Applied and Environmental Microbiology, Dec. 2010, p. 7708–7716 Vol. 76, No. 23
0099-2240/10/$12.00 doi:10.1128/AEM.01206-10

Kinetic Analysis of Strains of Lactic Acid Bacteria and Acetic Acid Bacteria in Cocoa Pulp Simulation Media toward Development of a Starter Culture for Cocoa Bean Fermentation[▽]

Timothy Lefeber, Maarten Janssens, Nicholas Camu, and Luc De Vuyst*

Research Group of Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bio-engineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

Received 20 May 2010/Accepted 26 September 2010

The composition of cocoa pulp simulation media (PSM) was optimized with species-specific strains of lactic acid bacteria (PSM-LAB) and acetic acid bacteria (PSM-AAB). Also, laboratory fermentations were carried out in PSM to investigate growth and metabolite production of strains of *Lactobacillus plantarum* and *Lactobacillus fermentum* and of *Acetobacter pasteurianus* isolated from Ghanaian cocoa bean heap fermentations, in view of the development of a defined starter culture. In a first step, a selection of strains was made out of a pool of strains of these LAB and AAB species, obtained from previous studies, based on their fermentation kinetics in PSM. Also, various concentrations of citric acid in the presence of glucose and/or fructose (PSM-LAB) and of lactic acid in the presence of ethanol (PSM-AAB) were tested. These data could explain the competitiveness of particular cocoa-specific strains, namely, *L. plantarum* 80 (homolactic and acid tolerant), *L. fermentum* 222 (heterolactic, citric acid fermenting, mannitol producing, and less acid tolerant), and *A. pasteurianus* 386B (ethanol and lactic acid oxidizing, acetic acid overoxidizing, acid tolerant, and moderately heat tolerant), during the natural cocoa bean fermentation process. For instance, it turned out that the capacity to use citric acid, which was exhibited by *L. fermentum* 222, is of the utmost importance. Also, the formation of mannitol was dependent not only on the LAB strain but also on environmental conditions. A mixture of *L. plantarum* 80, *L. fermentum* 222, and *A. pasteurianus* 386B can now be considered a mixed-strain starter culture for better controlled and more reliable cocoa bean fermentation processes.

Cocoa beans are the principal raw material for chocolate production (31, 36). Raw cocoa beans have an astringent, unpleasant taste and flavor and must be cured before they can be converted into good-tasting and full-flavored chocolate. Curing involves fermentation of the cocoa beans, followed by drying and roasting. Therefore, the process of fermentation is the foundation of the entire chocolate-making process and plays a significant role in determining flavor of chocolate and other cocoa-based products. Cocoa bean fermentation is a spontaneous fermentation process; it is carried out in heaps, boxes, baskets, or trays or on platforms and lasts about 3 to 6 days (2, 28, 31, 33, 36).

Cocoa pulp is a rich medium for microbial growth. It consists of 82 to 87% water, 10 to 15% mono- and disaccharides, 2 to 3% pentosans, 1 to 3% citric acid, and 1 to 1.5% pectin (20, 23). Proteins, free amino acids, vitamins, and minerals are present as well. The ratio of sucrose, glucose, and fructose present in the pulp varies with pod age. Pulp and beans within the ripe pods are microbiologically sterile. When these contents are removed from the opened pods, the pulp becomes accidentally contaminated with a variety of microorganisms from the surrounding environment, many of which contribute to the subsequent fermentation (4, 28, 30).

The heterogeneous natural cocoa bean fermentation process shows great variations in both microbial count and metabolite concentration, affecting the flavor and composition of cocoa and chocolate produced from fermented dry beans (1, 4–6, 28). Successful cocoa bean fermentation requires a succession of particular microbial activities. At the onset of cocoa bean fermentation, yeasts are the dominating microorganisms. They are responsible for liquefying the pulp through depectinization, which reduces pulp viscosity, and for fermentative production of ethanol from carbohydrates under anaerobic (due to tight packing of the beans), acidic (presence of citric acid in the pulp), and carbohydrate-rich (sucrose, glucose, and fructose in the pulp) conditions. Recent studies on Ghanaian cocoa bean heap fermentations show that *Hanseniaspora opuntiae* is the predominant yeast during the initial phase of fermentation (7, 10, 18, 19). Also, *Pichia kudriavzevii*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae* have been reported to play a role in the fermentation of cocoa beans (1, 7, 10, 27). As the pulp drains away, air penetrates into the fermenting mass, which creates ideal conditions for the growth of bacteria. Microaerophilic, acid-tolerant, and ethanol-tolerant lactic acid bacteria (LAB), in particular, *Lactobacillus plantarum* and *Lactobacillus fermentum*, first dominate the fermentation (1, 4, 11, 12, 19). Glucose, fructose, and citric acid are converted into acetic acid, lactic acid, and/or mannitol, resulting in a slight increase in the pH of the pulp (4). During the aerobic phase, created by enhanced pulp drainage and air ingress upon fermentation, acetic acid bacteria (AAB) dominate. *Acetobacter pasteurianus* is the most prevalent AAB species involved in spontaneous cocoa bean fermentation (4, 12, 19). AAB oxidize the ethanol produced by the yeasts into acetic acid.

* Corresponding author. Mailing address: Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussels, Belgium. Phone: 32 2 6293245. Fax: 32 2 6292720. E-mail: ldvuyst@vub.ac.be.

▽ Published ahead of print on 1 October 2010.

Information on the functioning of yeasts during cocoa bean fermentation is widely available (1, 7, 10, 19, 28). Fewer studies focus on the functioning of LAB and AAB in addition to species diversity and identification (1, 4, 5, 18, 28). To fully understand the functioning and role of LAB and AAB during cocoa bean fermentation, their kinetics of growth, substrate consumption, and metabolite production should be studied in more detail. As for other complex ecosystems, such as meat and cereal fermentations, this can be done by the use of simulation media during laboratory fermentations (14, 17). Pettipher was the first to formulate a cocoa pulp simulation medium (PSM) that could be used for microbiological and biochemical studies of laboratory cocoa bean fermentations in general (20).

The aim of the present study was to optimize the chemical composition of cocoa pulp simulation media to be as close as possible to the actual chemical composition due to the action of LAB and AAB strains during fermentation and to perform a kinetic analysis of particular strains of LAB and AAB species in the appropriate cocoa pulp simulation media to gain more insight into their dynamics of substrate degradation and metabolite production in view of the development of a bacterial starter culture for cocoa bean fermentation.

MATERIALS AND METHODS

Selection and maintenance of microorganisms. Strains *Lactobacillus plantarum* LMG 6907^T, *Lactobacillus fermentum* LMG 6902^T, and *Acetobacter pasteurianus* LMG 1262^T were purchased from the BCCM/LMG Bacteria Collection (Ghent, Belgium) and were used as well-characterized species-specific strains to initiate optimization of growth on cocoa pulp simulation media and kinetic analysis of strain growth and metabolite production behavior. Next, cocoa-specific LAB and AAB strains were used. Three strains of *L. plantarum* (80, 270, and 297), *L. fermentum* (12, 48, and 222), and *A. pasteurianus* (386B, 417B, and 442) were selected out of 12 natural isolates each from spontaneous Ghanaian cocoa bean heap fermentations (4). The 12 strains were representative of different subclusters within the $(GTG)_5$-PCR banding patterns of all isolates that were available (4). The following selection criteria were used to reduce the number of strains per species to three: fast growth, high cell density, fast acidification (carbohydrate fermentation in the case of LAB and ethanol oxidation in the case of AAB), production of equal amounts (on a mass basis) of lactic acid and acetic acid (based on glucose, fructose, and citric acid fermentation), and mannitol production out of fructose in the case of LAB and (over)oxidation of acetic acid and lactic acid in the case of AAB, as well as acid, ethanol, and heat tolerance in MRS medium and PSM-LAB (see below) for LAB and in MYP medium and PSM-AAB (see below) for AAB (unpublished results).

LAB and AAB strains were stored at −80°C in MRS medium (Oxoid Ltd., Basingstoke, United Kingdom) and mannitol-yeast extract-peptone (MYP) medium (2.5% D-mannitol, 0.5% yeast extract, and 0.3% bacteriological peptone [Oxoid], wt/vol), respectively. All media were supplemented with 25% (vol/vol) glycerol as a cryoprotectant.

Formulation and validation of cocoa pulp simulation media and fermentation conditions. The compositions of the cocoa pulp simulation media (PSM) supporting good growth of LAB (PSM-LAB), representative of the composition of cocoa pulp at the start of fermentation, and AAB (PSM-AAB), representative of the composition of cocoa pulp after 2 days of fermentation, developed during this study are shown in Table 1. Ten-liter fermentations (see below) with monocultures of *L. fermentum* LMG 6902^T (PSM-LAB), *L. plantarum* LMG 6907^T (PSM-LAB), and *A. pasteurianus* LMG 1262^T (PSM-AAB) were carried out to optimize and validate medium composition and fermentation conditions. Therefore, the amounts of carbohydrates (sucrose, glucose, and fructose) and the levels of aeration and agitation (in the case of AAB) of the media were varied (unpublished results). For instance, sucrose was omitted from the final medium for LAB, because it was barely metabolized, and the concentration of glucose and fructose was adjusted to 25 g liter^{-1} to enable full fermentation within 48 h (time span of LAB growth during spontaneous cocoa bean fermentation). Citric acid, which is present in cocoa pulp at the start of the fermentation, was added as a cosubstrate for LAB (PSM-LAB), while ethanol and calcium lactate were added as energy sources for AAB, reflecting the cocoa pulp composition after 2 days of fermentation (PSM-AAB) (4). Vegetal-origin, neutralized soya peptone and granulated yeast extract ensured the availability of nitrogen, trace elements, and growth factors. Moreover, the presence of yeast extract mimicked yeast cell lysis, releasing vitamins and other nutrients, upon cocoa bean fermentation (4). Magnesium and manganese were added to support good growth of lactobacilli (PSM-LAB). Tween 80 facilitated nutrient uptake by lactobacilli in PSM-LAB and served as an antifoam agent in PSM-AAB. Continuous aeration (see below) of PSM-AAB simulated infiltration of air and turning during a natural cocoa bean heap fermentation process. Finally, a medium validation based on a coculture fermentation of *L. plantarum* LMG 6907^T and *L. fermentum* LMG 6902^T was performed.

TABLE 1. Compositions of the cocoa pulp simulation media for LAB (PSM-LAB) and AAB (PSM-AAB)

Component[a]	Amt[b] (initial concn) in:	
	PSM-LAB	PSM-AAB
Fructose	25 (139 mM)	0
Glucose	25 (139 mM)	0
Calcium lactate-pentahydrate	0	10 (75 mM lactate)
Ethanol	0	10 (220 mM)
Citric acid	10 (52 mM)	0
Yeast extract	5	10
Soya peptone	5	5
Magnesium sulfate-heptahydrate	0.5	0
Manganese sulfate-monohydrate	0.2	0
Tween 80	1	1

[a] All chemicals were purchased from VWR International (Darmstadt, Germany), except neutralized soya peptone, which was purchased from Oxoid.
[b] All amounts are given in grams per liter, except that for Tween 80, which is given in milliliters per liter. The pH values were 3.5 to 4.0 over 48 h for PSM-LAB and 4.5 for PSM-AAB.

Fermentation experiments. Fermentations with the LAB and AAB strains, both type strains and selected strains (three cocoa-specific strains per species investigated), were carried out using computer-controlled, 15-liter laboratory fermentors (Biostat C; Sartorius AG/B. Braun Biotech International, Melsungen, Germany) containing 10 liters of PSM-LAB or 8 liters of PSM-AAB, respectively. The pH values of PSM-LAB and PSM-AAB were adjusted to pH 3.5 and 4.5, respectively, before sterilization. The vessels were sterilized *in situ* at 121°C and 110,000-Pa overpressure for 20 min. Glucose, fructose, and citric acid solutions were autoclaved (121°C, 210,000 Pa, 20 min) separately, and ethanol was filter sterilized (0.2-μm-pore-size filters, Minisart RC 4; Sartorius AG, Göttingen, Germany), followed by aseptic transfer to the media. For inoculum buildup, strains were initially transferred from −80°C to MRS (LAB) or MYP (AAB) medium and incubated at 37°C for 12 h or 30°C for 24 h, respectively. Afterwards, the strains were propagated twice in the appropriate PSM with the energy source of interest and finally inoculated into the fermentor. The transfer inoculum was always 1% (vol/vol), except for the final inoculum with PSM-AAB, which was 6% (vol/vol). During LAB fermentation, a linear pH profile was applied using 10 M NaOH or 1.5 M HCl, starting from pH 3.5 (pH of fresh cocoa pulp) and ending at pH 4.0 (pH of cocoa pulp after 48 h of fermentation) over 48 h (time span of most important microbial activities during spontaneous cocoa bean heap fermentation). A constant pH of 4.5 (pH after 2 days of fermentation) was used for the AAB fermentations. The AAB fermentations were carried out aerobically by continuous sparging of the medium with 15 liters min^{-1} sterile air for 48 h. Agitation (100 rpm for LAB to keep the fermentation medium homogeneous and 300 rpm for AAB to improve aeration), temperature (37°C for LAB and 30°C for AAB), and pH (see above) were controlled online (MFCS/win 2.1 software; Sartorius AG).

For detailed kinetic analysis of the *L. plantarum* 80 and *L. fermentum* 222 fermentations, the initial concentrations of glucose (139 mM) and fructose (139 mM) of PSM-LAB were kept constant, while the initial concentration of citric acid (26, 39, 52, or 104 mM) was changed to investigate the effect of citric acid on the fermentation kinetics of the cocoa-specific LAB strains studied. Also, fermentations in modified PSM-LAB were performed with glucose (139 mM), fructose (139 mM), glucose (139 mM) plus fructose (139 mM), or citric acid (52 mM) as the sole energy source. An extra fermentation in PSM-LAB was performed with glucose (139 mM), fructose (139 mM), and citric acid (26 mM) at a

constant pH of 5.5. Also, a coculture fermentation of *L. plantarum* 80 and *L. fermentum* 222 in PSM-LAB was performed.

Similarly, for detailed kinetic analysis of *A. pasteurianus* 386B fermentations, the influence of differing initial concentrations of lactic acid (38, 75, or 150 mM) of PSM-AAB was examined, while the initial concentration of ethanol was kept constant (220 mM). Two additional fermentations in modified PSM-AAB were performed with ethanol (220 mM) or lactic acid (75 mM) as the sole energy source.

During all fermentations, samples were withdrawn aseptically from the fermentor at regular time intervals for further analysis. All fermentations were performed in triplicate, and the graphs in the figures display representative results.

Analysis of growth. Growth was measured as optical density at 600 nm (OD_{600}) (Uvikon 923; Kontron Instruments, Milan, Italy). CFU of LAB and AAB were determined through plating of serial 10-fold dilutions of samples in saline (0.85%, wt/vol, NaCl) on MRS and MYP agar, respectively.

Analysis of metabolites. Concentrations of lactic acid, acetic acid, and ethanol were determined by high-performance liquid chromatography (HPLC) analysis with a Waters chromatograph (Waters Corp., Milford, MA) equipped with a 2414 differential refractometer, a 600S controller, a column oven, and a 717plus autosampler. An ICSep ICE ORH-801 column (Interchim, Montluçon, France) was used with 10 mN H_2SO_4 as the mobile phase at a flow rate of 0.4 ml min^{-1}. The column temperature was kept at 35°C. Samples were centrifuged (16,060 × *g* for 15 min), and equal volumes of 20% (vol/vol) trichloroacetic acid were added to the cell-free culture supernatants to remove proteins. After centrifugation (16,060 × *g* for 5 min), the supernatant was filtered (0.2-μm-pore-size filters; Minisart RC4) before injection. External standards were used for quantification.

Concentrations of glucose, fructose, and mannitol were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA) with a DX500 chromatograph using a CarboPac PA10 column. The mobile phase, at a flow rate of 1.0 ml min^{-1}, consisted of ultrapure water (0.015 μS cm^{-1}) (eluent A), 167 mM NaOH (eluent B), and 500 mM NaOH (eluent C). The following gradient was applied: 0 min, 87% eluent A and 13% eluent B; 20 min, 87% eluent A and 13% eluent B; 30 min, 100% eluent C; 32 min, 100% eluent C; and 35 min, 87% eluent A and 13% eluent B. To remove proteins, fermentation samples (700 μl) were treated with 700 μl of acetonitrile. After centrifugation (16,060 × *g* for 15 min), the supernatant was filtered (0.2 μm; Minisart RC 4) prior to injection. External standards were run together with the samples for quantification.

The concentration of citric acid was determined by HPAEC with conductivity monitoring under ion suppression (CIS) on an ICS3000 chromatograph, using an AS-19 column (Dionex). 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 samples were treated with acetonitrile for protein removal as described above, appropriately diluted, filtered (0.2 μm; Minisart RC 4) prior to injection, and run together with the appropriate external standards.

All samples were analyzed in triplicate, and the average values and standard deviations are presented.

The percent carbon recovery of the carbohydrate and citric acid conversions during the fermentations was calculated by dividing the total amount of carbon recovered in the sugar metabolites by the total amount of carbon present in the added energy sources.

RESULTS

Formulation and validation of cocoa pulp simulation media and fermentation conditions with the type strains of *L. plantarum*, *L. fermentum*, and *A. pasteurianus*. The compositions of optimal PSM-LAB and PSM-AAB (Table 1) supported good growth of the investigated type strains of LAB and AAB, respectively. In PSM-LAB, *L. plantarum* LMG 6907^T (homofermentative) utilized both glucose and fructose simultaneously (not completely consumed after 48 h of fermentation), slightly converted citric acid near the end of the fermentation, and produced primarily lactic acid (and very small amounts of acetic acid out of citric acid) (Fig. 1A). *Lactobacillus fermentum* LMG 6902^T (strictly heterofermentative) did not convert citric acid and utilized both glucose (almost exclusively during the exponential growth phase) and fructose of PSM-LAB (Fig. 1B). Glucose and fructose were fermented into lactic acid, acetic acid, and ethanol, whereas some of the fructose was reduced to mannitol (fructose was depleted after 24 h of fermentation). In the coculture fermentation of *L. plantarum* LMG 6907^T and *L. fermentum* LMG 6902^T in PSM-LAB, glucose and fructose (partially converted into mannitol) were both utilized incompletely, citric acid was slightly converted, and

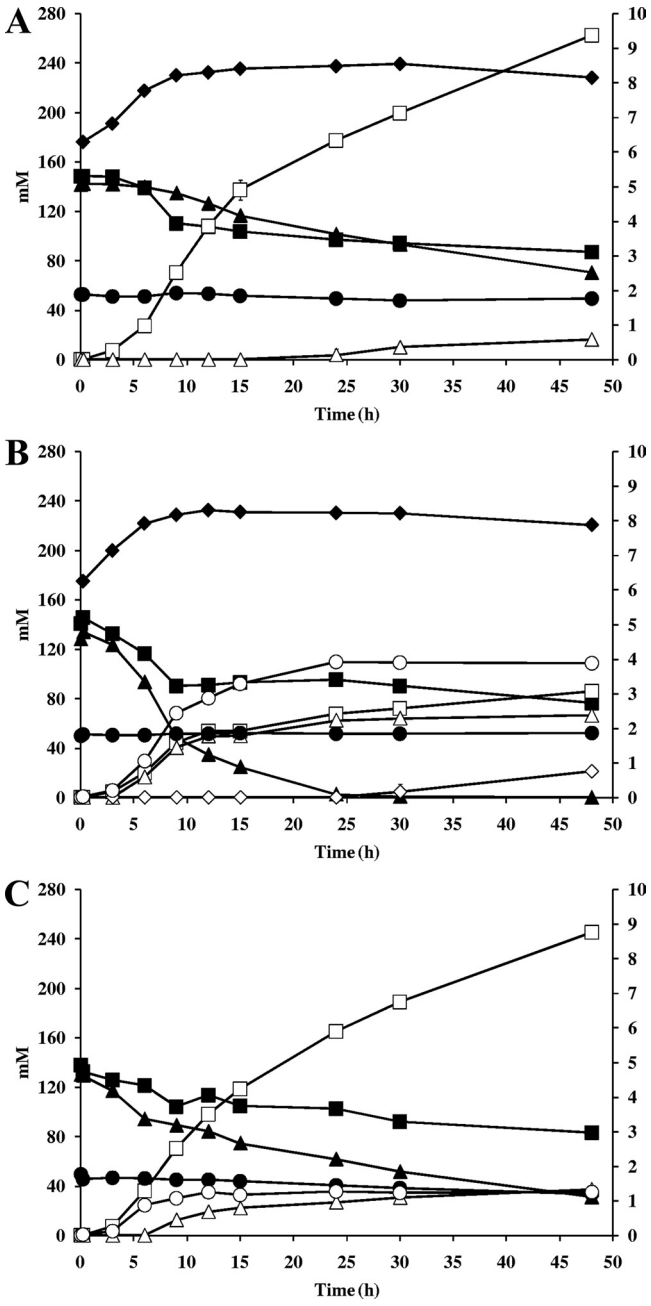

FIG. 1. Monoculture fermentations of *L. plantarum* LMG 6907^T (A) and *L. fermentum* LMG 6902^T (B) and coculture fermentation of these two strains (C) in cocoa pulp simulation medium for lactic acid bacteria (PSM-LAB). Right axis: ◆, log CFU ml^{-1}. Left axis (concentrations in mM): ■, glucose; ▲, fructose; ●, citric acid; □, lactic acid; △, acetic acid; ○, mannitol; ◇, ethanol.

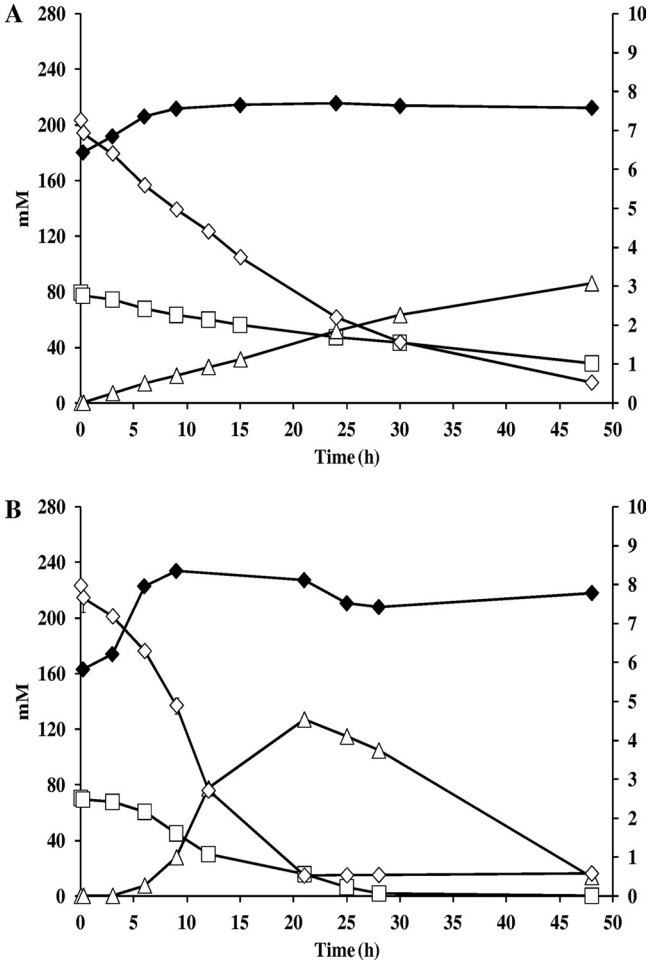

FIG. 2. Monoculture fermentations of *A. pasteurianus* LMG 1262^T (A) and the cocoa-specific strain *A. pasteurianus* 386B (B) in cocoa pulp simulation medium for acetic acid bacteria (PSM-AAB). Right axis: ◆, log CFU ml^{-1}. Left axis (concentrations in mM): □, lactic acid; △, acetic acid; ◇, ethanol.

lactic acid (mainly) and acetic acid (small amount) were produced (Fig. 1C). In PSM-AAB, *A. pasteurianus* LMG 1262^T oxidized ethanol to acetic acid over a time span of 48 h and simultaneously oxidized about 60% of the initial lactic acid to carbon dioxide and water (Fig. 2A).

Comparison of selected cocoa-specific LAB and AAB strains on fermentor scale regarding kinetics of growth, substrate consumption, and metabolite production. The selected cocoa-specific *L. plantarum* strains reached maximum cell counts after 9 or 12 h of fermentation in PSM-LAB for strain 80 (Fig. 3A) or strains 270 and 297 (data not shown), respectively. In the case of strains 80 (Fig. 3A) and 270 (data not shown), the cell counts remained stable upon further fermentation, whereas a decrease was seen for strain 297 (data not shown). All three strains metabolized glucose and fructose at the same time; in the case of strains 270 and 297, this occurred after a lag phase of 6 h (Fig. 3A and data not shown). In no case were glucose and fructose depleted after 48 h of fermentation (Fig. 3A and data not shown). Lactic acid was the major metabolite produced during growth on glucose and fructose, while citric

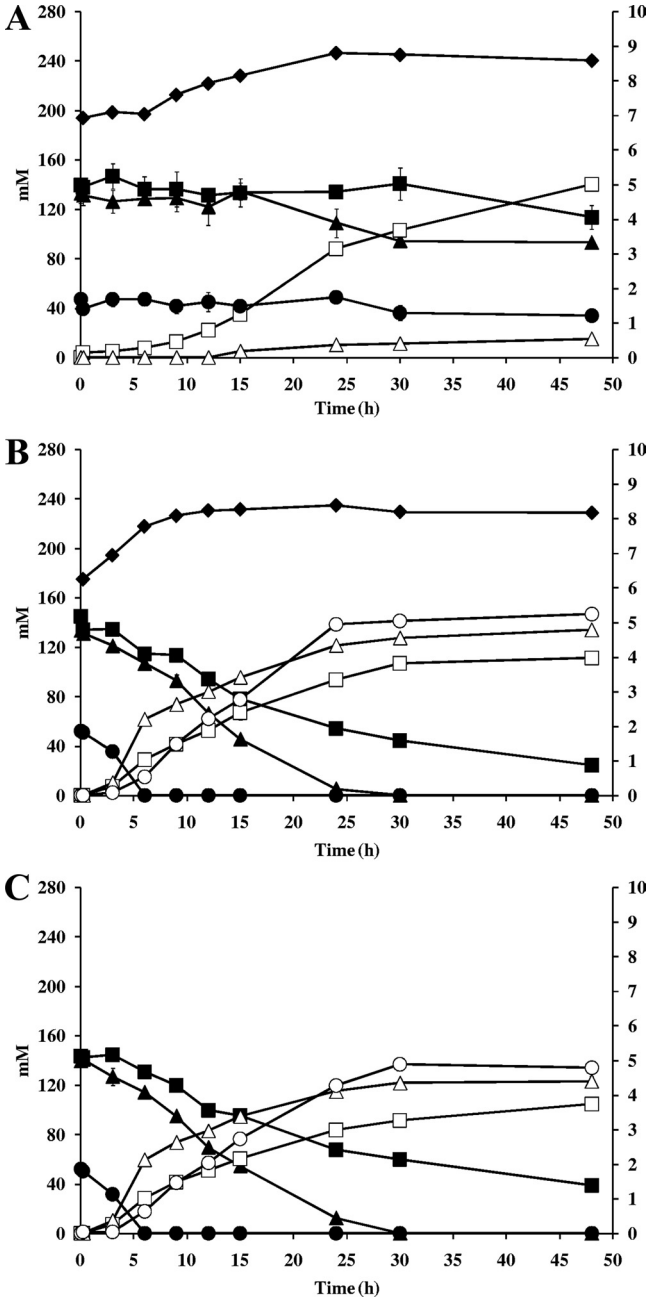

FIG. 3. Monoculture fermentations of the cocoa-specific strains *L. plantarum* 80 (A) and *L. fermentum* 222 (B) and coculture fermentation of these two strains (C) in cocoa pulp simulation medium for lactic acid bacteria (PSM-LAB). Right axis: ◆, log CFU ml^{-1}. Left axis (concentrations in mM): ■, glucose; ▲, fructose; ●, citric acid; □, lactic acid; △, acetic acid; ○, mannitol.

acid was fermented only slightly at the end of the fermentation (Fig. 3A; Table 2).

The selected cocoa-specific *L. fermentum* strains reached maximum cell counts after 9 to 12 h of fermentation in PSM-LAB (Fig. 3B and data not shown). In the case of strain 222, the cell counts plateaued after 12 h of fermentation (Fig. 3B), while growth of *L. fermentum* 12 declined (data not shown). All three strains consumed glucose and fructose simultaneously;

TABLE 2. Bacterial growth, substrate consumption, and metabolite production, as well as carbon recovery, for fermentations performed with cocoa-specific strains of *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus*, carried out under different conditions of substrate source availability

Strain and fermentation conditions[a]	Maximum cell population (log CFU ml^{-1})	Consumption (mM)[b]			Production (mM)[b]				Carbon recovery (%)[c]
		Glucose	Fructose	Citric acid	Lactic acid	Acetic acid	Ethanol	Mannitol	
L. plantarum 80									
139 G, 139 F, 26 C	8.03	36.0 ± 0.7	77.7 ± 0.6	5.50 ± 0.61	187 ± 1	5.50 ± 0.3	0.90 ± 0.16	0.0	80
139 G, 139 F, 26 C (pH 5.5)	9.88	122 ± 1	125 ± 1	26.0 ± 1.9	522 ± 13	31 ± 0.5	6.89 ± 0.26	0.0	99
139 G, 139 F, 39 C	8.66	32.3 ± 7.8	75.2 ± 7.7	14.2 ± 6.6	170 ± 8	5.91 ± 0.55	1.37 ± 0.32	0.0	72
139 G, 139 F, 52 C	8.90	25.8 ± 1.2	39.9 ± 1.0	12.9 ± 2.9	140 ± 0	15.2 ± 0.5	0.0	0.0	96
139 G, 139 F, 104 C	7.86	30.0 ± 0.7	63.6 ± 1.0	5.30 ± 2.34	177 ± 0	7.13 ± 0.63	0.0	0.0	92
52 C	7.78			52.0 ± 0.7	14.4 ± 1.2	66.6 ± 0.5	0.0	0.0	56
139 G	8.27	94.1 ± 2.9			173 ± 3	2.73 ± 0.13	1.48 ± 0.44	0.0	93
139 F	7.90		86.0 ± 3.6		158 ± 4	2.96 ± 0.20	0.81 ± 1.02	0.0	93
139 G, 139 F	8.44	18.0 ± 2.8	39.0 ± 1.4		106 ± 6	0.0	0.0	0.0	107
L. fermentum 222									
139 G, 139 F, 26 C	8.39	24.3 ± 9.1	40.2 ± 5.5	26.0 ± 0.7	45.7 ± 2.5	60.7 ± 1.8	0.67 ± 1.14	49.1 ± 1.2	102
139 G, 139 F, 26 C (pH 5.5)	9.95	129 ± 1	124 ± 1	25.0 ± 3.4	156 ± 7	105 ± 3	99.7 ± 5.6	115 ± 2	91
139 G, 139 F, 39 C	8.67	26.8 ± 4.1	29.5 ± 7.1	42.5 ± 5.5	29.8 ± 1.1	55.8 ± 1.2	0.99 ± 0.8	21.7 ± 0.6	56
139 G, 139 F, 52 C	8.41	114 ± 2	139 ± 3	52.0 ± 1.0	111 ± 3	135 ± 4	0.96 ± 1.05	153 ± 3	85
139 G, 139 F, 104 C	8.51	80.1 ± 0.5	110 ± 2	104 ± 1	108 ± 2	179 ± 2	1.67 ± 0.84	90.7 ± 0.7	70
52 C	8.49			52.0 ± 0.1	12.5 ± 0.3	56.8 ± 1.6	0.0	0.0	50
139 G	8.46	122 ± 2			140 ± 1	4.96 ± 0.10	129 ± 6	0.0	94
139 F	8.37		136 ± 5		54.1 ± 3.6	50.1 ± 3.1	2.77 ± 3.00	81.9 ± 1.6	93
139 G, 139 F	8.42	126 ± 8	124 ± 3		118 ± 3	80.3 ± 1.9	38.5 ± 1.2	91.6 ± 17.1	76
A. pasteurianus 386B									
38 L, 220 E	7.81				35.0 ± 2.5	87.3 ± 0.2	220 ± 2		
75 L, 220 E	7.63				73.6 ± 0.4	96.1 ± 6	220 ± 1		
150 L, 220 E	7.70				148 ± 1	96.0 ± 0.8	220 ± 1		
75 L	8.12				75.7 ± 0.7	7.31 ± 0.13			
220 E	7.52					101 ± 1	220 ± 3		

[a] Fermentation conditions are given as the concentration (in mM) followed by the abbreviated component: G, glucose; F, fructose; C, citric acid; L, lactic acid; or E, ethanol.
[b] The amount of substrate consumed or metabolite produced is the difference in concentrations of the samples taken at 0 and 48 h of fermentation. In the case of PSM-AAB fermentations, lactic acid and ethanol were consumed; the acetic acid concentration was the maximum concentration produced during fermentation.
[c] In both types of fermentations, PSM-LAB and PSM-AAB, carbondioxide was not taken into account.

glucose was not depleted after 48 h of fermentation, whereas fructose was exhausted within 30, 48, and 48 h of fermentation for strains 12 (data not shown), 48 (data not shown), and 222 (Fig. 3B), respectively. Glucose was fermented into lactic acid, acetic acid, and carbon dioxide (Fig. 3B; Table 2); in the case of strain 48, a small amount of ethanol was also formed (data not shown). Fructose was mainly reduced to mannitol, but a small portion was fermented into lactic acid, acetic acid, and carbon dioxide (Fig. 3B; Table 2). Citric acid was completely converted to mainly acetic acid and carbon dioxide within 6 h (strains 48 and 222 [data not shown and Fig. 3B, respectively]) to 9 h (strain 12 [data not shown]) of fermentation.

The selected cocoa-specific *A. pasteurianus* strains grew to maximum cell densities in the order 386B > 417B > 442 (data not shown). Strains 386B and 417B oxidized all ethanol within 21 h of fermentation, with an accompanying accumulation of acetic acid (up to 21 h) that was then further oxidized to carbon dioxide and water, to be completely depleted after 48 h of fermentation (Fig. 2B and data not shown). Lactic acid was oxidized simultaneously into carbon dioxide and water and was depleted after 28 h of fermentation (Fig. 2B and data not shown). Strain 442 oxidized almost all ethanol within 48 h of fermentation, while the concentration of lactic acid remained constant during the first 22 h of fermentation, followed by a decrease upon further fermentation (data not shown); after 22 h of fermentation, production of acetic acid was found (data not shown).

All of these fermentation data resulted in the choice of three strains for further kinetic analysis, mainly based on the cell densities obtained. For LAB, strain *L. fermentum* 222 was chosen because of its ability to achieve a high cell density within a reasonable fermentation time, its rapid conversion of citric acid, its production of almost equal amounts (on a mass basis) of lactic acid and acetic acid, and its production of mannitol. Strain *L. plantarum* 80 was chosen for its ability to achieve a high cell density within a reasonable fermentation time but also, in contrast to *L. fermentum* 222, for its production of mainly lactic acid. For AAB, strain *A. pasteurianus* 386B was chosen because this strain achieved a high cell density within a reasonable fermentation time and consumed lactic acid at the same time that ethanol was oxidized into acetic acid.

Influence of citric acid on the kinetics of *L. plantarum* 80 and *L. fermentum* 222 fermentations. With glucose (139 mM), fructose (139 mM), or citric acid (52 mM) as the sole energy source during fermentations with *L. plantarum* 80, glucose or fructose was not depleted within 48 h of fermentation, while citric acid was (Table 2). Both glucose and fructose were fermented mainly into lactic acid (Table 2). More fructose was fermented

than glucose (data not shown). In the presence of glucose, fructose, and citric acid, low (26 and 39 mM) and 52 mM or higher (104 mM) initial concentrations of citric acid were hardly consumed, except with fermentation at a constant pH of 5.5 in the presence of 26 mM citric acid, while cofermentation of glucose and fructose resulted in the production of solely lactic acid (Table 2). In all cases, no mannitol was formed; fructose was always fermented (Fig. 3A; Table 2).

With glucose (139 mM), fructose (139 mM), or citric acid (52 mM) as the sole energy source during fermentations with *L. fermentum* 222, glucose or fructose was not completely consumed within 48 h of fermentation, while citric acid was depleted within 9 h (Table 2); a lag phase of 24 h was observed in the case of glucose and fructose, which could be ascribed to cell death until a pH of about 4.0 (after 15 h of fermentation) was reached (data not shown). Glucose and fructose were fermented to mainly lactic acid/ethanol/carbon dioxide and lactic acid/acetic acid/carbon dioxide, respectively, within 48 h of fermentation; fructose was partially converted into mannitol (Table 2). Citric acid conversions resulted in the production of more acetic acid/carbon dioxide than lactic acid (Table 2). In the presence of both glucose and fructose, growth of *L. fermentum* 222 was not delayed (data not shown) and glucose and fructose were both fermented to lactic acid, acetic acid, and carbon dioxide (Table 2). In addition, part of the fructose was reduced to mannitol (Table 2), and it was depleted within 24 h of fermentation, after which ethanol production started (data not shown and Table 2). In the presence of glucose, fructose, and citric acid (26, 39, 52, and 104 mM), growth was not delayed and citric acid was converted first, whereas glucose and fructose consumption started after 3 h of fermentation (Fig. 3B and data not shown). When citric acid was exhausted, carbohydrate fermentation was delayed for 22 h with an initial concentration of 26 mM citric acid compared to fermentation with higher initial citric acid concentrations (data not shown). Whereas mainly lactic acid, acetic acid, and carbon dioxide were formed, mannitol was produced in all cases, in particular in the presence of an initial citric acid concentration of 52 mM (Fig. 3B; Table 2) and at a constant pH of 5.5 with 26 mM citric acid (Table 2). The production of different mannitol concentrations in the presence of 26 mM and 39 mM citric acid was due to differences in fructose consumption (Table 2). In all cases, fructose was both fermented and converted into mannitol (Fig. 3B; Table 2).

A coculture fermentation of *L. plantarum* 80 and *L. fermentum* 222 resulted in the production of almost equal amounts (on a mass basis) of lactic acid/acetic acid (no ethanol) and mannitol, due to fermentation of glucose (not to depletion) and citric acid (converted within 6 h) and depletion of fructose within 30 h of fermentation, respectively (Fig. 3C).

Influence of lactic acid on the kinetics of *A. pasteurianus* 386B fermentations. During fermentations with *A. pasteurianus* 386B in the presence of ethanol as the sole energy source, ethanol was oxidized to acetic acid, which reached a maximum concentration after 24 h of fermentation, followed by complete oxidation to carbon dioxide and water within 24 h (Table 2 and data not shown). With lactic acid as the sole energy source, growth of *A. pasteurianus* 386B was favored and lactic acid was oxidized to carbon dioxide and water, while a small amount of acetic acid accumulated after 6 h of fermentation and again decreased thereafter by oxidation to carbon dioxide and water (Table 2 and data not shown). During the fermentation with *A. pasteurianus* 386B in the presence of both ethanol (220 mM) and lactic acid (38, 75, and 150 mM), almost all ethanol was oxidized to acetic acid after 24 h (in the case of 38 mM and 75 mM initial lactic acid, which was oxidized to carbon dioxide and water within 15 and 30 h of fermentation, respectively) or 30 h (in the case of 150 mM initial lactic acid, which was oxidized to carbon dioxide and water within 48 h) of fermentation. After this, acetic acid was further oxidized to carbon dioxide and water, indicating a preference for ethanol as the oxidation substrate (Fig. 2B; Table 2).

DISCUSSION

Based on the data from the community dynamics and metabolite target analyses of natural cocoa bean heap fermentation processes (4–6) as well as the optimization of the chemical composition of cocoa pulp simulation media and the kinetics of growth, substrate consumption, and metabolite production of selected, possibly dominant LAB and AAB strains during laboratory fermentations in cocoa pulp simulation media (this study), a potential starter culture formulation could be developed. This starter culture formulation is composed of functional strains of *L. plantarum*, *L. fermentum*, and *A. pasteurianus*. Starter cultures have long been used in the dairy, meat, and wine industries; the industrial applicability of functional starter cultures with spontaneously fermented foods is currently under investigation (13). Whereas fermentative and pectinolytic yeast starter cultures have been tried before to speed up cocoa bean fermentation with respect to pulp volume and air ingress, the use of a mixed starter culture of LAB and AAB strains for this fermentation is promising as well (3, 8, 24, 26, 29).

During the present study, after validation of the potential of the cocoa pulp simulation media with species-specific strains of *L. plantarum*, *L. fermentum*, and *A. pasteurianus*, initial fermentation experiments were performed to compare several cocoa-specific strains of LAB and AAB isolated from natural cocoa bean fermentations. Yeasts were not taken into account, as sampling results from natural cocoa bean heap fermentations indicate that their initial number is quite high, concomitant with the absence of residual sucrose at the start of fermentation and hence reflecting the maturation stage of the harvested pods (4). This first screening was necessary to define a correct starter culture mixture, as it has been shown before that the starter strains most competitive to perform in a particular fermented food ecosystem are derived from their natural environment (13, 15). Therefore, appropriate growth-supporting pulp simulation media for species-specific LAB (PSM-LAB) and AAB (PSM-AAB) were formulated during the present study based on (i) the chemical composition of natural cocoa pulp as a function of fermentation time, omitting compounds that influence the transparency of the medium due to low solubility (e.g., polysaccharides, such as pectin) and hence interfere with growth measurements, and (ii) the desirable energy sources (glucose/fructose and ethanol, respectively) and appropriate nutrients allowing good bacterial growth of species-specific strains (4, 20, 23, 27). The omission of pectin from the cocoa pulp simulation media was accept-

able, because pectinolytic activity during cocoa bean fermentation is due to yeasts (27). Citric acid was included in PSM-LAB, as it has been shown that citric acid conversion occurs at the beginning of the cocoa bean fermentation and determines, together with acid and ethanol tolerance, the dominance of certain LAB species during spontaneous Ghanaian cocoa bean heap fermentations (4, 5). Lactic acid was included in PSM-AAB, as it has been shown that dominance of *A. pasteurianus* throughout Ghanaian cocoa bean heap fermentation is due to growth on ethanol and lactic acid, combined with tolerance to acid, ethanol, and heat (4, 5). The concentrations of all medium components used did not precisely reflect a certain time point of the composition of cocoa pulp, because cocoa bean fermentation is a dynamic process, but they were chosen to result in good growth of LAB and AAB dominating cocoa bean fermentation under appropriate physical circumstances to understand the functional role of these bacterial species (1, 4, 5, 28).

Interestingly, all cocoa-specific *L. fermentum* strains tested converted citric acid, in contrast to the type strain of *L. fermentum*. This indicates strain-dependent differences within this species and reflects an adaptation of indigenous strains to their ecosystem, as cocoa pulp is rich in citric acid (1 to 3%) and possesses a low pH (3.0 to 3.5) (20, 23, 27). The strain-dependent metabolism of citric acid by *L. fermentum* has been noticed before (21). As consumption of citric acid results in the production of organic acids with a higher pK_a value, it hence increases the pH of the environment, which allows better bacterial growth and microbiological control of the environment (4, 28).

This study proposes a starter culture, composed of *L. plantarum* 80 (originating from heap 3 [4]), *L. fermentum* 222 (heap 6 [4]), and *A. pasteurianus* 386B (heap 5 [4]), for heap fermentation of Ghanaian cocoa beans, based on predictable good performance during cocoa bean fermentation, as validated through the laboratory fermentations in the appropriate cocoa pulp simulation media mentioned above. In all cases investigated in the present study, the acid-tolerant strains *L. plantarum* 80 (not citric acid consuming) and *L. fermentum* 222 (citric acid consuming) performed homolactic and heterolactic fermentation, respectively, in the presence of glucose or fructose as the sole energy source. The higher acid sensitivity of *L. fermentum* 222 (as revealed by death of the cells at the onset of the fermentations, when the pH was 3.5, with glucose and fructose as the sole energy source) is in agreement with the natural succession of LAB species during Ghanaian cocoa bean heap fermentations. Indeed, during a spontaneous Ghanaian cocoa bean heap fermentation process, *L. plantarum* occurs mainly in the beginning of the fermentation (the initial pH of the cocoa pulp is 3.5 due to the presence of citric acid), and *L. fermentum* survives and dominates throughout the fermentation process (with a concomitant increase of the pH of cocoa pulp up to 4.0) (4).

L. plantarum and *L. fermentum* are generally associated with vegetable-based and cereal fermentations (9). With both glucose and fructose as the energy source, as is the case during spontaneous cocoa bean fermentation, both components were fermented simultaneously by these LAB species, fructose being completely consumed before glucose, which might be due to its simultaneous use as an energy source and an external electron acceptor, in particular in the case of *L. fermentum*. Indeed, in the case of heterofermentative LAB, fructose can serve as an alternative external electron acceptor, being reduced to mannitol by a mannitol dehydrogenase (35). In the presence of fructose as the sole energy source, a maximal conversion to mannitol of 66.7% (60% in the case of *L. fermentum* 222) can be expected, as in general 1 mol of fructose is fermented while 2 mol of fructose are reduced to mannitol. In the presence of both fructose and other carbohydrates, higher fructose-into-mannitol conversions can be expected (82% in the case of *L. fermentum* 222 with both glucose and fructose). Alternatively, fructose-loving bacteria, such as *Fructobacillus pseudoficulneus*, participate in the initial LAB growth phase of cocoa bean fermentation processes as well (18; unpublished results). The formation of equal amounts (on a mass basis) of lactic acid and acetic acid—besides mannitol out of fructose—within a certain time span, as was the case for the chosen *L. fermentum* strain, is of importance for controlled cocoa bean fermentation processes, as less lactic acid (nonvolatile) will positively affect the acidity of fermented dry cocoa beans and hence the taste of chocolate made thereof (6). Also, more acetic acid (instead of ethanol) is produced by the reduction of fructose to mannitol and the conversion of citric acid into pyruvate, from a physiological point of view to regenerate the NAD^+ cofactor and to produce extra ATP, explaining good growth of the strains at low pH. Fast citric acid conversion at the onset of fermentation confirms the role of acid-tolerant LAB during a natural cocoa bean fermentation process, namely, (i) initial growth on citric acid, avoiding competition with depectinizing (citrate-negative) yeasts that degrade carbohydrates to ethanol anaerobically; (ii) pH regulation of cocoa pulp upon fermentation (replacing citric acid with acetic acid and lactic acid), ensuring better growth and microbiological stability of the ecosystem; (iii) fermentative production of flavor precursor molecules (acetic acid, pyruvate catabolites, and amino acid conversion products); and (iv) appropriate conditions for proteolysis, flavor precursor formation, and color development within the beans (4, 5, 7).

The fermentation behavior of *L. fermentum* 222 in the presence of different initial concentrations of citric acid can be explained by a preference for citric acid as a cosubstrate and a possible link between citric acid fermentation and mannitol production, most likely due to the NADH requirement of mannitol dehydrogenase, which is in turn dependent on central carbohydrate metabolism. Accordingly, citric acid consumption stimulates amino acid conversions in LAB (30, 34), indicating that metabolic connections may exist between citric acid conversion and processes such as amino acid interconversion (34) and mannitol production (this study). Similarly, *in vitro* experiments have shown that lactate dehydrogenase activity catalyzes the reduction of phenylpyruvic acid into phenyllactic acid (16). However, in all cases in the present study, citric acid fermentation was optimal in the presence of an initial concentration of 52 mM citric acid, at least at the pH of cocoa bean pulp. The corresponding high mannitol production levels, compared with those seen with lower (26 and 39 mM) or higher (104 mM) initial concentrations of citric acid, were probably the result of lack of stimulation of mannitol production or inhibition of the metabolic flux toward mannitol formation, respectively. It is likely that when carbohydrates and citric acid

are consumed simultaneously, NAD^+ regeneration is performed by the reduction of pyruvate, coming from citric acid, to lactic acid. However, a shift in the conversion of pyruvate leads to acetic acid (from acetyl-phosphate) instead of ethanol, as was the case for *L. fermentum* 222, which enables extra ATP generation. Moreover, once citric acid fermentation was finished, a metabolic shift to lactic acid/acetic acid production occurred.

The acid- and ethanol-tolerant *A. pasteurianus* 386B strain oxidized lactic acid and ethanol simultaneously, as well as acetic acid intermediately formed out of lactic acid and ethanol, to carbon dioxide and water. This means that during a natural cocoa bean heap fermentation process, lactic acid produced by LAB will favor growth of *A. pasteurianus*, enabling generation of reducing equivalents that are needed in many synthetic pathways and redox reactions and of ATP production by means of its oxidation together with oxidation of ethanol to acetic acid. The former takes place through lactate dehydrogenase, pyruvate decarboxylase, and acetaldehyde dehydrogenase activities (22), while the latter depends on the sequential action of pyrroquinoline-quinone-dependent alcohol dehydrogenase and acetaldehyde dehydrogenase activities (32). However, so-called overoxidation of acetic acid to carbon dioxide and water in both cases decreases the amount of volatile acetic acid and increases the amount of heat produced upon oxidation. Heat and acetic acid production cause the end of a natural cocoa bean fermentation process, and penetration into the seeds kills the embryo (28). In addition, acetic acid acts as a flavor precursor molecule and initiates biochemical processes inside the beans that, for instance, produce additional flavor precursor molecules. Whereas overoxidation of acetic acid may be disadvantageous, the amount of acetic acid formed by both LAB and AAB activities is enough to produce well-fermented cocoa beans and acceptable chocolate made thereof, provided that the right strains are in place (natural fermentation process) or added (starter culture formulation). Indeed, the use of *Gluconacetobacter xylinus* subsp. *xylinus* as the starter strain for the fermentation of cocoa beans, without an intermediate LAB fermentation phase, resulted in fermented cocoa beans with lower pH and higher levels of acetic acid but less cocoa flavor than naturally fermented cocoa beans (25).

To conclude, the present study contributes to the explanation of the competitiveness of particular strains of *L. plantarum* (homolactic and acid tolerant), *L. fermentum* (heterolactic, citric acid fermenting, mannitol producing, and less acid tolerant), and *A. pasteurianus* (ethanol and lactic acid oxidizing, acetic acid overoxidizing, acid tolerant, and moderately heat tolerant) during the natural cocoa bean heap fermentation process. In particular, it reveals that citric acid fermentation is important for the onset of the cocoa bean fermentation process by an appropriate LAB strain. Also, mannitol production and lactic acid and acetic acid production and further overoxidation determine the properties of well-fermented cocoa beans. This study further indicates that, by setting the initial conditions, defined inocula of yeast, LAB, and AAB can now be considered mixed-strain starter cultures that should lead to better controlled and more reliable cocoa bean fermentation processes and hence good-tasting and flavorful chocolates.

ACKNOWLEDGMENTS

This research was funded by the Research Council of the Vrije Universiteit Brussel (OZR, GOA, and IOF projects), the Fund for Scientific Research Flanders, the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT projects 040043, 050818, and 080357), the Federal Research Policy (Action for the Promotion of and Co-operation with the Belgian Coordinated Collections of Microorganisms, BCCM), and Barry Callebaut N.V.

The technical assistance of Tom De Winter is gratefully acknowledged.

REFERENCES

1. **Ardhana, M., and G. Fleet.** 2003. The microbial ecology of cocoa bean fermentations in Indonesia. Int. J. Food Microbiol. **86:**87–99.
2. **Baker, D. M., K. I. Tomlins, and C. Gray.** 1994. Survey of Ghanaian cocoa farmer fermentation practices and their influence on cocoa flavour. Food Chem. **51:**425–431.
3. **Buamah, R., V. P. Dzogbefia, and J. H. Oldham.** 1997. Pure yeasts culture fermentation of cocoa (*Theobroma cacao* L.): effect on yield of sweatings and cocoa bean quality. World J. Microbiol. Biotechnol. **13:**457–462.
4. **Camu, N., T. De Winter, K. Verbrugghe, I. Cleenwerck, P. Vandamme, J. S. Takrama, M. Vancanneyt, and L. De Vuyst.** 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Appl. Environ. Microbiol. **73:**1809–1824.
5. **Camu, N., Á. González, T. De Winter, A. Van Schoor, K. De Bruyne, P. Vandamme, J. S. Takrama, and L. De Vuyst.** 2008. Influence of turning and environmental contamination on the dynamics of lactic acid bacteria and acetic acid bacteria populations involved in spontaneous cocoa bean heap fermentation in Ghana. Appl. Environ. Microbiol. **74:**86–98.
6. **Camu, N., T. De Winter, S. K. Addo, J. S. Takrama, H. Bernaert, and L. De Vuyst.** 2008. Fermentation of cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of chocolate. J. Sci. Food Agric. **88:**2288–2297.
7. **Daniel, H.-M., G. Vrancken, J. S. Takrama, N. Camu, P. De Vos, and L. De Vuyst.** 2009. Yeast diversity of Ghanaian cocoa bean heap fermentations. FEMS Yeast Res. **9:**774–783.
8. **Dzogbefia, V. P., R. Buamah, and J. H. Oldham.** 1999. The controlled fermentation of cocoa (*Theobroma cacao* L.) using yeasts: enzymatic process and associated physico-chemical changes in cocoa sweatings. Food Biotechnol. **13:**1–12.
9. **Hammes, W. P., and R. F. Vogel.** 1995. The genus *Lactobacillus*, p. 19–54. *In* B. J. Wood and W. H. Holzapfel (ed.), The genera of lactic acid bacteria. Blackie Academic & Professional, Glasgow, United Kingdom.
10. **Jespersen, L., D. S. Nielsen, S. Hønholt, and M. Jakobsen.** 2005. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast Res. **5:**441–453.
11. **Kostinek, M., L. B. an-Koffi, M. Ottah-Atikpo, D. Teniola, U. Schillinger, W. H. Holzapfel, and C. M. A. P. Franz.** 2008. Diversity of predominant lactic acid bacteria associated with cocoa fermentation in Nigeria. Curr. Microbiol. **56:**306–314.
12. **Lagunes-Gálvez, S., G. Loiseau, J. L. Paredes, M. Barel, and J.-P. Guiraud.** 2007. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. Int. J. Food Microbiol. **114:**124–130.
13. **Leroy, F., and L. De Vuyst.** 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. **15:**67–78.
14. **Leroy, F., and L. De Vuyst.** 2005. Simulation of the effect of sausage ingredients and technology on the functionality of the bacteriocin-producing *Lactobacillus sakei* CTC 494 strain. Int. J. Food Microbiol. **100:**141–152.
15. **Leroy, F., J. Verluyten, W. Messens, and L. De Vuyst.** 2002. Modelling contributes to the understanding of the different behaviour of bacteriocin-producing strains in a meat environment. Int. Dairy J. **12:**247–253.
16. **Li, X., B. Jiang, P. Beilei, M. Wanmeng, and T. Zhang.** 2008. Purification and partial characterization of *Lactobacillus* species SK007 lactate dehydrogenase (LDH) catalyzing phenylpyruvic acid (PPA) conversion into phenyllactic acid (PLA). J. Agric. Food Chem. **56:**2392–2399.
17. **Messens, W., P. Neysens, W. Vansieleghem, J. Vanderhoeven, and L. De Vuyst.** 2002. Modeling growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in response to temperature and pH values used for sourdough fermentations. Appl. Environ. Microbiol. **68:**1431–1435.
18. **Nielsen, D. S., O. D. Teniola, L. Ban-Koffi, M. Owusu, T. S. Andersson, and W. H. Holzapfel.** 2007. The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. Int. J. Food Microbiol. **114:**168–186.
19. **Nielsen, D. S., S. Hønholt, K. Tano-Debrah, and L. Jespersen.** 2005. Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). Yeast **22:**271–284.
20. **Pettipher, G. L.** 1986. Analysis of cocoa pulp and the formulation of a standardized artificial cocoa pulp medium. J. Sci. Food Agric. **37:**297–309.

21. **Radler, F., and K. Bröhl.** 1984. The metabolism of several carboxylic acids by lactic acid bacteria. Z. Lebensm. Unters. Forsch. **179:**228–231.
22. **Raj, K. C., L. O. Ingram, and J. A. Mauphin-Furlow.** 2001. Pyruvate decarboxylase: a key enzyme for the oxidative metabolism of lactic acid by *Acetobacter pasteurianus*. Arch. Microbiol. **176:**443–451.
23. **Roelofsen, P. A.** 1958. Fermentation, drying and storage of cacao beans. Adv. Food Res. **8:**225–296.
24. **Samah, O. A., M. F. Ptih, and J. Selamat.** 1992. Biochemical changes during fermentation of cocoa beans inoculated with *Saccharomyces cerevisiae* (wild strain). J. Food Sci. Technol. **29:**341–343.
25. **Samah, O. A., M. F. Puteh, J. Selemat, and H. Alimon.** 1993. Fermentation products in cocoa beans inoculated with *Acetobacter xylinum*. ASEAN Food J. **8:**22–25.
26. **Sanchez, J., G. Daguenet, J. P. Guiraud, J. C. Vincent, and P. Galzy.** 1985. A study of the yeast flora and the effect of pure culture seeding during the fermentation of cocoa beans. Lebensm. Wiss. Technol. **18:**69–76.
27. **Schwan, R. F., A. H. Rose, and R. G. Board.** 1995. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. J. Appl. Bacteriol. Symp. Suppl. **79:**96S–107S.
28. **Schwan, R. F., and A. E. Wheals.** 2004. The microbiology of cocoa fermentation and its role in chocolate quality. Crit. Rev. Food Sci. Nutr. **44:**205–221.
29. **Schwan, R. F.** 1998. Cocoa fermentations conducted with a defined microbial cocktail inoculum. Appl. Environ. Microbiol. **64:**1477–1483.
30. **Tanous, C., A. Kieronczyk, S. Helinck, E. Chambellon, and M. Yvon.** 2002. Glutamate dehydrogenase activity: a major criterion for the selection of flavour-producing lactic acid bacteria strains. Antonie Van Leeuwenhoek **82:**271–278.
31. **Thompson, S. S., K. B. Miller, and A. S. Lopez.** 2007. Cocoa and coffee, p. 837–849. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers, 2nd ed. ASM Press, Washington, DC.
32. **Thurner, C., C. Vela, L. Thony-Meyer, L. Meile, and M. Teuber.** 1997. Biochemical and genetic characterization of the acetaldehyde dehydrogenase complex from *Acetobacter europaeus*. Arch. Microbiol. **168:**81–91.
33. **Tomlins, K. L., D. M. Baker, P. Daplyn, and D. Adomako.** 1993. Effect of fermentation and drying practices on the chemical and physical profiles of Ghana cocoa. Food Chem. **46:**257–263.
34. **Vermeulen, N., M. G. Gänzle, and R. F. Vogel.** 2006. Influence of peptide supply and co-substrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM 20451^T and *Lactobacillus plantarum* TMW 1.468. J. Agric. Food Chem. **54:**3832–3839.
35. **Wisselink, H. W., R. A. Weusthuis, G. Eggink, J. Hugenholtz, and G. J. Grobben.** 2002. Mannitol production by lactic acid bacteria: a review. Int. Dairy J. **12:**151–161.
36. **Wood, G. A. R., and R. A. Lass.** 2001. Cocoa, 4th ed. Longman Group Limited, London, United Kingdom.