalensis*"(9)	1	S5
	2	S3
	4	S5
	6	S1, S2, S3, S6
	7	S11

[a] Sample numbers S0, S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, and S16 represent samples taken after 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 84, 96, 120, and 144 h of fermentation, respectively. Isolates from cocoa pods, baskets, machetes, banana/plantain leaves, and farmers' hands were taken as swabs.

Winter, K. Vandemeulebroecke, P. De Vos, and L. De Vuyst, unpublished results). DNA/DNA hybridizations between isolates 108B (cluster III) and 420A (cluster IV) revealed a DNA homology value of 75%. Hybridizations of both isolates against *A. tropicalis* LMG 21419^T and the newly proposed species "*Acetobacter senegalensis*" LMG 1617^T (B. Ndoye, I. Cleenwerck, K. Engelbeen, R. Dubois-Dauphin, A. T. Guiro, S. Van Trappen, A. Willems, and P. Thonart, unpublished results), their phylogenetically closest neighbors, revealed respective DNA hybridization values of 54 to 58%, which is below species level, and 79 to 81%, which is above the accepted limit (70%) for species delineation (69). This indicates that rep-PCR clusters III and IV represent two clusters of the newly proposed "*A. senegalensis*" isolates. The G+C contents of DNA from isolates 108B and 420A were 55.6 and 55.9 mol%, respectively. These values were similar to the G+C content of DNA obtained for the type strain of "*A. senegalensis*." Further, isolates 108B and 420A showed the same phenotypic features as the type strain of "*A. senegalensis*": growth on yeast extract and 30% (wt/vol) D-glucose, growth with ammonium as the sole nitrogen source and ethanol as the energy source, growth in the presence of 10% (vol/vol) ethanol, growth with glycerol as the sole energy source but not with maltose or methanol, and the ability to produce 2-keto-D-gluconic acid from D-glucose but not 5-keto-D-gluconic acid.

Cluster analyses of the rep-PCR profiles according to farm and season did not reveal significantly different results (data not shown).

Population dynamics of Ghanaian cocoa bean heap fermentations: culture-independent approach. Figure 5 shows representative fermentation courses for all seven spontaneous cocoa bean heap fermentations as obtained by a community analysis of DNA samples through 16S rRNA PCR-DGGE with the LAC1-LAC2 primer pair (only heaps 2 and 5 are shown). The number and intensity of visible bands that varied among samples with fermentation time and among fermentations could be related to shifts of the bacterial compositions and hence a reflection of the impact of certain strains on fermentation (cf. infra) as well as heterogeneous samplings. Concerning the microbial succession as determined by DGGE in general, it turned out that *L. fermentum* (band i) was the most dominant species throughout fermentation in all heaps (Fig. 5A and B), although *L. plantarum* (band iii) was detected in heap fermentations 2, 6, and 7 mainly during the first part of the fermentation (Fig. 5A). In general, the intensity of the band corresponding with *L. fermentum* increased upon fermentation (Fig. 5A and B), in some fermentations still visible after 66 h (Fig. 5B) but in others already disappeared before that time (Fig. 5A). *L. pseudomesenteroides* (band ii) was detected during the first 24 h of fermentation in heaps 2, 3, 4, 5, and 7 (Fig. 5A and B). Also, an unidentified bacterium (band iv), of which the partial 16S rRNA gene sequence was identical to that of the "*W. ghanaensis*" isolates, appeared throughout heap fermentations 2, 3, 4, 5, and 6 up to 48 h (Fig. 5B). Lanes without bands as in heap 2 indicate time points below the detection limit of DGGE (Fig. 5A), due to a too-low number of LAB as a result of death or lysis of the cells (as it was difficult to recover LAB and AAB towards the end of the fermentations, too) (Table 1), and the increasing possibility of interference by inhibiting compounds for DNA extraction and PCR amplification present in the samples upon fermentation time. Finally, PCR-DGGE analyses performed on DNA extracted from cell mass recovered from MRS agar plates where colonies were picked from revealed the same species identities (Fig. 5C).

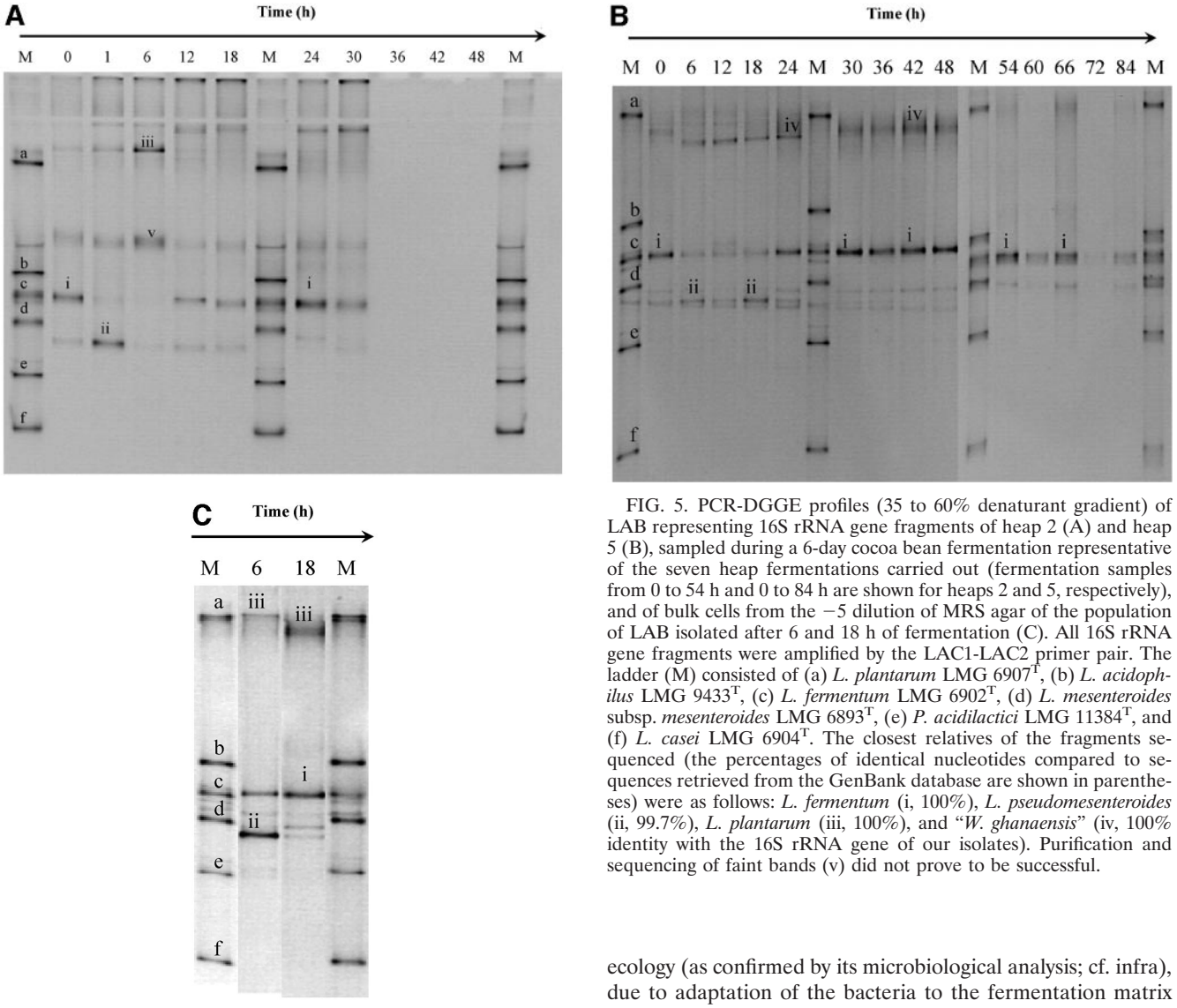


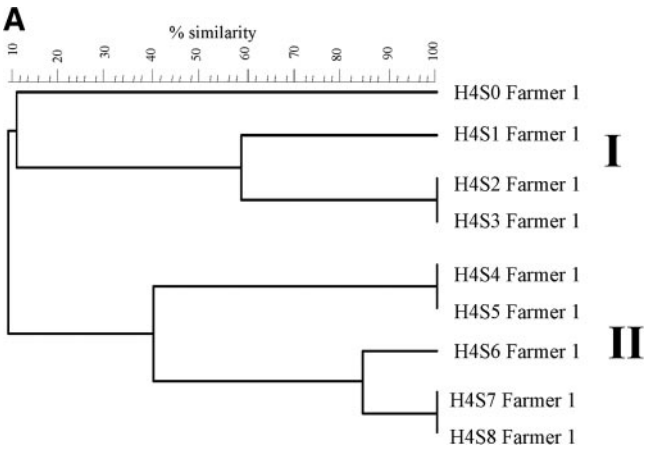
FIG. 5. PCR-DGGE profiles (35 to 60% denaturant gradient) of LAB representing 16S rRNA gene fragments of heap 2 (A) and heap 5 (B), sampled during a 6-day cocoa bean fermentation representative of the seven heap fermentations carried out (fermentation samples from 0 to 54 h and 0 to 84 h are shown for heaps 2 and 5, respectively), and of bulk cells from the −5 dilution of MRS agar of the population of LAB isolated after 6 and 18 h of fermentation (C). All 16S rRNA gene fragments were amplified by the LAC1-LAC2 primer pair. The ladder (M) consisted of (a) *L. plantarum* LMG 6907^T, (b) *L. acidophilus* LMG 9433^T, (c) *L. fermentum* LMG 6902^T, (d) *L. mesenteroides* subsp. *mesenteroides* LMG 6893^T, (e) *P. acidilactici* LMG 11384^T, and (f) *L. casei* LMG 6904^T. The closest relatives of the fragments sequenced (the percentages of identical nucleotides compared to sequences retrieved from the GenBank database are shown in parentheses) were as follows: *L. fermentum* (i, 100%), *L. pseudomesenteroides* (ii, 99.7%), *L. plantarum* (iii, 100%), and "*W. ghanaensis*" (iv, 100% identity with the 16S rRNA gene of our isolates). Purification and sequencing of faint bands (v) did not prove to be successful.

Using the WBAC primers, PCR-DGGE analyses revealed again *L. plantarum* and *L. fermentum* as the dominating species, while no AAB species were detected, although PCR-DGGE analysis of AAB reference strains was successful (data not shown). Taking into account the totality of PCR-DGGE runs performed, no pronounced differences were observed between the repetitions.

Cluster analysis of the PCR-DGGE profiles revealed no influence of the season (data not shown). However, it revealed influences of the farm (heaps). For instance, cluster analysis of the PCR-DGGE profiles of heap fermentations 4 and 5 revealed a difference in bacterial ecology between both farms, as shown by the 60% similarity percentage limit (Fig. 6A). Also, a shift in the bacterial ecology from day 1 (samples 1 to 3) to day 2 (samples 4 to 8) occurred within a heap (clusters I and II), respecting the differences between the farms (subclusters A and B). The start sample that occurred as an outlier, according to the clustering performed, showed a shift in bacterial ecology (as confirmed by its microbiological analysis; cf. infra), due to adaptation of the bacteria to the fermentation matrix (Fig. 6B).

Metabolite target analyses of Ghanaian cocoa bean heap fermentations. Figure 3C to H are representative of the seven spontaneous cocoa bean heap fermentations performed. For comparison, the lower and higher values of the seven fermentations performed are included below. Variations within one fermentation and between different fermentations were due mainly to sample collection, as the fermenting mass was not turned and hence was not homogeneous.

In general, almost all free sucrose in the pulp was consumed at the start of fermentation (Fig. 3C). The citric acid of the pulp was rapidly consumed as well (within 30 to 48 h of fermentation) and paralleled LAB growth (Fig. 3F), causing the pH of the pulp to increase. The initial decrease in citric acid of the beans was less than that of the pulp (Fig. 3F). At the end of all fermentations, citric acid slightly increased or stabilized in both pulp and beans. Fructose and glucose disappeared simultaneously in the pulp and were almost exhausted after 36 to 48 h of fermentation, whereas mannitol increased from 20 h of fermentation and stabilized from 70 h of fermentation onward (Fig. 3C). Erythritol was not found. Sucrose was hydro-



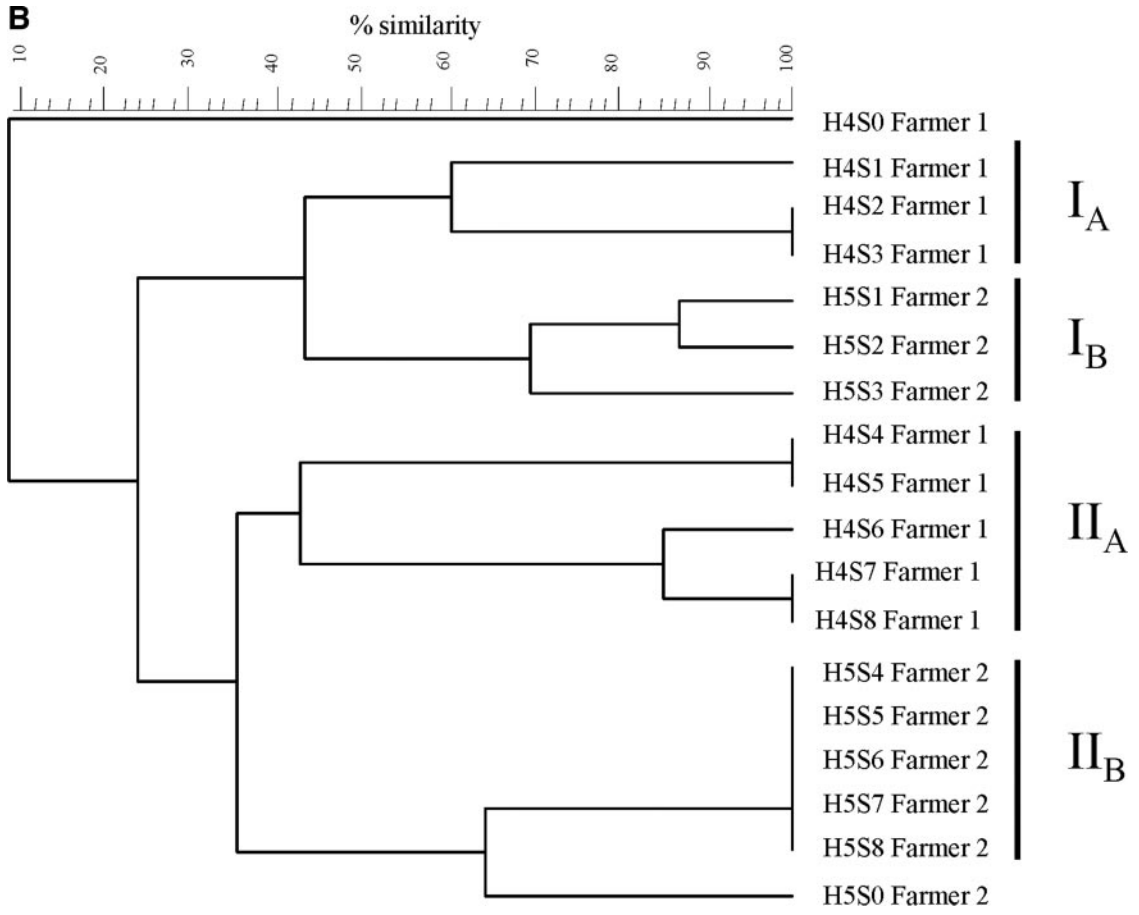


FIG. 6. Illustrations of the cluster analysis of the PCR-DGGE profiles of spontaneous cocoa bean heap fermentations. Dendrograms were based on the Dice coefficient of similarity (weighted) and obtained with the UPGMA clustering algorithm. Samples are indicated by fermentation heap H, sample number S (see Table 1), and farmer (Farmer 1 and Farmer 2). The identified clusters are indicated by numerals to the right of each panel. (A) Bacterial shift in cocoa bean heap fermentation 4 (H4) at farmer 1. (B) Bacterial shift in and effect of the heap on fermentations 4 (H4) and 5 (H5) at farmers 1 and 2, respectively.

lyzed into glucose and fructose in the beans (Fig. 3D). Ethanol increased upon fermentation, almost simultaneously in pulp and beans, and reached a maximum of 10 ± 0.40 to 25 ± 0.40 mg g^{-1} in the pulp after 30 to 36 h of fermentation, after which it declined (Fig. 3H). Acetic acid levels increased after 6 h of fermentation. Concentrations of 10 ± 0.11 to 15 ± 0.11 mg g^{-1} of acetic acid were found in the fermented cocoa bean pulp after 90 h of fermentation.

The total amount of organic acids differed from heap to heap (data not shown). Whereas the amounts of ethanol and acetic acid were always slightly higher in the pulp than in the beans, the amounts of lactic acid were considerably higher in the pulp than in the beans. Lactic acid reached a maximum concentration of 1.0 ± 0.06 to 9.0 ± 0.06 mg g^{-1} in the pulp after 48 h of fermentation. Succinic acid increased up to 0.10 ± 0.0025 to 0.25 ± 0.0025 mg g^{-1} and 0.23 ± 0.0035 to 0.65 ± 0.0035 mg g^{-1} in pulp and beans, respectively, after 40 to 60 h of fermentation. Ketogluconic acid, malic acid, and fumaric

acid were not found; gluconic acid (2 ± 0.005 to 4 ± 0.005 mg g^{-1} in pulp and 0.2 ± 0.005 to 0.4 ± 0.005 mg g^{-1} in beans) and oxalic acid (1 ± 0.004 mg g^{-1} in pulp and 1 ± 0.004 to 2 ± 0.004 mg g^{-1} in beans) remained stable as a function of time. Fatty acids (except for acetic acid), methanol, acetaldehyde, diacetyl, and acetoin were not found under the analysis conditions.

There was an increase in all free amino acids upon fermentation from 1.1 ± 0.003 to 3.5 ± 0.003 mg g^{-1} to 4.4 ± 0.003 to 4.8 ± 0.003 mg g^{-1} in the pulp and from 1.7 ± 0.002 to 2.1 ± 0.002 mg g^{-1} to 5.2 ± 0.002 to 5.9 ± 0.002 mg g^{-1} in the beans (depending on the amino acid), except for lysine and glutamine in the pulp, asparagine in pulp and beans, and aspartic acid in the beans, which all decreased. In general, hydrophobic amino acids increased and acid amino acids decreased upon fermentation time (data not shown). The glutamic acid content increased in the pulp and first decreased and then increased in the beans (data not shown). Arginine showed a decrease followed by an increase in the pulp, while histidine showed an increase followed by a decrease in the beans (data not shown). Aromatic amino acid metabolites such as phenyllactic acid and OH-phenyllactic acid were not found under the analysis conditions.

DISCUSSION

Cocoa bean fermentations continue to be conducted in a traditional manner, resulting in a great diversity in production methods and organoleptic characteristics of the end products (4, 67). Studies have been performed on cocoa bean fermentations in Belize, Brazil, Ghana, Indonesia, Malaysia, and Trinidad using classical microbiological methods (3, 8, 11, 51, 53–55, 72). Only in a few cases have molecular techniques been used to study cocoa bean fermentations, in particular with respect to the yeast microbiota (30, 49). In the present paper, a multiphasic approach was used for the first time to systematically study the biodiversity, population dynamics, and metabolomics of spontaneous cocoa bean heap fermentations in Ghana.

Population dynamics. The studied Ghanaian cocoa bean heap fermentations were characterized by a high initial level and rapid growth of both yeasts and LAB, a simultaneous but slower growth of AAB, and a lower maximum temperature compared with those of other cocoa bean fermentations reported in the literature. In other reports, counts are generally from an initial 3 to 7 and a maximum 7 to 9 to finally 2 to 3 log CFU g^{-1}, from 4 to 5 and 8 to 9 to 1.5 log CFU g^{-1}, and from 3 to 4 and 6 to 7 to 0 log CFU g^{-1} for yeast, LAB, and AAB, respectively, and the maximum fermentation temperature is often up to 50°C (3, 66). A clear three-phase fermentation process of a well-ordered succession of microbial groups and a timely production of metabolites was difficult to recognize. In general, the succession of microorganisms during cocoa bean fermentation actually reflects the environmental factors (temperature, pH, and oxygen tension) and the metabolism of substrates of the cocoa bean pulp or originating from the cocoa beans (depending on composition and harvest conditions), thus resulting in the production of significant amounts of ethanol, lactic acid, and acetic acid (3, 66, 67). It is likely to assume that pod ripeness and postharvest pod age (due to transportation and transient storage) and hence the pH of the cocoa bean pulp determine the initial amounts of yeasts and LAB. Moreover, yeast metabolism favors the growth of aciduric LAB (55). The speed of AAB growth probably determines the time course within which a maximum fermentation temperature is reached and hence of the whole fermentation, as the heat generated through acetic acid formation will be responsible for the death of almost all microorganisms involved in a desirable fermentation. This depends mainly on the size of the heap and the influence of turning, as both factors probably influence sweating and aeration of the heap and hence full growth of yeasts, LAB, and AAB (4, 28).

Biodiversity. Although the microbial ecology might be influenced by cocoa cultivar, pod age, fermentation method and site, and sample collection, the present study revealed that the biodiversity of both LAB and AAB in the fermentations analyzed was rather restricted, in contrast with a rich and varied yeast microbiota reported so far for (Ghanaian) cocoa bean fermentations (3, 30, 49, 63, 66). *Lactobacillus fermentum*, *L. plantarum*, and *A. pasteurianus* were found to be the dominant species. Although earlier studies report on a broad diversity of both LAB and AAB as well (8, 53–55, 72), recent reports mention the dominance of *L. fermentum*, *L. plantarum*, *L. mesenteroides*, *L. lactis*, *A. pasteurianus*, and *Acetobacter aceti* (3, 67). This limited number of taxa can be explained by an updated taxonomy of both LAB and AAB during the last years (18) as well as an adaptation of only a few dominant species to the cocoa bean fermentation conditions (cf. infra). For instance, *Lactobacillus cellobiosus* was considered an important microorganism in cocoa bean fermentation (3), but this species has been transferred to *L. fermentum* (14). Similarly, *Acetobacter rancens* has been included in the species *A. pasteurianus* (13). Interestingly, new taxa of both LAB (e.g., species of *Weissella*) and AAB (e.g., species of *Acetobacter*) were found in the present study.

Dominant strains and microbial successions. The present study showed that *L. plantarum* and *L. fermentum* were the most dominant LAB species in the Ghanaian cocoa bean heap fermentations performed. Moreover, *L. plantarum* decreased and *L. fermentum* increased upon fermentation time, the latter species often dominating the whole fermentation course. These data were supported by both PCR-DGGE and enumeration on MRS, M17, and KAA, the latter medium being selective for *L. plantarum* under the conditions applied. Both species have been found in cocoa bean fermentations before (8, 54, 55, 72). To our knowledge, this is the first paper to show their individual succession. Culture-dependent microbiological analysis of AAB species further indicated that *A. pasteurianus* fulfilled a key role in the Ghanaian cocoa bean heap fermentations performed, given its isolation and metabolic activity throughout fermentation. "*A. ghanaensis*" and "*A. senegalensis*" strains were not always isolated, and the latter strains disappeared faster than the former strains. Although AAB isolated from fermenting material are difficult to grow under laboratory conditions (3, 48), they possibly grew or remained viable under the anaerobic conditions of the heaps, as has been found in wine fermentations (23). DGGE analyses of AAB did not produce satisfactory results; again, *L. plantarum* and *L. fermentum* were detected with the WBAC primers used. These primers were actually developed to monitor both LAB and

AAB during wine fermentation (44). This underlines the importance of the detection limit to carry out PCR-DGGE, which is generally between 4 and 6 log CFU g^{-1} or higher, depending on the bacteria investigated, and hence the importance of detecting the >90% most numerous species of a community without discriminating living from dead cells or cells in a viable but not cultivable state (16, 19). The cultivable amount of the total AAB during the fermentations performed was never higher than 7 log CFU g^{-1}, and this occurred only temporarily in the middle of the fermentations. Also, it may be that the detection limit of AAB in a mixed ecosystem is higher and/or that differential or preferential PCR amplification of DNA of LAB instead of AAB occurred. A possible solution for this monitoring problem could be the use of different species-specific primers targeting other regions of the 16S rRNA gene or other genes (74) or increasing the intensity of the PCR amplicons produced from DNA by applying a nested PCR (23). Both the agar medium used for isolation and the high temperature of the heaps might be responsible for the selective isolation of *A. pasteurianus*, as this species may prefer calcium lactate and mannitol and is more heat resistant and ethanol tolerant (3, 8). It has been shown before that more-complex and more-differentiated media are necessary for the isolation of non-*Acetobacter* species (42). *A. pasteurianus* has been found during cocoa bean fermentation before (3, 53); *A. syzygii* (41) and *A. tropicalis* (40) have not. As mentioned before, the clusters corresponding with "*A. ghanaensis*" (cluster II) and "*A. senegalensis*" (a newly proposed heat-resistant AAB species isolated from mango fruit; clusters III and IV) (B. Ndoye et al., unpublished results) represent new species of *Acetobacter*.

In the beginning of the fermentations, *L. pseudomesenteroides* and *E. casseliflavus* were present, but they disappeared rather rapidly. These species are often associated with plant material (6, 20). As most species of *Weissella* and some other LAB were found only in one or a few samples in the beginning of the fermentations, they represent contaminants from the environment. They could be associated with pod surfaces, banana/plantain leaves, the materials used, and farmers' hands (51). However, they did not adapt to the matrix upon further fermentation (as revealed by their absence through isolation and analysis of PCR-DGGE profiles) and hence are not regarded as important for cocoa bean fermentation. In contrast, although "*W. ghanaensis*" could hardly be recovered by plating (in one sample only), it appeared throughout some of the fermentations, as revealed by PCR-DGGE. Therefore, it may be speculated that "*W. ghanaensis*" plays a role in the Ghanaian cocoa bean heap fermentation process.

All dominant microorganisms mentioned above come from the environment as well. *L. plantarum* and *L. fermentum* are indeed associated with plant material (50). *A. pasteurianus*, *A. syzygii*, and *A. tropicalis* are generally isolated from fermented foods, flowers and fruits, and fruits and fermented foods, respectively, almost always from tropical countries (40–42). Some of these heat-resistant strains are interesting for industrial fermentations at higher temperatures (48). Interestingly, only a few strains of the better-adapted populations of *L. plantarum*, *L. fermentum*, "*W. ghanaensis*," and *A. pasteurianus* outnumbered the rest of the microbiota and were responsible for spontaneous fermentation of the cocoa beans, as only a limited number of strains within a heap and among heaps were found. Indeed, isolates with highly similar or even identical $(GTG)_5$ PCR fingerprints were frequently found within the set of LAB and AAB isolates recovered from the same fermentation heap, whereas the samples were always taken at the same depth but in different points of the heap, indicating clones of the same strain and a possible microbial succession during fermentation at the strain level. Moreover, as no differences were observed between the season and only slight differences could be detected between the farms (as revealed by cluster analysis of the PCR-DGGE profiles), it can be anticipated that the heap fermentations performed during this study were dominated by certain strains and were hence very reproducible, which may support the general high quality standard of Ghanaian fermented cocoa beans. This is in accordance with the results of Nielsen et al. (49) on yeast diversity. Furthermore, they showed grouping of the fermentations with respect to fermentation method, site, time, and season. Microbial succession at both species and strain level within a fermentation course is often reported for spontaneous food fermentations (30).

Metabolomics. Metabolite target analysis during this study revealed that sugars, in particular sucrose, were utilized by the yeasts through invertase activity, being converted to ethanol and carbon dioxide, while glucose, fructose, and citrate were used by LAB to be converted into lactic acid, acetic acid, ethanol, and mannitol. During fermentation, sucrose inversion took place in the beans due to cotyledon invertase activity (26) and/or induced acid hydrolysis as a result of acetic acid penetration into the beans upon fermentation. Glucose was preferentially fermented above fructose following sucrose hydrolysis (60). Although citrate has been mentioned as an important carbon source for yeasts during cocoa bean fermentation (65), only a few yeasts (e.g., *Pichia fermentans*) can assimilate citrate and of the dominant species, such as *Candida krusei*, found in Ghanaian cocoa bean heap fermentations, only some isolates are able to assimilate citrate within a reasonable time (30). This indicates that citrate assimilation was due to LAB growth as hypothesized before (3, 73), probably being favored at low pH values (52). Hence, it may be an important selective parameter for LAB strains to be used in cocoa bean fermentation. *Leuconostoc* spp., *Enterococcus* spp., and *L. plantarum* metabolize citrate (52, 59, 70). This may explain their survival during the first part of the fermentation course. Although citrate consumption has been shown to enhance the growth of *Leuconostoc* spp. and not that of *L. plantarum* (52, 70), the latter species is more acid and ethanol tolerant than the former one (3, 45), possibly explaining its dominance against *L. pseudomesenteroides* during the first part of the fermentation course. However, citrate consumption caused the pH of the pulp to increase from pH 3.5 to about pH 4.3, which is slightly lower than reported elsewhere (3, 67) but important for the proteolysis stage of the fermentation (5). The production of succinic acid may be ascribed to citrate-fermenting LAB as well or to the conversion of fumaric acid and malic acid (36). The aciduric and ethanol-tolerant character of *L. fermentum* (3, 7, 68) may explain its survival and dominance of the whole cocoa bean fermentation process. In addition, strains of *L. plantarum* and *L. fermentum* are able to produce antimicrobial substances that play a role in bacterial ecology (17). The increased population of *L. fermentum* upon fermentation ex-

plains the simultaneous accumulation of mannitol, as *L. fermentum* can use fructose as an alternative electron acceptor, being completely converted to mannitol. The part of available fructose that was used as an alternative electron acceptor by *L. fermentum* to produce mannitol could hence not be converted to nonvolatile lactic acid (undesired for cocoa beans) through glycolysis by this or other LAB species. Reversely, the production of mannitol enabled the production of extra acetic acid and ATP, contributing to both volatile acidity (desired for cocoa beans) and enhanced growth competitiveness, respectively (80). Both physiological characteristics likely influence the quality of fermented cocoa beans (29, 34). Finally, AAB, in particular *A. pasteurianus*, grew on ethanol, mannitol, and lactate and converted ethanol into acetic acid. This oxidation process was responsible for the increase in temperature inside the heap (67), and hence, a maximum population of AAB corresponded with a maximum fermentation temperature. Part of the acetic acid volatilized and part penetrated into the cotyledons of the beans and was, together with part of the ethanol and the heat, responsible for the killing of the cocoa seed embryo and changes in the subcellular structure of the beans (10), being an important end point of fermentation.

Free amino acids increased upon fermentation time, although they were present in smaller amounts than reported elsewhere (27, 38, 62). However, the use of aqueous extracts in the present study reflects the bioavailability of nutrients for microbial fermentation of the pulp instead of their total extractable concentrations, which is important for further processing of the beans. Different patterns have frequently been observed for different amino acids (10, 27, 38). While the increase of hydrophobic amino acids and hydrophilic oligopeptides is due to cocoa bean proteolytic activity (5, 26, 77), it is well known that LAB and AAB use free (acid) amino acids as a carbon/nitrogen source and a nitrogen source, respectively (24, 35, 37, 64). In general, proteolysis depends primarily on the fermentation conditions, namely, the duration and intensity of acidification, temperature, and aeration (10, 38). Also, oxidation, condensation, and complexation (with polypeptides) of polyphenols occur (10, 31). Consequently, the fermentation conditions determine the amount of free amino acids, oligopeptides, reducing sugars, and polyphenols of fermented, dried cocoa beans, which all play an important role in aroma precursor formation that is further developed during cocoa processing (10, 73). The metabolite results presented in this paper in combination with literature data indicate that a successful fermentation process is reached after about 72 h of spontaneous fermentation.

Conclusions. The use of a multiphasic approach as applied during this study increases our understanding of spontaneous food fermentation processes. The combination of both microbiological analyses, encompassing culture-dependent and -independent methods, and metabolite analyses, encompassing pure culture and fermentation samples, allows us to profile population dynamics and fermentation courses. This approach permits the identification of specific populations and metabolites useful in improving fermentation (with respect to reproducible and standardized end products and fermentation time) and organoleptic (with respect to reproducible consistency, color, flavor, and taste) profiles. Although cocoa bean fermentation is a heterogeneous process per se, the organoleptic quality of Ghanaian fermented cocoa beans is frequently reported as excellent (12, 81). Yet, fermentation of cocoa beans depends on production methods, batch sizes, pod ripeness and storage, and fermentation conditions. As shown in the present paper, particular competitive strains of both LAB and AAB dominate the Ghanaian cocoa bean heap fermentation process. With the purpose of selecting starter cultures for controlled cocoa bean fermentations (65, 67), we indicate that these should be acid-tolerant, ethanol-tolerant, and citrate-utilizing strains in the case of LAB (preferably a combination of *L. plantarum* and *L. fermentum*) and acid-tolerant, ethanol-tolerant, and heat-resistant strains in the case of AAB (preferably *A. pasteurianus*). Nevertheless, further research is needed to determine whether these species are indeed essential or necessary to develop fermented, dried, and roasted cocoa beans for good chocolate quality.

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