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Systematic and Applied Microbiology 28 (2005) 527–540
SYSTEMATIC AND APPLIED MICROBIOLOGY
www.elsevier.de/syapm

# Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of Gari, a traditional African food

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## Abstract

Traditional fermentation of cassava is dominated by a lactic acid bacteria (LAB) population. Fermentation is important for improving product flavour and aroma as well as safety, especially by reduction of its toxic cyanogenic glucosides. The production of Gari from cassava in Benin typically occurs on a household or small industrial scale, and consequently suffers from inconsistent product quality and may not always be safe for consumption. Therefore, the diversity of LAB from a typical cassava fermentation for the preparation of Gari, and their technologically relevant characteristics were investigated with a view towards selection of appropriate starter cultures. A total of 139 predominant strains isolated from fermenting cassava were identified using phenotypic tests and genotypic methods such as rep-PCR and RAPD-PCR. DNA–DNA hybridisation and sequencing of the 16S rRNA genes were done for selected strains. *Lactobacillus plantarum* was the most abundantly isolated species (54.6% of isolates), followed by *Leuconostoc fallax* (22.3%) and *Lactobacillus fermentum* (18.0%). *Lactobacillus brevis*, *Leuconostoc pseudomesenteroides* and *Weissella paramesenteroides* were sporadically isolated. The *L. plantarum* strains were shown to be better acid producers and capable of faster acid production than the *L. fallax* or *L. fermentum* strains. The incidence of β-glucosidase (linamarase) activity was also highest among strains of this species. Production of antagonistic substances such as $H_2O_2$ and bacteriocins, however, was more common among *L. fallax* and *L. fermentum* strains. Strains of all three species were capable of utilising the indigestible sugars raffinose and stachyose. Therefore, a starter culture containing a mixture of strains from all three species was recommended.


***Keywords:*** Fermented food; Cassava; Linamarase; Gari; Lactic acid bacteria; Genotyping

## Introduction

Cassava, the enlarged root of *Manihot esculenta* Crantz, is a staple food for over 500 million people in the developing world [11], including about 80 million in Nigeria alone [36]. Cassava has important agronomic

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doi:10.1016/j.syapm.2005.03.001

advantages [15], but it has two important deficiencies. Firstly, the bitter varieties contain the toxic cyanogenic glucosides linamarin and (to a lesser extent) lotaustralin, which have fatal consequences when consumed in unprocessed foods [14,42]. Secondly, Gari is very poor in protein, containing only about 1% [15,42]. Cassava may be processed by boiling, roasting, drying or by fermentation, depending on the variety [23]. The most popular processing method, however, especially for the varieties high in cyanogenic glucosides, is by fermentation. One of the most popular foods derived from fermented cassava is Gari, which is consumed by nearly 200 million people across West Africa [36]. Gari is prepared by grating the cassava root, followed by dewatering, fermentation for 2 days at ambient temperature and roasting ('garification') of the fermented mash [50]. During grating, the endogenous linamarase in the root is released and degrades the linamarin. However, the endogenous linamarase is not sufficient to break down all the cyanogenic glucosides in the root, and traces are usually carried into the Gari [35]. It is now fairly well accepted that the flavour of Gari results from the fermentative activities of lactic acid bacteria (LAB) and yeasts, many of which also produce linamarase [32,36,37]. The microbiology of cassava fermentation for Gari production was originally considered a two-stage process, in which *Corynebacterium* spp. and *Geotrichum candidum* strains were reported to be responsible for acid and flavour production [12,13]. Later studies have shown that among the microorganisms isolated from fermenting cassava, *Lactobacillus plantarum* produced the most typical Gari flavour [32]. However, the involvement of five different genera of microorganisms in the fermentation was reported, i.e., *Leuconostoc*, *Alcaligenes*, *Corynebacterium*, *Lactobacillus* and *Candida*, and it was concluded that strains of the genus *Leuconostoc* were the most frequently occurring microorganisms [35].

To date, various investigations on the microbiology of cassava fermentation and Gari production have been done. Most investigations done so far identified microorganisms associated with the fermentation by phenotypic means. In this study, LAB isolated throughout the fermentation of grated cassava for Gari preparation were characterised and identified at both the phenotypic and genotypic levels.

## Materials and methods

### Microbiological sampling and culture conditions

Five samples of fermenting cassava (ca. 200 g material) used for the production of Gari were used in this investigation and were obtained at a small, semi-industrial (women's enterprise) Gari production facility in Benin. Four of these were taken after 48 h fermentation and before the roasting. One ('unfermented') was taken before the fermentation stage but was subsequently transported and despite cooling, it was not possible to determine to which extent a fermentation may have taken place during transport. For transport, all samples were transferred from Benin to Germany by airflight under cooled conditions (temperature increased from 4 to approximately 10 °C during transportation). From the five batches, a 10 g sample each was diluted 1:10 using 90 ml quarter-strength Ringer's solution (VWR International, Bruchsal, Germany) and homogenised for 2 min using a stomacher Blender (VWR International). The samples were further diluted using 10-fold dilutions and plated on MRS agar, Rogosa agar, Kanamycin Esculin Azide agar and M17 Agar (all from Merck, Darmstadt, Germany) to obtain LAB. Plates were incubated at 30 °C for 48 h under aerobic conditions. Colonies were randomly picked from the agar plates of the highest dilutions ($10^{-6}$ and $10^{-7}$) which still showed growth, to obtain predominant strains associated with the fermentation. After picking, the strains were grown in the same type of culture medium from which they were isolated, and streaked out repeatedly to check for purity. Further cultivation of the isolates was performed in MRS broth (Merck, Darmstadt, Germany) and cultures were incubated aerobically at 30 °C for 18 h.

### Phenotypic characterisation

Presumptive LAB strains isolated from the different batches and media (Table 1) were further characterised by determination of cell morphology using phase contrast microscopy, growth at 15 and 45 °C, gas ($CO_2$) production from glucose, determination of the presence of D-*meso*-diaminopimelic acid (mDAP) in the cell wall and determination of the type of lactic acid enantiomer produced, using the methods as described by Schillinger und Lücke [43]. All strains were tested for their sugar fermentation patterns using 20 key sugars [26]. Based on the results of the phenotypic characterisation, the strains were divided into three groups, i.e., obligately heterofermentative rods, facultatively heterofermentative and obligately homofermentative rods and obligately heterofermentative cocci.

### DNA isolation

The total genomic DNA from LAB was isolated according to the method of Pitcher et al. [40] as modified by Björkroth and Korkeala [8] for Gram-positive bacteria.

**Table 1.** Strains isolated from fermented cassava used in this study

| Strains (BFE No.) | Medium of isolation | Ferm. batch[a] | BFE Strains belonging to same rep-PCR-group | BFE strains belonging to same RAPD-PCR group |
|---|---|---|---|---|
| *Heterofermentative cocci* ($n = 34$) | | | | |
| 6641, 6642, 6643, 6644, 6645, 6647, 6648, 6649, 6650 | MRS | A | I (6649), II (6644) all others IIIA | i (6649), ii (6644) all others iii* |
| 6691, 6692, 6693, 6694, 6695, 6696, 6697, 6698 | Rogosa | A | II (6693), all others IIIA | ii (6693), all others iii |
| 6721, 6722, 6723 | KEA | A | IIIA | iii |
| 6746, 6747, 6749, 6750 | M17 | A | IIIA (6746, 6747), IIIB (6749, 6750) | iii |
| 6607, 6608 | MRS | B | IIIA | iii |
| 6657, 6660 | Rogosa | B | IIIA | iii |
| 6726, 6728 | M17 | B | IIIA | iii |
| 6611; 6612 | MRS | C | IIIA | iii |
| 6630 | MRS | D | IIIA | iii |
| 6743 | M17 | E | IIIA | iii |
| *Heterofermentative rods* ($n = 27$) | | | | |
| 6700 | Rogosa | A | i | I |
| 6603, 6604, 6609, 6610 | MRS | B | i | I |
| 6658, 6659 | Rogosa | B | i | I (6658), II (6659) |
| 6615, 6618, 6620 | MRS | C | i | I |
| 6662 | Rogosa | C | i | I |
| 6623, 6625, 6626, 6627, 6628 | MRS | D | i | I |
| 6672, 6673, 6674 | Rogosa | D | i (6672, 6674), ii (6673) | I (6672, 6674), II (6673) |
| 6740 | M17 | D | i | I |
| 6637, 6638, 6639, 6640 | MRS | E | ii (6638), all others i | II (6638), all others I |
| 6682, 6683, 6687 | Rogosa | E | i | I |
| *Facultatively heterofermentative rods* ($n = 76$) | | | | |
| 6646 | MRS | A | A | a |
| 6724, 6725 | KEA | A | A (6725) D (6724) | a (6725) d (6724) |
| 6729 | M17 | A | A | a |
| 6730 | M17 | A | B | b |
| 6601, 6602, 6605, 6606 | MRS | B | B | a |
| 6701, 6702, 6703, 6704, 6705 | KEA | B | B | a (6704) b (6701, 6702, 6705), c (6703) |
| 6651, 6652, 6653, 6654, 6655, 6656 | Rogosa | B | A (6651, 6655, 6656), all others B | a (6655) all others b |
| 6613, 6614, 6616, 6617 | MRS | C | B | a |
| 6706, 6707, 6708, 6709, 6710 | KEA | C | A (6710), all others B | a (6710) all others b |
| 6661, 6663, 6664, 6665, 6666, 6667, 6668, 6669, 6670 | Rogosa | C | A (6663, 6666, 6670) all others B | a (6661, 6663, 6666, 6667, 6668) b (6664, 6669, 6670) c (6665) |
| 6732, 6733, 6734, 6735 | M17 | C | A (6732, 6734) B (6733) C (6735) | a (6734, 6735) b (6732, 6733) |
| 6621, 6622, 6624, 6629 | MRS | D | A (6629) B (6622) C (6621) D (6624) | a (6621, 6624, 6629) b (6622) |
| 6671, 6675, 6676, 6678, 6679, 6680 | Rogosa | D | A (6680) all others B | a (6676, 6678, 6679, 6680) b (6671) c (6675) |
| 6737, 6738, 6739 | M17 | D | A (6738, 6739), B (6737) | a |
| 6631, 6632, 6633, 6635, 6636 | MRS | E | A | a |
| 6716, 6717, 6718, 6719, 6720 | KEA | E | A | a |
| 6684, 6685, 6686, 6688, 6689, 6690 | Rogosa | E | B | a (6688, 6689, 6690) b (6684, 6685, 6686) |
| 6741, 6742, 6744, 6745, 6748 | M17 | E | A (6741, 6742, 6748) B (6744, 6745) | a (6741, 6742, 6744, 6748) b (6745) |

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[a]Ferm. batch: fermentation batch; A: unfermented cassava; B-D: different cassava fermentation batches; MRS: MRS agar; Rogosa: Rogosa Agar; KEA: *Kanamycin Esculin Azide* agar; M17: M17 Agar.

## Rep-PCR

This was used for identification of strains using methods as previously described by Gevers et al. [21]. DNA was amplified in 50 µl volumes containing 100 ng template DNA, 1 × *Taq* DNA polymerase buffer (Amersham Pharmacia, Freiburg, Germany), 200 µM dNTPs, 50 pM of each primer, 4% dimethyl sulphoxide (Sigma, Steinheim, Germany) and 1.5 U *Taq* DNA polymerase (Amersham Pharmacia).

## RAPD-PCR strain typing

This was done using the primer M13 (5′-GAG GGT GGC GGT TCT-3′) [24]. DNA was amplified using methods and amplification conditions described by Andrighetto et al. [4].

## Gel-electrophoresis

PCR products were separated by electrophoresis on 1.8% (w/v) agarose gels using 1 × TBE puffer [41] and a GNA 200 electrophoresis chamber (Amersham Pharmacia). Rep-PCR and RAPD-PCR products were subjected to electrophoresis at 48 V for 17 and 16.5 h, respectively. The gels were stained in ethidium bromide and visualised with a UV transilluminator. Gel images were captured using the Fluorchem Imager 5500 system (Alpha Innotech, USA). The digitised images were normalised and subsequently analysed using the Bionumerics (version 2.5) software package (Applied Maths, Sint-Martens-Latem, Belgium). Groupings of the RAPD-PCR and rep-PCR fingerprints were performed by means of the Pearson product-moment correlation coefficient ($r$) and the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA) [44].

## Species-specific *recA* gene-multiplex PCR

Seventy-six strains, which were identified as presumptive *L. plantarum* by phenotypic tests, were subjected to species-specific *recA* gene-multiplex PCR analysis to distinguish between the three closely related species, *L. plantarum*, *L. paraplantarum* and *L. pentosus* which, together with *L. arizonensis*, belong to the so-called '*L. plantarum* group'. The species-specific *recA* gene-multiplex PCR was done according to the methods of Torriani et al. [49].

## Determination of mol% G + C content of the DNA, DNA–DNA hybridisation and ribotyping

DNA was isolated and purified according to the method of Marmur [27] as modified by Stackebrandt and Kandler [45]. The DNA base composition was estimated from the thermal melting point of DNA ($T_M$) as described by Marmur and Doty [28] using a spectrophotometer (Varian Cary 100 Bio UV-Visible Mulgrave, Australia). DNA homology was determined spectrophotometrically by using the modified [25] method of De Ley et al. [18]. Ribotyping was done at DSMZ (Braunschweig, Germany) using an automatic ribotyping system.

## 16S rDNA sequencing

The almost complete 16S rDNA of selected strains was amplified by PCR using the primers 16Sseqfw (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16Sseqrev (5′-GGN TAC CTT GTT ACG ACT TC-3′) corresponding to positions 8–27 and 1511–1491 of the corresponding 16S rDNA gene of *Escherichia coli* [10], respectively. DNA was amplified in 32 cycles (94 °C, 1 min; 56 °C, 1 min; 72 °C, 2 min) in a 50 µl reaction volume containing *Taq* DNA polymerase (Amersham Pharmacia), polymerase buffer (Amersham Pharmacia), primers and dNTPs at the same concentrations as described for rep-PCR. The PCR products were cleaned using quantum prep-PCR clean columns (Biorad, München, Germany) and sequenced bi-directionally at GATC Biotech (GATC, Konstanz, Germany). The partial 16S sequences were submitted to GenBank and given the accession nos. AY929279, AY929280, AY929281, AY929282 and AY929283 for *Lactobacillus fermentum* strains BFE 6618, BFE 6620, BFE 6628, BFE 6658, BFE 6625, AY929284 for *Lactobacillus brevis* BFE 6673, AY929285, AY929288, AY929286 and AY929287 for *Leuconostoc fallax* strains BFE 6643, BFE 6696, BFE 6749, BFE 6750 and AY929289 for *Leuconostoc pseudomesenteroides* BFE 6644.

## Bacteriocin activity

Bacteriocin activity was detected by the deferred inhibition assay [1] with LAB strains from fermented cassava as the producing organisms and *Weissella paramesenteroides* DSM 20288, *Lactobacillus sake* DSM 20017, *Lactobacillus buchneri* LMG 11439 and *L. fermentum* DSM 20052 as indicator bacteria. Indicator bacteria were inoculated (1%) into soft (0.75%) MRS agar, which was used to overlayer MRS agar plates [20].

## Production of hydrogen peroxide

The production of hydrogen peroxide was tested according to the method of Marshall [29] using MRS agar containing 0.5 mM of 2-2′-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma,

Deisenhofen, Germany) and 3 mg/l horseradish peroxidase (Sigma).

### Utilisation of non-digestible α-galactoside sugars

The fermentation of the sugars raffinose and stachyose was tested according to the method of Jayne-Williams [26].

### Production of β-glucosidase

β-Glucosidase was tested according to the method of Weagant et al. [51] using 4-nitrophenol-β-D-glucopyranoside as substrate.

### Production of α-amylase and tannase

To test for α-amylase production, a single streak of a test culture was made on modified MRS agar plates that contained 0.2% soluble starch instead of glucose. The plates were incubated at 30 °C overnight, after which they were flooded with iodine. A colourless area around the growth indicated a positive test. *Lactobacillus amylovorus* strain DSM 20531 was used as a positive control. Production of tannase was tested on Brain Heart Infusion agar (Oxoid) according to the method of Osawa [38].

### Acid production

The test strains were inoculated (1% of an overnight culture) into MRS broth adjusted to pH 6.5 before autoclaving (pH 6.2 after autoclaving) and grown aerobically at 30 °C. Acid production was determined by measuring the pH of the culture after 6, 24 and 48 h. MRS broth medium for all acid production tests was prepared from a single batch which was pH adjusted and then dispensed into tubes of 10 ml each before autoclaving.

## Results

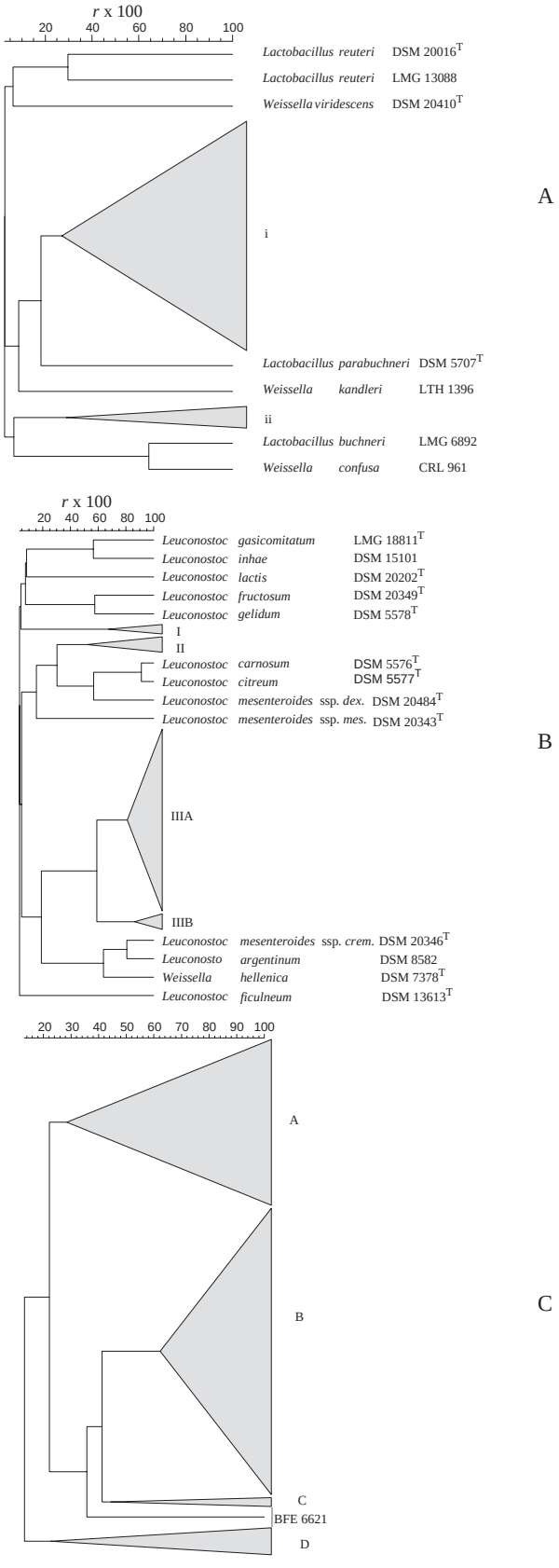
### LAB counts in fermented cassava

Numbers of bacteria from the five fermented cassava samples ranged from $2.4 \times 10^8$ to $1.8 \times 10^9$ CFU/g on MRS agar, from $3.4 \times 10^8$ to $2.8 \times 10^9$ CFU/g on Rogosa agar, from $1.0 \times 10^6$ to $5.5 \times 10^8$ CFU/g on Kanamycin Esculin Azide agar and from $7.4 \times 10^7$ to $6.1 \times 10^8$ CFU/g on M17 agar.

### Obligately heterofermentative rods

Twenty-seven strains exhibited rod-shaped morphology, produced gas from glucose fermentation and DL-lactate and thus were classified as obligately heterofermentative rods belonging either to the genus *Lactobacillus* or *Weissella* [7,22]. Of these 59.3% were isolated from MRS, 37% from Rogosa and 3.7% from M17 agar media. These 27 strains were further characterised using rep-PCR and two reference strains of *L. fermentum* (LMG 8900 and the type strain DSM 20052$^T$), two *L. reuteri* strains (the type strain DSM 20016$^T$ and LMG 13088) as well as various other heterofermentative strains from culture collections were used as reference strains. The two *L. fermentum* reference strains were grouped together in the analysis and moreover showed a high correlation value ($r = 86.7\%$) while the two *L. reuteri* reference strains also grouped together in the rep-PCR (Fig. 1). All obligately heterofermentative, rod-shaped strains from fermented cassava, except BFE 6638 and BFE 6673, grouped together with the reference strains of *L. fermentum* in group i (Fig. 1). The strains BFE 6638 and BFE 6673 grouped together with the type strain of *L. brevis* (DSM 20054) in group ii. Thus, the majority of the heterofermentative, rod-shaped strains isolated from fermented cassava could be characterised as *L. fermentum*, while *L. brevis* also occurred among the predominant isolated strains, albeit at a low incidence. To confirm the rep-PCR characterisation, the 16S rDNA of one of the strains (BFE 6673) grouping together with the *L. brevis* DSM 20054 type strain, and five representative strains (BFE 6618, BFE 6620, BFE 6628, BFE 6625 and BFE 6658) from the *L. fermentum* cluster (group i) was amplified and sequenced. 16S rDNA sequencing showed high similarity of all strains from the *L. fermentum* cluster to the *L. fermentum* type strain DSM 20052 (strains BFE 6618, BFE 6620, BFE 6628, and BFE 6658 exhibiting 99.5% and strain BFE 6625 showing 99.2% similarity to the type strain, respectively). The 16S sequence of the representative isolate from the *L. brevis* cluster (BFE 6673) showed a similarity to that of the *L. brevis* type strain DSM 20054 of 99.6%.

The sugar fermentation pattern of the strains identified as *L. fermentum* in this study compared well to that as described previously [22] for this species. The majority (>90%) of these strains fermented glucose, gluconate, maltose, melibiose, raffinose and sucrose, while less than 10% of strains fermented esculin, cellobiose, glycerol, mannitol, melizitose and rhamnose. *L. fermentum* strains have previously been described as positive (>90% of strains) for maltose, melibiose, raffinose, ribose and sucrose, and negative (<10% of strains positive) for esculin and melizitose [22].




RAPD-PCR was used to determine possible clonal relationships among the *L. fermentum* strains from fermented cassava. For RAPD-PCR analysis, three reference strains of *L. fermentum* were used (LMG 8900, DSM 20052$^T$ and LMG 11441). The DNA of the *L. brevis* type strain (DSM 20054$^T$) was isolated in duplicate as to determine the reproducibility of RAPD-PCR analyses and running conditions. These fingerprints obtained from the duplicate isolates clustered at $r = 92.2\%$ (Fig. 2). Ten of the isolates from fermented cassava clustered together with the *L. fermentum* reference strains in group I (Table 1, Fig. 2). Some isolates which were characterised as *L. fermentum* with rep-PCR, clustered with the reference strains *L. buchneri* (LMG 6892), *L. reuteri* (DSM 20016 and DSM 20015), *W. confusa* (CRL 961), *W. viridescens* DSM 20410 or *L. brevis* (LMG 11438) in RAPD-PCR strain typing (Fig. 2). The strains BFE 6603, BFE 6620, BFE 6626 and BFE 6628 were all isolated from MRS agar and clustered together closely ($r = 96.1\%$) in the RAPD analysis. Moreover, strains BFE 6626 and BFE 6628 were isolated from the same fermentation batch (Table 1), indicating a possible clonal relationship especially for these latter two strains. Also, the strains BFE 6658, BFE 6682, BFE 6604 and BFE 6637 clustered closely ($r = 94.0\%$). These strains stemmed from either fermentation batch B (BFE 6604 and BFE 6658) or batch E (BFE 6637 and BFE 6682) (Table 1) and thus could be clonally related, even though these strain pairs from the same batch were isolated from different media (MRS and Rogosa agar, Table 1). However, to confirm such possible clonal relationships a second strain typing method would need to be performed. The occurrence of different clusters of *L. fermentum* strains with different fingerprint patterns in the RAPD-PCR analysis suggests that *L. fermentum* is a genetically quite heterogeneous species.

## Heterofermentative cocci

Thirty-four heterofermentative strains showing coccoid morphology all produced D-lactate and gas from glucose metabolism, indicating that they belong either to the genus *Leuconostoc* or *Weissella*. Of these, 41.2%

**Fig. 1.** Dendrogram obtained by UPGMA of correlation value *r* of rep-PCR fingerprint patterns of heterofermentative, rod-shaped *Lactobacillus* and *Weissella* isolates (A), heterofermentative, coccoid *Leuconostoc* and *Weissella* isolates (B) and facultatively heterofermentative *Lactobacillus* isolates (C) from fermented cassava and reference strains obtained with primer GTG5. Cluster i contained the *L. fermentum* DSM 20052T and LMG 8900 reference strains, cluster ii contained the *L. brevis* DSM 20054 type strain (A), clusters I contained the *W. paramesenteroides* DSM 20288 type strain, cluster II the *L. pseudomesenteroides* DSM 20193 type strain (B).

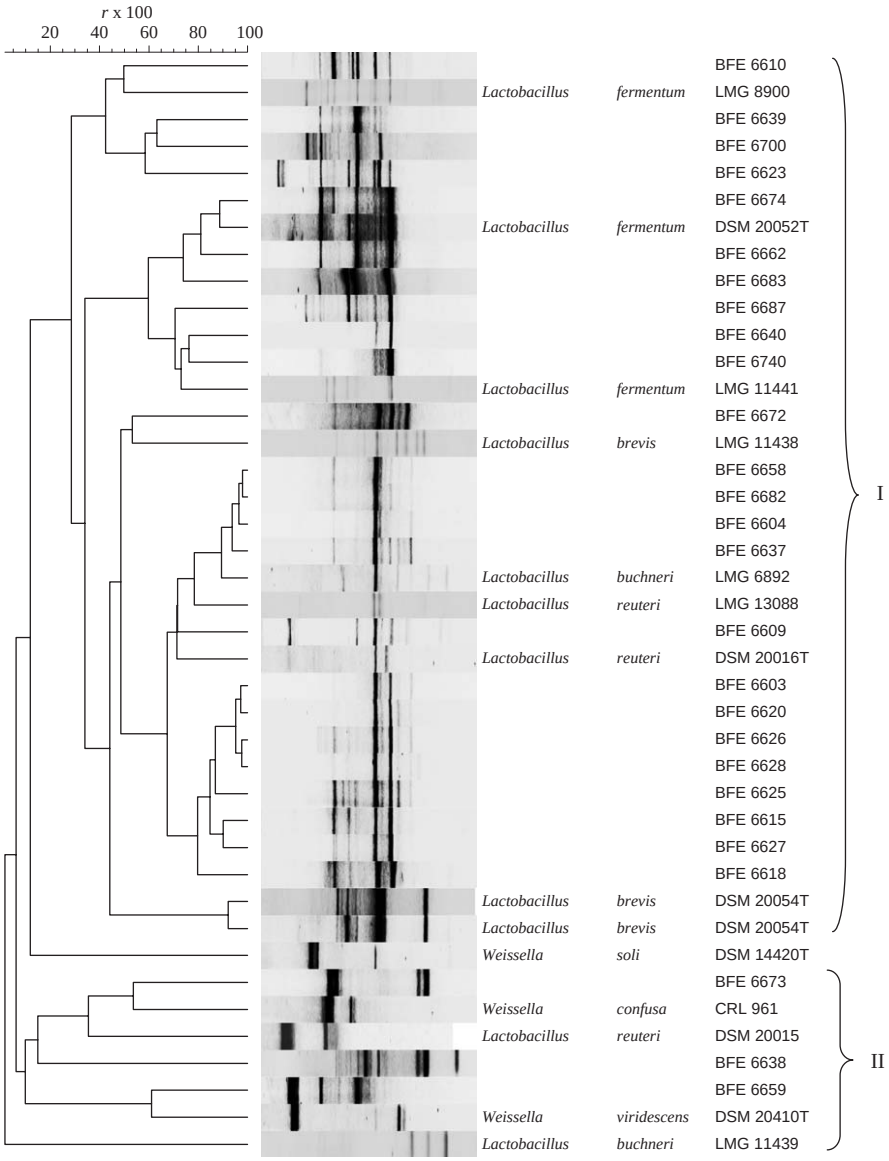



**Fig. 2.** Dendrogram obtained by UPGMA of correlation value *r* of RAPD-PCR fingerprint patterns of rod-shaped heterofermentative *Lactobacillus* and *Weissella* isolates from fermented cassava and reference strains obtained with primer M13.

were isolated from MRS, 29.4% from Rogosa, 8.8% from Kanamycin Esculin Azide and 20.6% from M17 agar media. Characterisation by rep-PCR showed that most of these cocci grouped together with the *L. fallax* DSM 20189 type strain at a correlation value of $r = 83.7\%$ in subgroup IIIA, while two of the cassava strains (BFE 6750 and BFE 6749) clustered in subgroup IIIB at $r = 86.3\%$ together with the *L. fallax* reference strain DSM 10615 (Fig. 1). All *L. fallax* strains from fermented cassava and the two reference strains DSM $20189^T$ and DSM 10615 grouped together at $r = 81.0\%$ in group III. From this *L. fallax* group, some strains were selected for 16S rDNA sequencing (BFE 6643, BFE 6749, BFE 6750 and BFE 6696) and DNA–DNA hybridisation (BFE 6647, BFE 6743 and BFE 6696) to confirm the rep-PCR characterisation. 16S rDNA sequencing showed high similarity of all strains from fermented cassava to the 16S rDNA gene of the type strain *L. fallax* DSM 20189 (BFE 6643, 99.4%; BFE 6749, 99.4%; BFE 6750, 99.4%; and BFE 6696, 99.7% similarity to the type strain, respectively). DNA–DNA hybridisation experiments also showed that the strains had high similarity to the *L. fallax* type strain DSM 20189. The strains BFE 6647, BFE 6743 and BFE 6696 showed reassociation levels of 91%, 88% and 89% with strain DSM 20189, respectively, confirming the identification of these isolated strains as *L. fallax*. The two *L. fallax* reference strains (DSM $20189^T$ and DSM 10615) which grouped into two subclusters (Fig. 1) had a DNA–DNA reassociation value of 81%. Three of the

heterofermentative cocci isolates (BFE 6649, BFE 6693 and BFE 6644) were not grouped together with *L. fallax* in the rep-PCR typing experiment, but grouped in group I (BFE 6649) or group II (BFE 6693 and BFE 6644), respectively (Fig. 1, Table 1).

The sugar fermentation pattern of the strains identified as *L. fallax* in this study compared to that described previously [7] for this species. All strains fermented glucose, while 16–87% of these strains fermented esculin, arabinose, cellobiose, maltose, mannitol, raffinose, rhamnose, ribose, sucrose, salicin and trehalose. Less than 10% of strains fermented galactose, gluconate, glycerol, lactose, melizitose, melibiose, sorbitol and xylose.

Based on rep-PCR, the strain BFE 6649 was characterised as *W. paramesenteroides* as it clustered together with the *W. paramesenteroides* DSM 20288 type strain in group I (Fig. 1). The strains BFE 6693 and BFE 6644 grouped with the *L. pseudomesenteroides* type strain DSM 20193$^T$ in group II. This characterisation was confirmed with ribotyping, in which the strain BFE 6693 clustered together with the *L. pseudomesenteroides* type strain (results not shown) and 16S rDNA sequencing, in which the 16S rDNA sequence of strain BFE 6644 showed 99.8% homology to that of *L. pseudomesenteroides* DSM 20193$^T$. The results of the RAPD-PCR analysis of the heterofermentative cocci from fermented cassava indicated that the majority of the cocci (except the four strains BFE 6644, BFE 6607, BFE 6693 and BFE 6649) were closely related and occurred in one cluster (Table 1).

## Obligately homo- and facultatively heterofermentative rods

The majority (78 strains) of the strains isolated from fermented cassava were rod-shaped, produced either D-, L- or DL-lactate and did not produce gas ($CO_2$) from glucose fermentation. Two strains (BFE 6634 and BFE 6699) were obligately homofermentative as they did not ferment the 5-carbon sugars tested, i.e., xylose, ribose or arabinose. Seventy-six strains were facultatively heterofermentative, as they fermented at least one of the 5-carbon sugars mentioned above. Of these, 23.7% were isolated from MRS, 35.5% from Rogosa, 22.4% from Kanamycin Esculin Azide and 18.4% from M17 agar media. These 76 strains produced DL-lactate and possessed mDAP in the cell wall, and were thus presumptively characterised as belonging to the *L. plantarum* group. By using rep-PCR analysis it was possible to distinguish between the strains characterised as *L. plantarum* (also characterised as *L. plantarum* by phenotypic tests and DNA–DNA hybridisation, see below) and the *L. arizonensis* DSM 13273 type strain, which occurred in groups A and B, and the *L. pentosus* and *L. paraplantarum* strains which clustered in groups C and D, respectively (Fig. 1, Table 1). Three different reference strains of *L. pentosus* were used (LTH 2067, LTH 2792 and the type strain DSM 20314$^T$). The rep-PCR fingerprinting technique was unable to distinguish between the *L. plantarum* and *L. arizonensis* species, as the type strains of both species grouped closely at a high correlation value ($r = 81.2\%$) in the same cluster (cluster B, Fig. 1). Two well-delineated genomic subgroups were detected within the *L. plantarum*/*L. arizonensis* group when using rep-PCR characterisation. Cluster A contained nearly half of the strains from fermented cassava, while the remainder and the type strains of *L. plantarum* and *L. arizonensis* grouped into cluster B (Fig. 1, Table 1). Only four of the fermented cassava strains (BFE 6624, BFE 6724, BFE 6735 and BFE 6621) were not included in these clusters. The strains BFE 6624 and BFE 6724 clustered together with *L. pentosus* in group D and may thus be considered as presumptive *L. pentosus* strains. Strain BFE 6735 grouped together with the *L. paraplantarum* type strain DSM 10667$^T$ in group C and thus could be characterised as a presumptive *L. paraplantarum* strain. The strain BFE 6621 grouped between the *L. plantarum* and *L. pentosus* clusters (Fig. 1); for this reason, the 16S rDNA gene was sequenced and this strain was identified as *L. pentosus*.

To confirm the characterisation of strains from the two *L. plantarum*/*L. arizonensis* genomic subgroups (A and B), DNA–DNA hybridisation was performed for representative strains from both subgroups. DNA from two strains of genomic subgroup B (BFE 6654 and BFE 6688) were hybridised to DNA from the *L. plantarum* type strain DSM 20174$^T$ and showed a reassociation value of 94.5% and 75.8% similarity, respectively. DNA from two strains of genomic subgroup A (BFE 6742 and BFE 6739) was hybridised to DNA from strains of group B (BFE 6654 and BFE 6688) and showed levels of 77.5% (BFE 6739 vs. BFE 6654) or 98.8% (BFE 6742 vs. BFE 6688) reassociation, respectively. This demonstrated that the representative strains from genomic subgroups A and B all could be identified as *L. plantarum*. To investigate the relatedness of the two type strains *L. plantarum* DSM 200174$^T$ and *L. arizonensis* DSM 13272$^T$, DNA–DNA hybridisation was also done between the DNA of these two strains and showed a reassociation value of 100.7%. This result was confirmed in a duplicate experiment, in which the reassociation value was 89.4%, using a different *L. arizonensis* type strain DNA extraction and furthermore the *L. arizonensis* type strain was obtained from a different institute. Thus, we could not distinguish between the *L. plantarum* and *L. arizonensis* type strains using either rep-PCR or DNA–DNA hybridisation.

The sugar fermentation patterns of the strains identified as *L. plantarum*/*L. arizonensis* in this study compared well to those described previously for these

species [22,48]. The majority (>90%) of these strains fermented glucose, esculin, cellobiose, galactose, lactose, maltose, mannitol, melibiose, ribose, sucrose, salicin and trehalose, while less than 10% of strains fermented glycerol, rhamnose and xylose.

Fig. 3 shows the results of the typing of homofermentative and facultatively heterofermentative rods by RAPD-PCR. This genotypic method again appeared to be too sensitive for characterisation to the species level, because some strains characterised as *L. plantarum* by rep-PCR (e.g., BFE 6703 and BFE 6665), grouped together with the *L. paraplantarum* and *L. pentosus* reference strains in groups c and d, respectively (Fig. 3, Table 1). Nevertheless, the technique was useful to show that the *L. plantarum* strains appeared to be genetically very heterogeneous (Fig. 3) and was useful also for investigating potential clonal relationships. Thus, strains BFE 6725, BFE 6718, BFE 6710, BFE 6716, BFE 6629, BFE 6666, BFE 6737, BFE 6744, BFE 6742, BFE 6741, BFE 6738, BFE 6739, BFE 6734, BFE 6606, BFE 6635, BFE 6717 may possibly be clonally related. Such a presumptive clonal relationship should, however, be confirmed using a second strain typing technique. Furthermore, the strains BFE 6632, BFE 6636, BFE 6655, BFE 6646, BFE 6663 and BFE 6735, the strains BFE 6701 and BFE 6733 and the strains BFE 6671, BFE 6708 and BFE 6664 may also be clonally related. Interestingly, all the above strains that showed a possible clonal relationship were isolated from different agar media and fermentation batches. This may be explained by the fact that they were possibly environmental strains, which persisted in the small Gari production facility where all the batches were prepared.

The species-specific *recA* gene-multiplex PCR method of Torriani et al. [49] was used to investigate its usefulness for distinguishing among the different species of the *L. plantarum* group. In the RAPD-PCR, a group (13 strains of presumptive clonal relationship with very similar RAPD-PCR fingerprints) was detected, which probably consisted of multiple isolates of the same strain. In RAPD-PCR (Fig. 3), these strains (BFE 6725, BFE 6718, BFE 6710, BFE 6716, BFE 6629, BFE 6666, BFE 6737, BFE 6744, BFE 6742, BFE 6741, BFE 6738, BFE 6739, BFE 6734) clustered at $r = 95.4\%$. The RAPD-PCR results of the multiple isolates were compared with the results of the species-specific *recA* gene-multiplex PCR for distinguishing the species *L. plantarum*, *L. pentosus* and *L. paraplantarum*. DNA from five strains gave no product with the *recA* gene-derived primers, DNA from three strains reacted with the *L. plantarum* primers, DNA from four strains reacted with the *L. paraplantarum* primers and DNA from one strain reacted with the *L. pentosus* primers. Furthermore, comparison of the *recA* results with the groupings obtained with rep-PCR, 16S rDNA sequencing and DNA–DNA hybridisation showed clearly that species-specific *recA* gene-multiplex PCR in our study led to incorrect identification of numerous strains.

### Technological properties of predominant LAB strains from fermented cassava

None of the predominant LAB strains isolated from fermented cassava showed α-amylase activity. The results obtained for technological properties were compared between strains of the three major groups of bacteria isolated in this study, i.e., heterofermentative cocci (majority characterised as *L. fallax*), obligately heterofermentative rods (majority characterised as *L. fermentum*) and obligately homo- and facultatively heterofermentative rods (majority characterised as *L. plantarum*). Production of tannase was a rare trait exhibited by only 6.4% of strains belonging to the obligately homo- and facultatively heterofermentative rod group (Fig. 4), i.e., mainly strains characterised as *L. plantarum*. The incidence of β-glucosidase activity was high among the obligately homo- and facultatively heterofermentative rods (98.7%; Fig. 4), while it was low among the heterofermentative rods and cocci at approximately 11%. The incidence of $H_2O_2$ production was quite high for the obligately heterofermentative strains, at 79.4% for the cocci and 63% for the rods, but considerably lower for the obligately homo- and facultatively heterofermentative rods (7.7%) (Fig. 4). Bacteriocin production (indicated by a zone of clearing of more than 1 mm against at least one of the indicator bacteria tested) also occurred at a higher incidence for the heterofermentative cocci (ca. 12%) and rods (63%), while only few strains (3.9%) of the obligately homo- and facultatively heterofermentative rods produced bacteriocin (Fig. 4). The obligately heterofermentative rods and the obligately homo- and facultatively heterofermentative rods showed a high incidence for raffinose utilisation, while stachyose was utilised by a low proportion of strains in all three LAB groups in this study (Fig. 4). Clearly, the obligately heterofermentative rods and cocci were not as good acid producers as strains from the obligately homo- and facultatively heterofermentative group (mostly *L. plantarum*), while a large percentage (>80%) of the obligately homo- and facultatively heterofermentative strains were capable of lowering the pH of the medium to pH 5.3 or 3.9 after 6 and 48 h of growth, respectively (Fig. 4).

## Discussion

Using a polyphasic taxonomy approach, the diversity of predominant LAB occurring in fermented cassava for production of Gari could be accurately described. The most predominant LAB (56.1% of all strains) associated

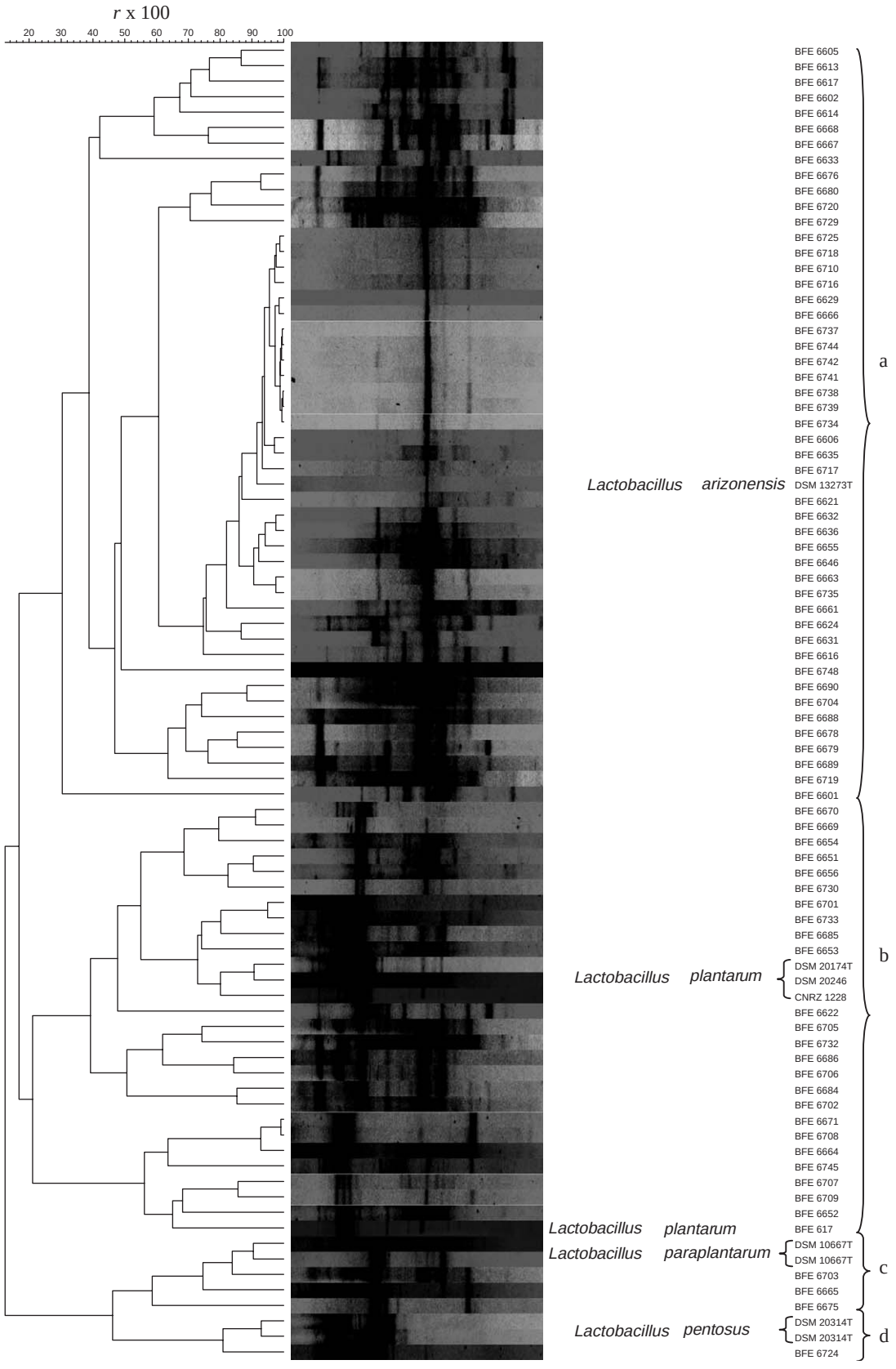



**Fig. 3.** Dendrogram obtained by UPGMA of correlation value *r* of RAPD-PCR fingerprint patterns of facultatively heterofermentative *Lactobacillus* isolates from fermented cassava and reference strains obtained with primer M13.

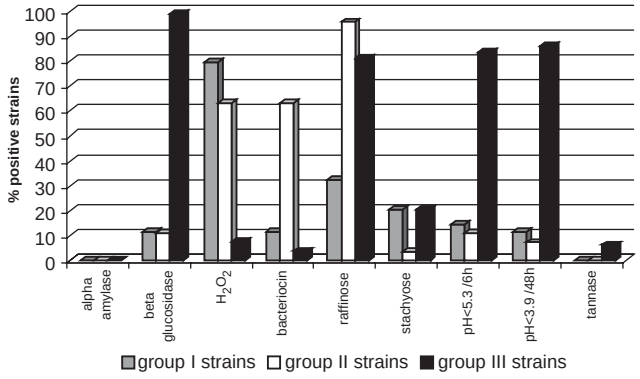



**Fig. 4.** Percentage of strains isolated from fermented cassava belonging to the obligately heterofermentative cocci group which mainly consisted of *L. fallax* strains (group I), the obligately heterofermentative group which mainly consisted of *L. fermentum* strains (group II) and the facultatively heterofermentative/obligately homofermentative rods group (group III) which mainly consisted of *L. plantarum* strains, which were positive for technologically important traits for Gari production.

with the fermentation were obligately homo- or facultatively heterofermentative rods, which mostly consisted of *L. plantarum*. These could be isolated from all four media used in the study. The second and third most predominant LAB groups were the heterofermentative cocci (24.5%) and obligately heterofermentative rods (19.4%), which largely constituted *L. fallax* and *L. fermentum* strains, respectively. Other heterofermentative species such as *L. brevis*, *L. pseudomesenteroides* and *W. paramesenteroides* also occurred among the predominant isolates, although at a low incidence. The heterofermentative cocci and rods were mostly isolated from MRS and Rogosa media, but less frequently from Kanamycin Esculin Azide and M17 agar. Speculatively, reasons for this may be that these bacteria are more susceptible to the kanamycin and azide in the Kanamycin Esculin Azide agar, when compared to the *L. plantarum* strains. M17 medium on the other hand, contains lactose, a carbohydrate source which may not be utilised by *L. fallax* and *L. fermentum* strains as readily as by the *L. plantarum* strains. Our data on sugar fermentation patterns indeed confirmed this (data not shown).

Our results clearly showed that neither RAPD-PCR nor rep-PCR alone would have sufficed as a technique for identifying these bacteria from fermented cassava. Gevers et al. [21] showed that rep-PCR, especially with primer GTG5, is a powerful technique for accurate *Lactobacillus* speciation. Although we also found that rep-PCR with this primer had a good discriminatory power to group both *Leuconostoc* and *Lactobacillus* species, there were some misidentifications, which were only resolved once the 16S rDNA of the questionably identified strain was sequenced. Thus, we found that only a polyphasic taxonomic approach, based on phenotypic methods and genotyping fingerprinting techniques, as well as sequencing of 16S rDNA genes and DNA–DNA hybridisations of representative strains, could lead to accurate species determination.

Other studies on fermented cassava products from Africa or Columbia have reported the involvement of various *Lactobacillus* species (e.g., *L. plantarum*, *L. fermentum*, *L. delbrueckii* and *L. manihotivorans*) as well as other Gram-positive and Gram-negative bacteria and yeasts and moulds [3,6,9,30–32,34,35] in the fermentation. Amoa-Awua et al. [2] reported that among LAB from agbelima, a fermented cassava product from West Africa, *L. plantarum* strains constituted 51% of the isolates, while *L. mesenteroides* and *L. brevis* strains were isolated at frequencies of 15% and 16%, respectively. The common occurrence of *L. plantarum* in fermenting cassava has also been reported earlier by Oyewole and Odunfa [39], who studied the succession of LAB species and observed that *L. plantarum* predominated after 3 days of fermentation.

While *L. plantarum*, *L. fermentum* and *Leuconostoc* spp. could also be isolated and identified as the dominant LAB in fermenting cassava in our study, the association of some of the LAB (i.e., *Pediococcus* spp., *L. manihotivorans*) reported in previous studies could not be confirmed. This is probably a result of differences in geographic regions, type of raw material, i.e., cassava varieties, products and production technologies practiced in the different studies. The involvement of *L. fallax* in cassava fermentation for the production of Gari, however, has not previously been reported, but this may be a result of the fact that this species is difficult to distinguish from *L. mesenteroides* by phenotypic and biochemical characteristics [5]. Recently, *L. fallax* has been shown to be involved in Sauerkraut fermentations and to occur in fermented rice cakes, although traditional knowledge indicated that *L. mesenteroides* was the only *Leuconostoc* associated with these products. Barrangou et al. [5] postulated that the involvement of *L. fallax* in these fermentations only became apparent, once relevant molecular biological tools for the correct identification of this species, and consequently the reliable methodology to distinguish this species from *L. mesenteroides*, were developed.

According to Figueroa et al. [19], two different LAB groups, which occur in succession, are involved in cassava fermentation for Gari production. The obligately heterofermentative strains, mainly *Leuconostoc* spp., initiate the fermentation and these are replaced by the facultatively heterofermentative lactobacilli, mainly *L. plantarum*. The high incidences of both *L. plantarum* and *L. fallax* strains in our study confirm this. Also, our finding that *L. plantarum* occurred at a much higher incidence when compared to the other predominant

LAB strains (i.e., *L. fallax* and *L. fermentum* strains) correlates well to the fact that samples were taken at the end of the fermentation when *L. plantarum* should predominate. *L. plantarum* is known to be more acid resistant than *Leuconostoc* spp. or many other *Lactobacillus* spp., which is one of the reasons why strains of *L. plantarum* often predominate in the late stages of vegetable fermentations [19,46]. This was also reflected in our results, showing that the majority of the *L. plantarum* strains were characterised by a faster and higher acid production (resulting in a faster and stronger reduction in pH of the growth medium) when compared to *Leuconostoc* spp. or heterofermentative lactobacilli.

*Lactobacillus* strains belonging to the *L. plantarum* group (i.e., *L. plantarum*, *L. paraplantarum*, *L. pentosus* and *L. arizonensis*) are very difficult to distinguish both by phenotypic and genotypic methods. They share certain key phenotypic characteristics as they are all Gram-positive, catalase-negative, rod-shaped, produce DL-lactate and possess mDAP in the cell wall. They are facultatively heterofermentative, do not produce gas from glucose fermentation and are able to ferment five carbon sugars such as ribose, arabinose or xylose. Xylose and glycerol fermentation are key phenotypic criteria to distinguish between *L. plantarum* and *L. pentosus* as *L. pentosus* strains generally produce acid from xylose whereas *L. plantarum* and *L. paraplantarum* strains do not. In our study, we found that *L. plantarum* strains generally could utilise ribose, some utilised arabinose, but xylose was generally not fermented. In contrast, the few *L. pentosus* strains characterised in our study generally fermented ribose, arabinose and xylose (results not shown).

Members of the *L. plantarum* group are also difficult to distinguish with molecular biological methods since, for example, they share high similarity in the 16S rDNA sequence. Furthermore, strains of *L. plantarum* are genetically quite heterogeneous [17] as is also shown in our study with RAPD-PCR typing. Nevertheless, various typing methods such as RAPD-PCR and AFLP have been used successfully to discriminate between *L. plantarum*, *L. paraplantarum* and *L. pentosus* [6]. DNA–DNA similarity studies can also effectively distinguish between the species [16,47,48,52]. However, DNA–DNA hybridisation is tedious and costly and was therefore done for only a few selected strains in our study in order to confirm their identity. Using DNA–DNA hybridisation, we found a high similarity (89.4% and 100.7%) between the *L. arizonensis* and *L. plantarum* type strains. This result was supported also by rep-PCR, in which *L. arizonensis* grouped together with *L. plantarum* in one cluster (Fig. 4). Thus, the identification of *L. arizonensis* is doubtful and further investigations should be done to determine whether *L. arizonensis* is a junior synonym of *L. plantarum*.

Species-specific *recA* gene-multiplex PCR was also done to distinguish between *L. plantarum*, *L. paraplantarum* and *L. pentosus*. This technique was previously reported to be successful for discriminating between these species [49]. However, the primers for the species-specific *recA* gene amplification were constructed on the basis of *recA* gene sequences determined for only a few reference strains (up to three) of each of the species *L. plantarum*, *L. pentosus* and *L. paraplantarum*, one of which was the type strain [49]. Thus, failure of species-specific *recA* gene amplification in our study may have resulted, because many of the field strains may share lower similarity in their *recA* gene sequences.

Some technologically relevant properties were investigated for strains in this study in order to select strains for possible use as starter cultures. The use of starter cultures for fermented cassava products was suggested to optimise flavour and texture, as well as for reasons of adequate detoxification and safety assurance [33]. Therefore, $\beta$-glucosidase activity was investigated in order to select strains which can aid in the degradation of cyanogenic glucosides. Furthermore, rapid acid production and production of antimicrobial compounds such as bacteriocin or $H_2O_2$ were also considered as useful technological traits. Generally, *L. plantarum* strains produced acid more rapidly when compared to strains belonging to the obligately heterofermentative rods or cocci (mostly *L. fermentum* and *L. fallax*, respectively). For this reason, *L. plantarum* strains should be selected as part of a starter preparation. However, the pH-reducing characteristics would need to be tested in model cassava fermentations to confirm the extent of pH-reducing ability in the actual product. Suitable *L. fallax* and *L. fermentum* strains should also be chosen, partly because the *Leuconostoc* strains and the heterofermentative rods would presumably start off the fermentation and create the right environment for *L. plantarum* growth, as is the case in many vegetable and cereal fermentations [33,47]. In addition, the trait of $H_2O_2$ production occurred at a high incidence among *Leuconostoc* strains, while bacteriocin activity was detected at a high frequency among the heterofermentative rods. Selection of starter strains with such suitable characteristics may therefore lead to improvement of the hygiene of the product. Suitable starter strains are currently being selected and their technological properties investigated in more detail for the development of a starter culture preparation that is to be produced at an industrial scale.

## Acknowledgements

Financial support from the EU Commission within the framework of the INCO RTD programme is gratefully acknowledged. This study was carried out

under the project "Improving the quality and nutritional status of GARI through the use of starter cultures and fortification with soybean, palm oil and coconut milk" (ICA4-CT-2002-10058). The work does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area. We also thank DSMZ for ribotyping of selected strains.

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