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Diversity of Predominant Lactic Acid Bacteria Associated with Cocoa Fermentation in Nigeria

Melanie Kostinek · Louis Ban-Koffi · Margaret Ottah-Atikpo · David Teniola · Ulrich Schillinger · Wilhelm H. Holzapfel · Charles M. A. P. Franz

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Abstract The fermentation of cocoa relies on a complex succession of bacteria and filamentous fungi, all of which can have an impact on cocoa flavor. So far, few investigations have focused on the diversity of lactic acid bacteria involved in cocoa fermentation, and many earlier investigations did not rely on polyphasic taxonomical approaches, which take both phenotypic and genotypic characterization techniques into account. In our study, we characterized predominant lactic acid bacteria from cocoa fermentations in Nigeria, using a combination of phenotypic tests, repetitive extragenic palindromic PCR, and sequencing of the 16S rRNA gene of representative strains for accurate species identification. Thus, of a total of 193 lactic acid bacteria (LAB) strains isolated from common media used to cultivate LAB, 40 (20.7%) were heterofermentative and consisted of either *L. brevis* or *L. fermentum* strains. The majority of the isolates were homofermentative rods (110 strains; 57% of isolates) which were characterized as *L. plantarum* strains. The homofermentative cocci consisted predominantly of 35 (18.1% of isolates) *Pediococcus acidilactici* strains. Thus, the LAB populations derived from these media in this study were accurately described. This can contribute to the further assessment of the effect of common LAB strains on the flavor characteristics of fermenting cocoa in further studies.

M. Kostinek · U. Schillinger · W. H. Holzapfel · C. M. A. P. Franz (✉)
Federal Research Centre for Nutrition and Food, Institute for Hygiene and Toxicology, Haid-und Neu-Strasse 9, D-76131 Karlsruhe, Germany
e-mail: Charles.Franz@bfel.de

L. Ban-Koffi
Centre National de Recherche Agronomique (CNRA), Km 17 Route de Dabou, 01 BP 1740 Abidjan, 01 Cote d'Ivoire

M. Ottah-Atikpo
CSIR Food Research Institute, P.O. Box M20, Accra, Ghana

D. Teniola
Federal Institute of Industrial Research, Oshodi (FIIRO), PMB 21023 Ikeja, Lagos, Nigeria

Introduction

Cocoa beans are the principal raw material for chocolate manufacture and originate as seeds from fruit pods of the cocoa tree *Theobroma cacao*, which are cultivated in the tropical regions of the world [1, 17]. The beans embedded in mucilaginous pulp are removed from the fruit pods and subjected to microbial fermentation as the first stage in the preparation of chocolate [1]. Fermentation is followed by a drying process, and both these production steps are important to initiate the formation of the precursors of chocolate flavor [1, 16, 17]. Two major events occur during fermentation: first, microbial action on the mucilaginous pulp produces alcohols and acids as well as heat; and second, complex biochemical reactions triggered by diffusion of metabolites from micro-organisms occur in the cotyledons [3]. These biochemical changes inside the beans contribute to the reduction of bitterness and astringency and the development of flavor precursors [3]. The seed embryo is killed and subsequently the fruit tissue degraded, which makes bean drying easier.

Cocoa beans are mainly fermented in heaps enveloped in plantain leaves or in wooden trays. The microbial succession of the fermentation process has been clearly established [8, 11, 13, 17]. Yeasts dominate at the beginning, and up to 24 h of fermentation. Their most important

roles are to break down the citric acid in the pulp, which leads to an increase in pH; to produce ethanol and organic acids, which kill the bean cotyledons to produce volatile organic compounds that contribute to precursors of chocolate flavor; and to secrete pectinases, which reduce the viscosity of the pulp and allow aeration of the pulp mass [15]. These resulting environmental conditions favor the growth of lactic acid bacteria (LAB), which exhibit fast growth from 16 to 48 h of fermentation and then reach a peak while the numbers of yeast are declining [17]. As aeration of the fermenting mass increases and the temperature rises above 37°C, acetic acid bacteria become the most predominant micro-organisms. The exothermic reactions of these bacteria raise the temperature of the fermenting mass to 50°C or more before they become inhibited themselves under these conditions [16, 17]. Finally, the increased aeration, increased pH of the cocoa pulp, and a rise in temperature to about 45°C in the cocoa mass in the later stages of fermentation favor the growth of aerobic spore-forming bacteria of the genus *Bacillus* or *Geobacillus* [17]. These produce a variety of chemical compounds which may contribute to acidity and, under certain conditions, also to off-flavors of the fermented cocoa beans [15].

Surprisingly, not much is known about the diversity of LAB involved in the fermentation of cocoa. Only relatively few studies have focused on these bacteria and these have not been based on modern, polyphasic taxonomical approaches, which utilize both phenotypic and genotypic investigation methods. This study serves to describe the diversity of predominant LAB occurring on common media for LAB cultivation, which were utilized to investigate cocoa fermentation in Nigeria.

Materials and Methods

Microbiological sampling, strains, and culture conditions LAB strains in this study were isolated from Nigerian tray and heap cocoa fermentation samples which were taken at from 0 up to 120 h of fermentation time (tray fermentation) or up to 144 h (heap fermentation). Fermentations were carried out at the Cocoa Research Institute of Nigeria (CRIN) in Ibadan.

Samples were taken from the middle of the fermenting mass for both the tray and the heap fermentations. The samples were transferred immediately to the laboratory for analysis. For isolation of LAB in Nigerian cocoa fermentation, 10-g samples from the various stages of fermentation were added to 90 ml quarter-strength Ringer's solution (QRS; 1:10 dilution; Merck, Darmstadt, Germany) and homogenized for 2 min using a Waring Blendor. The samples were further diluted in a 10-fold dilution series and 100-µl aliquots were spread-plated onto different agar media—MRS agar, Rogosa agar, kanamycin esculin azide (KEA) agar, and M17 agar (all from Merck)—to obtain the common LAB associated with fermenting cocoa capable of growing on these media. These media are all known to select for a variety of lactic acid bacterial species and were included in this study to obtain the widest possible species variety. Plates were incubated at 30°C for 24–48 h under anaerobic conditions. Colonies were randomly picked from the agar plates of the highest dilutions. After picking, the strains were sent to Germany for characterization. In Germany, they were grown aerobically in MRS broth at 30°C and streaked out repeatedly to check for purity. Stock cultures of the isolates were stored in MRS broth containing 15% glycerol (Merck) at −80°C.

Phenotypic characterization Cell morphology was observed using phase contrast microscopy at 1000 × magnification (Leitz, Jena, Germany), isolates were Gram-stained, and catalase activity was determined. Production of gas from glucose in MRS broth and determination of the presence of d-*meso*-di-aminopimelic acid in the cell wall (*m*DAP) and of the type of lactic acid enantiomer produced were done using the methods of Schillinger und Lücke [14]. The fermentation of specific sugars was tested according to the method of Jayne-Williams [4]. Growth of presumptive enterococci was tested in MRS broth containing 6.5% (w/v) NaCl or in MRS broth at pH 9.6, at 10°C or 45°C, respectively.

Genotypic characterization The total genomic DNA of all strains was isolated as described previously [5]. Repetitive extragenic palindromic (rep)-PCR-based typing used for presumptive identification of LAB strains was done using the primer (GTG)5, and the method and amplification conditions were as described previously by Kostinek et al. [6]. Gel electrophoresis, fingerprint analyses, and grouping of rep-PCR fingerprints was performed as described before [6]. The almost-complete 16S rRNA gene of selected strains was amplified by PCR as described by Kostinek et al. [6] and sequenced at GATC Biotech (Konstanz, Germany). The nucleotide sequences were imported into the Bionumerics (version 2.5) program (Applied Maths, Sint-Martens-Latem, Belgium). These sequences were aligned using multiple alignment and similarity was calculated by global cluster analysis, i.e., UPGMA clustering with Jukes and Cantor correction and discarding of unknown bases. The nucleotide sequences were deposited in the GenBank database and received the accession numbers EU147300 for *Lactobacillus fermentum* BFE 8253; EU147301, EU147302, EU147303, and EU147304 for *Lactobacillus brevis* strains BFE 8325, BFE 8266, BFE 8285, and BFE 8359, respectively; EU147305, EU147306,

EU147307, and EU 147308 for *Lactobacillus plantarum* strains BFE 8239, BFE 8200, BFE 8202, and BFE 8348, respectively; and EU147309, EU147310, EU147311, EU147312, EU147313, EU147314, EU147315, and EU147316 for *Pediococcus acidilactici* strains BFE 8245, BFE 8246, BFE 8387, BFE 8384, BFE 8230, BFE 8390, BFE 8260, and BFE 8262, respectively. The nucleotide accession numbers for the *L. plantarum* DSM 20174^T, *L. fermentum* ATCC 14931^T, *L. brevis* DSM 20054^T, and *P. acidilactici* DSM 20284^T type strains used for sequence comparisons were D79210, M58819, M58810, and M58833, respectively.

After phenotypic and genotypic characterization, the composition of the LAB biota isolated in the approximate first half of fermentation (0 to 60 h of fermentation) was compared to that isolated in the second half of fermentation (60 to 120 h for tray fermentation and 60 to 144 h for heap fermentation).

Results

Phenotypic Characterization

Gram-positive, catalase-negative bacteria growing on MRS agar were considered as presumptive LAB. A total of 193 LAB strains were isolated (Table 1). Numbers of LAB determined on MRS, Rogosa, KEA, and M17 agar were log 4 colony forming units (CFU)/g at the beginning of both the fermentations and increased to log 6.0 within 24 h for both types of fermentation. The LAB counts ranged from log 5.9 to log 8.2 from 24 h of fermentation till the end of the heap and tray fermentations, indicating that LAB constituted the majority of the isolates on these media.

Obligately Heterofermentative Rods

Forty strains (20.7%) exhibited a rod-shaped morphology and produced gas from glucose. All these strains produced DL-lactate and, thus, were characterized as obligately heterofermentative strains belonging either to the genus *Lactobacillus* or to *Weissella* (Table 1).

Obligately Homofermentative and Facultatively Heterofermentative Rods

One hundred eleven strains (57.2%) showed a rod-shaped morphology and produced no gas from glucose. One hundred ten strains produced DL-lactate and one strain produced L-lactate. The L-lactate-producing strain BFE 8382 was considered a facultatively heterofermentative rod, as it was able to ferment ribose. The group of 110 strains belonged to the *L. plantarum* group (Table 1), as they fermented at least one of the pentose sugars arabinose, ribose or xylose, and *m*DAP was present in the cell wall.

Presumptive Pediococci

Thirty-five (Table 1) strains (18.1%) showed a coccoid morphology and tetrad formation, and they produced DL-lactate and no gas from glucose metabolism, indicating that they belong to the genus *Pediococcus*.

Homofermentative Cocci

Seven (3.6%) coccus-shaped strains produced no gas from glucose and L-lactate (Table 1). These strains grew in MRS broth containing 6.5% (w/v) NaCl, in MRS broth with a pH of 9.6, and at 10°C and 45°C. Therefore, they were characterized as belonging to the genus *Enterococcus* [2].

Genotypic Characterization

Obligately Heterofermentative Rods

Forty obligately heterofermentative rods were characterized using genotypic methods. In the rep-PCR analysis,

Table 1 LAB strains isolated during cocoa heap and tray fermentation in Nigeria

Characterization/grouping	Number (%) of strains	
	Heap fermentation	Tray fermentation
LAB isolates ($n = 193$)	62 (32.1%)	131 (67.9%)
Obligately heterofermentative rods producing DL-lactate ($n = 40$)	16 (25.8%)	24 (18.3%)
Strains belonging to the *L. plantarum* group, producing DL-lactate, fermenting pentoses, and possessing *m*DAP in the cell wall ($n = 110$)	25 (40.3%)	85 (64.9%)
Obligately homofermentative rods producing L-lactate ($n = 1$)	0 (0%)	1 (0.8%)
Presumptive pediococci ($n = 35$)	21 (33.9%)	14 (10.7%)
Presumptive enterococci ($n = 7$)	0 (0%)	7 (5.3%)

eight strains grouped together at $r = 69.9\%$ with two different *L. fermentum* reference strains, i.e., *L. fermentum* LMG 8900 and the *L. fermentum* type strain DSM 20052^T (Fig. 1), indicating that these strains could be presumptively identified as *L. fermentum.* To confirm the rep-PCR results, the 16S rRNA gene of a representative strain (BFE 8253) from the rep-PCR cluster was sequenced and showed 99.5% similarity to the 16S rRNA gene sequence of the *L. fermentum* DSM 20052^T type strain (results not shown). The remaining 32 strains grouped together with the *L. brevis* type strain DSM 20054^T. To confirm their characterization as *L. brevis* strains, four representative strains (BFE 8325, BFE 8266, BFE 8285, and BFE 8359), which were distributed over the *L. brevis* rep-PCR cluster, were selected for 16S rRNA gene sequencing. These all shared at least 99.8% sequence similarity to the type strain.

L. plantarum-group Strains

One hundred ten strains were characterized as belonging to the *L. plantarum* group based on phenotypic characterization. In the rep-PCR analysis, all 110 strains grouped together with the *L. plantarum* type strain DSM 20174^T and the *L. plantarum* CNRZ 1228 reference strain at $r = 29.5\%$ (Fig. 2), indicating that these may also be characterized as *L. plantarum* strains. These 110 strains also showed a very similar and characteristic banding pattern, demonstrating their close relatedness. The relatively low correlation value can be explained by one outlying strain's fingerprint, strain BFE 8348. Rep-PCR fingerprinting was done in duplicate for the *L. paraplantarum* type strain LTH 5200^T and for the strain BFE 8348, to determine the reproducibility of the method. The reproducibility of PCR and running conditions was $r = 88.6\%$ and $r = 94.0\%$ for these strains, respectively. The *L. pentosus* type strain DSM 20314^T and the *L. paraplantarum* type strain LTH 5200^T clustered separately from the *L. plantarum* reference and type strain, indicating that these species could be successfully distinguished from *L. plantarum* strains. Because one *L. plantarum*-group strain (BFE 8348) clustered noticeably outside the coherent cluster which contained most other *L. plantarum*-group strains, the 16S rRNA gene was sequenced to identify this strain. Moreover, three *L. plantarum*-group strains (BFE 8239, BFE 8200, and BFE 8202), clustering near the type strain of *L. plantarum* DSM 20174^T, were also chosen for 16S rRNA gene sequencing to confirm the phenotypic characterization and rep-PCR analysis. The 16S rRNA gene of all four strains showed at least 99.5% similarity to the 16S rRNA gene of the type strain *L. plantarum* DSM 20174^T, showing that these could all be identified as *L. plantarum* strains.

Pediococcus Strains

The results of the rep-PCR fingerprinting of *Pediococcus* strains are shown in Fig. 3. All isolates grouped together at $r = 34.0\%$. Three different subclusters, each with a distinctive band pattern, could be distinguished. Thus, subcluster I contained 7 strains which grouped together with a correlation value of $r = 62.3\%$, subcluster II consisted of 13 strains which grouped at $r = 85.3\%$, and subcluster III consisted of 15 strains grouping closely at $r = 89.9\%$ (Fig. 3). The isolates did not show any close relationship to any of the *Pediococcus* type species fingerprint patterns, but it was noticed that they grouped more closely to the *P. claussenii*, *P. stilesii*, *P. parvulus*, and *P. acidilactici* type strains, at $r = 15.5\%$, than to any of the other *Pediococcus* type strains (Fig. 3). To better characterize the pediococci isolates, representative strains from sublusters I (BFE 8245, 8246, and 8262), II (BFE 8387, 8384, and 8230), and III (BFE 8390 and 8260) were chosen for 16S rRNA gene sequencing. The 16S rRNA gene sequences from all strains showed a high (at least 99.8%) similarity to that of the *P. acidilactici* DSM 20284^T type strain, indicating that all these strains from the three subclusters could be characterized as *P. acidilactici.*

Succession

In the heap fermentation, *L. plantarum*-group strains predominated (37.5%), followed by pediococci (35%) and obligately heterofermentative rods (27.5%), in the first half of fermentation (results not shown). In the second half of fermentation, the incidence of *L. plantarum* group strains increased slightly, to 45.5%, while the incidence of pediococci and obligately heterofermentative strains decreased slightly, to 31.8% and 22.7%, respectively. In the tray fermentation, *L. plantarum*-group strains also predominated, but at the even higher incidence of 63.6%, and they were also followed by pediococci (18.2%) and obligately heterofermentative rods (12.1%) as predominant isolates from these media. In addition, enterococci were isolated as predominant strains occurring on these media, at an incidence of 6.1% (results not shown). In the second half of the tray fermentation, the predominance of *L. plantarum*-group strains increased slightly, to 67.7%, while the incidence of obligately heterofermentative strains approximately doubled, to 24.6%. The incidence of pediococci and enterococci decreased to 3.1% and 4.6%, respectively.

Discussion

The succession of micro-organisms during the fermentation of cocoa beans has been investigated in detail [1, 7,

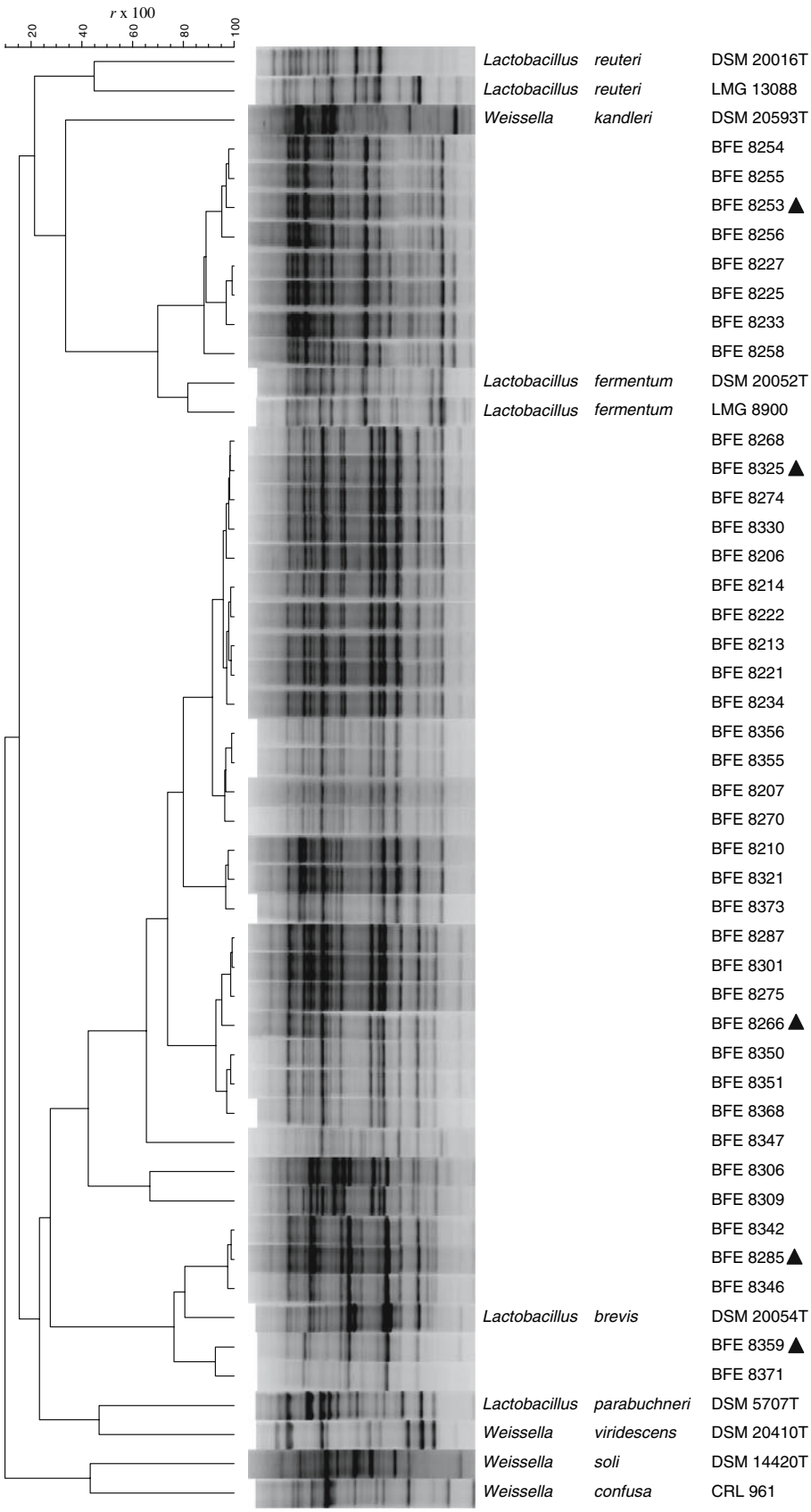

Fig. 1 Dendrogram obtained by UPGMA of correlation value *r* of rep-PCR fingerprint patterns of 40 obligately heterofermentative *Lactobacillus* strains producing DL-lactate isolated from heap and tray cocoa fermentations in Nigeria and several obligately heterofermentative *Lactobacillus* reference and type strains. Strains labeled with a triangle were selected for 16S rRNA gene sequencing

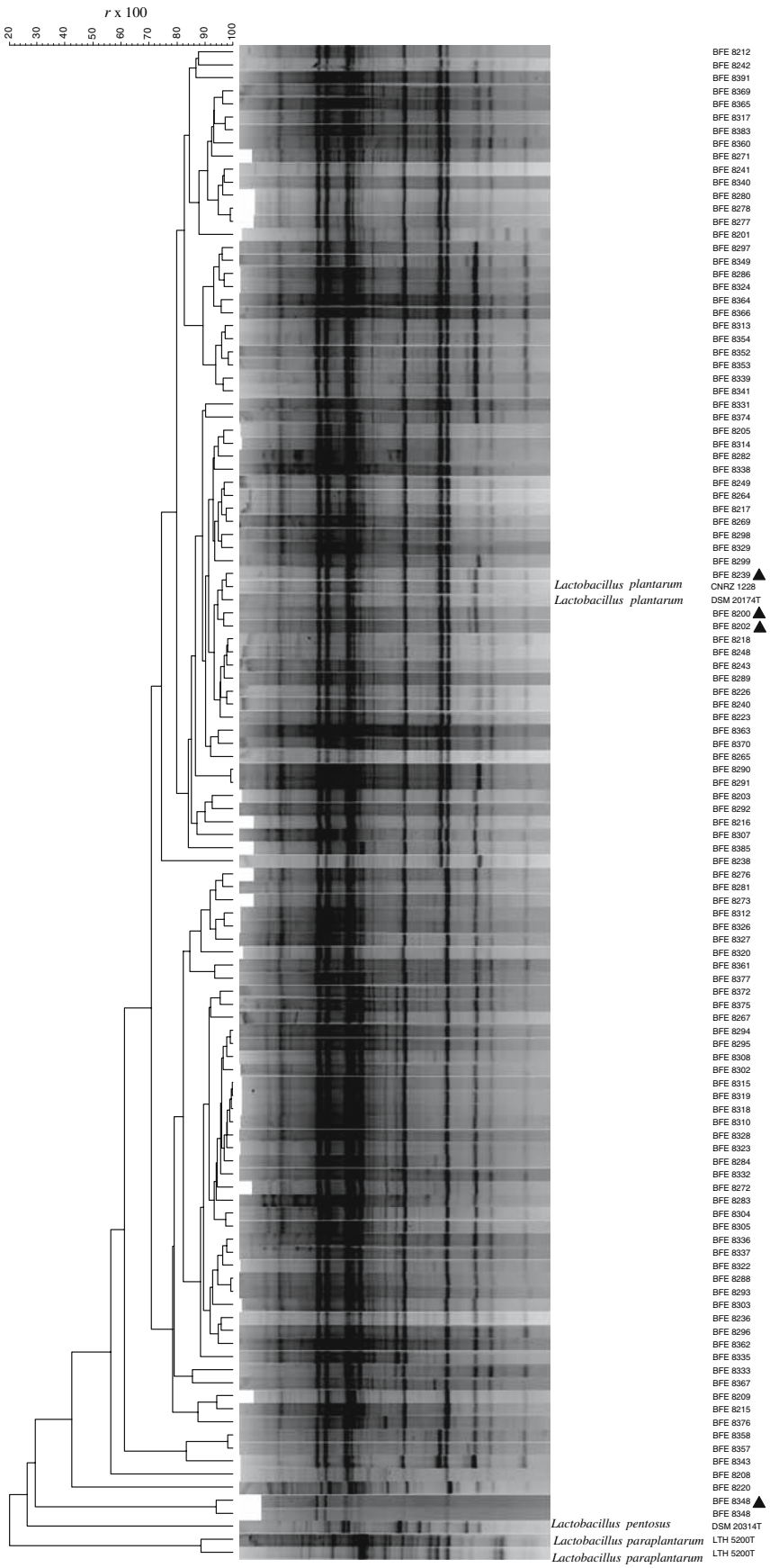

Fig. 2 Dendrogram obtained by UPGMA of correlation value *r* of rep-PCR fingerprint patterns of 110 strains belonging to the *L. plantarum*-group isolated from heap and tray cocoa fermentations in Nigeria and the *L. plantarum* DSM 20174^T and *L. plantarum* CNRZ 1228, *L. paraplantarum* LTH 5200^T, and *L. pentosus* DSM 20314^T type and reference strains. Strains labeled with a triangle were selected for 16S rRNA gene sequencing

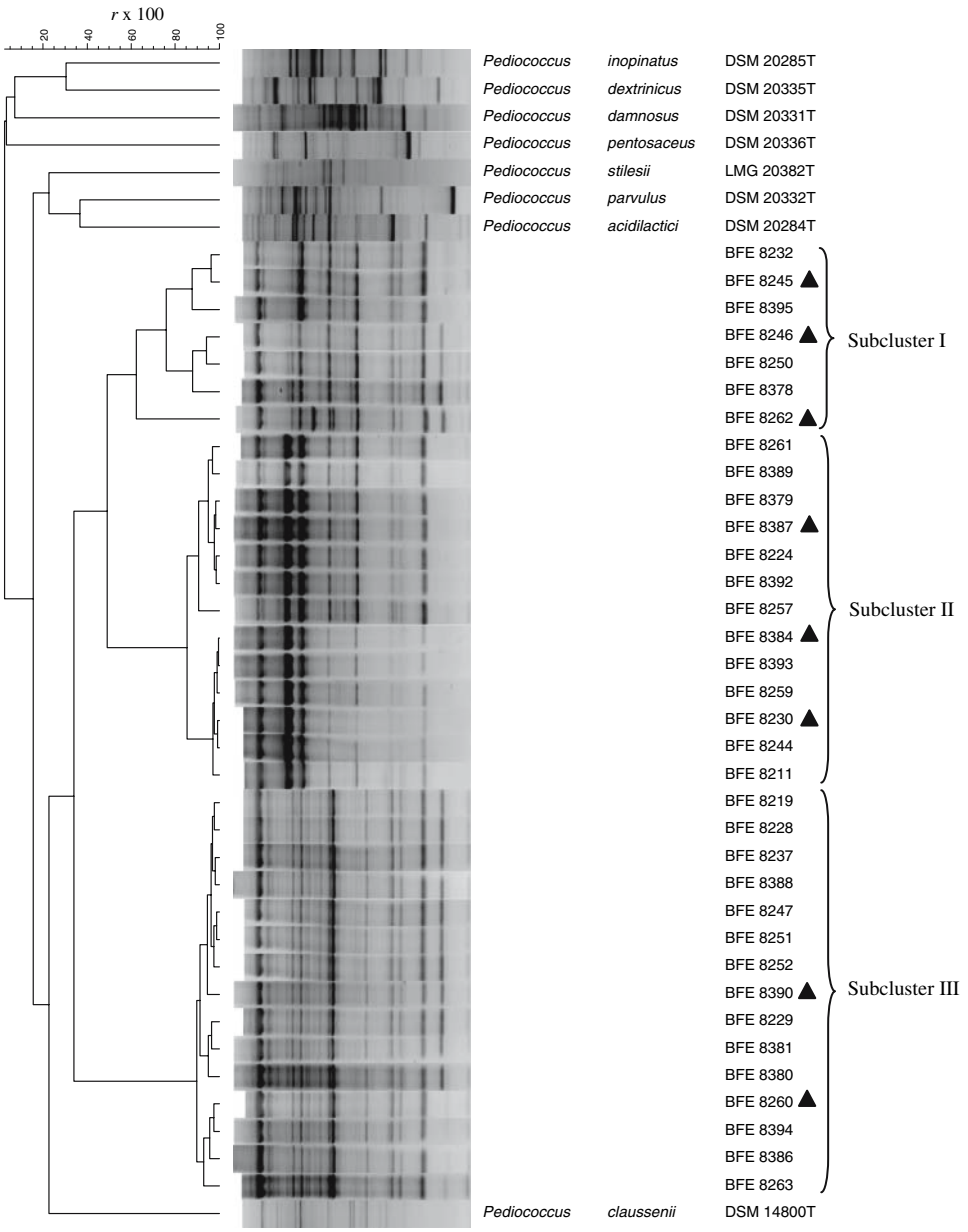

Fig. 3 Dendrogram obtained by UPGMA of correlation value *r* of rep-PCR fingerprint patterns of 35 presumptive strains belonging to the genus *Pediococcus* isolated from heap and tray cocoa fermentations in Nigeria and several *Pediococcus* type strains representing different species. Strains labeled with a triangle were selected for 16S rRNA gene sequencing

8, 11, 13, 17]. Also, the technological role that microorganisms of each of these groups play is superficially known, but certainly requires more study [15]. In our study we showed that, as expected, LAB were also involved in Nigerian cocoa bean fermentations carried out in heaps and trays. Broad characterization of these microorganisms to the genus level indicated that within the group of the LAB, there was quite a diversity of strains associated with the fermentation and isolated on the media used, inasmuch as strains of obligately heterofermentative rods, *L. plantarum*-group strains, pediococci, and enterococci could be characterized on the basis of phenotypic properties. By applying a polyphasic taxonomical approach, we were able to accurately identify the species isolated.

Surprisingly, not much recent and accurate information on the LAB diversity associated with cocoa fermentation, which is based on polyphasic taxonomical methodology and which includes modern genotypic fingerprinting methods, is available. *L. fermentum*, *L. plantarum*, *L. mesenteroides*, and *Lactococcus lactis* were described as the predominant LAB species isolated from cocoa fermentations in Trinidad during the first 24 h of fermentation [11]. Passos et al. [12] isolated and identified *L. casei*, *L. plantarum*, *L. delbrueckii*, *L. acidophilus*, *L. brevis*, *P. dextrinicus*, and *P. acidilactici* from cocoa fermentations in Brazil. Furthermore, in most studies on the bacterial diversity from cocoa fermentation, *L. plantarum* was described as the main representative [1]. In addition, *L. fermentum* was also described as belonging to the dominant LAB population in cocoa fermentations in India,

and could be isolated at an incidence of 60%–85% of LAB over a 48-h fermentation period. *L. plantarum* and *L. hilgardii* strains also predominated in the cocoa fermentations in India, but were only recovered during the first 24 h [1]. In a recent investigation, Nielsen et al. [9] characterized the succession and the predominant LAB from heap or tray cocoa fermentations in Ghana. These authors showed that LAB became dominant after 12 h of fermentation and remained predominant during the remainder of the fermentation period. *L. fermentum* was the dominant LAB species in most samples, while several other species, including *L. plantarum*, *L. pseudomesenteroides*, *L. pseudoficulneum*, *P. acidilactici*, and *L. ghanensis*, were detected during the fermentation [9, 10].

Similarly to most other studies on cocoa fermentation, *L. plantarum* strains were the LAB occurring at the highest incidence on the media used in our study to isolate the LAB from fermenting cocoa (57% of isolates). In addition, *L. brevis* and *L. fermentum* strains also commonly occurred at 16.6% and 4.1%, respectively. Although pediococci have been described to be associated with cocoa fermentation in Brazil, many other studies do not report these bacteria as a predominant part of the flora. Thus, unlike the Ghana fermentations in which no pediococci were reported [9], we could isolate these bacteria at a relatively high frequency (18.1%). In addition, enterococci were only seldom reported to occur as part of the cocoa fermentation micropopulation. Thus, there appears to be regional differences in the fermentation populations, although the presence of *L. fermentum*, *L. plantarum*, and *L. brevis* strains seem to be common for this type of fermentation. In our study, the composition of the LAB populations differed between heap and tray fermentation in that more pediococci were isolated from the heap fermentation (33.9% for heap fermentation compared to 10.7% for tray fermentation), and no enterococci were isolated from the heap fermentation. Furthermore, our succession investigation of LAB throughout the fermentation period clearly showed that *L. plantarum*-group strains occurred and increased in incidence throughout the fermentation period. Although the incidence of obligately heterofermentative rods decreased slightly in the heap fermentation, an increase in these bacteria to almost double the incidence could be observed in the tray fermentation. Thus, while *L. plantarum* and obligately heterofermentative rods like *L. brevis* and *L. fermentum* are predominant and thus very important in the cocoa fermentation, their incidence, and the presence of other LAB groups, may depend on the fermentation method, i.e., whether heap or tray fermentation is used.

Such diversity differences are considered to be important, especially as cocoa quality varies greatly depending on the bean quality and fermentation characteristics [3]. LAB, in cooperation with other micro-organisms, produce ethanol, organic acids, volatile compounds, and enzymes and, thus, are important for the flavor and aroma production in beans used for chocolate production. Even though these micro-organisms participate in the development of desirable changes in the beans, the physiological roles of each species in each group remain to be clarified. Thus, the accurate characterization of the common LAB species involved in cocoa fermentation which can be isolated from typical LAB growth media is an important first step in the EU project in order to select strains which may positively influence the quality of cocoa fermentation when used as adjunct starter cultures.

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