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Characterisation and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures

M. Kostinek [a], I. Specht [a], V.A. Edward [b], C. Pinto [b], M. Egounlety [c], C. Sossa [c], S. Mbugua [d], C. Dortu [e], P. Thonart [e], L. Taljaard [b], M. Mengu [f], C.M.A.P. Franz [a,*], W.H. Holzapfel [a]

[a] *Federal Research Centre for Nutrition and Food, Institute of Hygiene and Toxicology, Haid-und Neu-Strasse 9, D-76131 Karlsruhe, Germany*
[b] *CSIR Council for Scientific and Industrial Research, CSIR BioSciences, Meiring Naude Road, Brummeria, 0184 Pretoria, South Africa*
[c] *Universite Nationale de Benin, Abomey-Calavi, 01 Cotonou, Benin*
[d] *University of Nairobi, University Way, Ghandi Building, 2, Nairobi, Kenya*
[e] *Universite de Liege, Place du 20 Aout 7, 4000 Liege, Belgium*
[f] *Botswana Technology Centre, Private Bag 0082, Machel Drive 50654 Plot, Gaborone, Botswana*

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Abstract

A total of 375 lactic acid bacteria were isolated from fermenting cassava in South Africa, Benin, Kenya and Germany, and were characterised by phenotypic and genotypic tests. These could be divided into five main groups comprising strains of facultatively heterofermentative rods, obligately heterofermentative rods, heterofermentative cocci, homofermentative cocci and obligately homofermentative rods, in decreasing order of predominance. Most of the facultatively heterofermentative rods were identified by phenotypic tests as presumptive *Lactobacillus plantarum*-group strains, which also comprised the most predominant bacteria (54.4% of strains) isolated in the study. The next predominant group of lactic acid bacteria (14.1% of total isolates) consisted of obligately heterofermentative rods belonging either to the genus *Lactobacillus* or *Weissella*, followed by the heterofermentative cocci (13.9% of isolates) belonging to the genera *Weissella* or *Leuconostoc*. Homofermentative cocci were also isolated (13.3% of isolates). Biochemical properties such as production of α-amylase, β-glucosidase, tannase, antimicrobials (presumptive bacteriocin and H_2O_2-production), acidification and fermentation of the indigestible sugars raffinose and stachyose, were evaluated *in vitro* for selection of potential starter strains. A total of 32 strains with one or more desirable biochemical properties were pre-selected and identified using rep-PCR fingerprinting in combination with 16S rRNA sequencing of representative rep-PCR cluster isolates. Of these strains, 18 were identified as *L. plantarum*, four as *Lactobacillus pentosus*, two each as *Leuconostoc fallax*, *Weissella paramesenteroides* and *Lactobacillus fermentum*, one each as *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Weissella cibaria*, while two remained unidentified but could be assigned to the *L. plantarum*-group. These strains were further investigated for clonal relationships, using RAPD-PCR with three primers, and of the 32 a total of 16 strains were finally selected for the development as starter cultures for Gari production.

Keywords: Fermentation; Cassava; Gari; Lactic acid bacteria; β-glucosidase

1. Introduction

Cassava or manioc (*Manihot esculenta*), is a 1–2 m high shrub which, due to its high yields of starchy roots on marginal land, is a major staple crop for over 500 million people in the developing world (Cock, 1982, 1985). Cassava ranks fourth on the list of major food crops in developing countries after rice, wheat and maize (Mingli et al., 1992). Cassava grows in poor and acidic soils, which are often not suitable for other crops, and yields a harvest in times of drought when all other crops have failed for lack of water (Mingli, 1995). Despite these advantages, cassava has four major drawbacks which limit its utilisation as a food (Kimaryo et al., 2000). These are low energy density, low protein content, rapid postharvest deterioration and potential cyanide toxicity (Howlett et al., 1990; Mingli et al., 1991; Oyewole and Aibor, 1992; Mingli, 1995; Gidamis, 1988).

* Corresponding author. Tel.: +49 721 6625 225; fax: +49 721 6625 453.
E-mail address: Charles.Franz@bfel.de (C.M.A.P. Franz).

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In cassava, cyanide occurs as cyanogenic glucosides, mostly linamarin (>80%) and to a lesser extent lotaustralin (Kimaryo et al., 2000; Cereda and Mattos, 1996). The cyanogenic glucosides are present in all parts of the plant, with possible exception of the seeds (Vasconcelos et al., 1990). Bitter varieties, which contain higher amounts of cyanogenic glucosides, have to be processed to remove the toxic compounds before consumption, whereas sweet varieties, which have low levels of cyanogenic glucosides, can be eaten fresh (Rosling, 1990). Despite this, populations which use cassava as main staple food, mainly grow the bitter varieties due to their higher yields (Mozambique Ministry of Health, 1984), their resistance to insects and therefore rely on processing methods for detoxification.

Fermentation not only enhances detoxification, but may also improve the quality and hygienic safety of the food (Ogunsua, 1980). For the production of Gari, the cassava tuber is harvested, peeled and washed, grated and packed into coarsely knit bags. A weight is put on the bag to extract some of the juice. It is then left to undergo natural fermentation for several days at ambient temperatures. The grated cassava, after sieving to remove any coarse lumps and impurities, is heated by means of constant turning over a heated steel pan (Ogunsua, 1980). During this 'garification', the grated cassava is dried to about 10% moisture content and the starch is partially hydrolysed (Akinrele, 1964). At this stage, a small amount of palm oil may be added to give color, and the final dry granular product is called Gari (Moorthy and Mathew, 1998). Cassava fermentation for the production of Gari is associated with the fermentative activities of bacteria and yeasts (Moorthy and Mathew, 1998). The positive effects of these microorganisms are thought to include product preservation, flavour development, cyanide reduction and changes in functional properties (Akindahunsi et al., 1999). Lactic acid bacteria (LAB) were shown to predominate during fermentation (Okafor, 1977; Ngaba and Lee, 1979) and *Lactobacillus* (*L.*) *plantarum* produced the most typical Gari flavour (Ngaba and Lee, 1979).

In this study, the LAB that are responsible for the fermentation of cassava for Gari production were isolated and phenotypically characterised. Furthermore, some relevant biochemical properties were tested, in order to select suitable starter cultures. The selected starter cultures with appropriate biochemical characteristics were also genotypically characterised using rep-PCR, RAPD-PCR and 16S rDNA sequencing.

2. Materials and methods

2.1. Microbiological sampling, strains and culture conditions

LAB strains in this study were obtained from four different sources. A major part of the strains was isolated in Germany and originated from fermenting cassava samples collected in Benin. Furthermore, strains were isolated in South Africa, Benin and Kenya from cassava fermentations, and were sent to Germany. Concerning the strains that were isolated in Germany, five samples of fermented Gari (different fermentations, before the roasting stage after 48 h fermentation) and unfermented manioc were transferred to Germany by airflight under cooled conditions (temperature increased from 4 °C to approx. 10 °C during transportation). For isolation of all LAB both in Germany and Africa, 10 g samples from various stages of the fermentation were added to 90 ml quarter-strength Ringer's solution (QRS, Merck, Darmstadt, Germany) (1:10 dilution) and homogenised for 2 min using a stomacher Blender. The samples were further diluted in a ten-fold dilution series (10^{-2}–10^{-7}). Bacteria from the 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread plated onto four different agar media: MRS agar (Merck), Rogosa agar (Merck), Kanamycin Esculin Azide agar (Merck) and M17 agar (Merck), to obtain the predominant LAB associated with fermenting cassava. Plates were incubated at 30 °C for 48 h under aerobic conditions. One to five colonies were randomly picked from the agar plates of the highest dilutions. 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. Stock cultures of the isolates were stored in MRS broth containing 15% glycerol (Merck) at −80 °C.

For a succession study, 21 presumptive LAB strains were isolated from fermenting cassava in the first half of the fermentation (0–12 h), and 40 presumptive LAB strains in the second half of the fermentation (18–48 h), in order to determine any differences in the composition of the LAB population with progressing fermentation time.

2.2. Phenotypic characterisation

Cell morphology was observed by using phase contrast microscopy at 1000× magnification (Leitz, Jena, Germany) and isolates were Gram-stained and catalase activity was determined. Presumptive LAB were tested for growth at different temperatures (10 °C, 15 °C and 45 °C), and production of gas from glucose in MRS broth as described by Schillinger and Lücke (1987) and Dykes et al. (1994). Determination of the presence of D-*meso*-di-aminopimelic acid in the cell wall (*m*DAP) and the type of lactic acid enantiomer produced, was done using the methods of Schillinger and Lücke (1987). Selected strains were tested for sugar fermentation patterns using the API 50 CHL test kit system (Biomerieux, Nürtingen, Germany) and their range of hydrolytic enzymes produced was tested using the APIZYM test kit (Biomerieux).

2.3. Investigation of biochemical properties of the LAB

Production of α-amylase, tannase, heme-dependent catalase and hydrogen peroxide by LAB strains was determined as previously described by Kostinek et al. (2005a). Presumptive bacteriocin activity was detected by the deferred inhibition assay as described by Ahn and Stiles (1990) with LAB strains from Gari as the producing organisms and *Weissella* (*W.*) *paramesenteroides* DSM 20288, *L. sakei* DSM 20017, *L. plantarum* DSM 20174, *L. buchneri* LMG 11439 and *L. fermentum* DSM 20052 as indicator bacteria. Indicator bacteria were inoculated (1%) into soft MRS agar containing 0.75% agarose, which was used to overlayer MRS agar plates (Franz et al., 1999). Plates were incubated at 30 °C for 18 h and examined for zones of inhibition surrounding the producer colony. The

fermentation of the non-digestible sugars raffinose and stachyose was tested according to the method of Jayne-Williams (1976). Strains were also investigated for acid production in MRS broth. For this purpose, each strain was 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. The pH of the culture was determined after 6, 24 and 48 h. The MRS broth medium used for all test strains was prepared from a single batch which was pH adjusted before autoclaving.

A total of 32 presumptive LAB strains were pre-selected as possible starter cultures on the basis of suitable biochemical properties.

2.4. Genotypic characterisation

The total genomic DNA from the pre-selected starter strains was isolated according to the method of Pitcher et al. (1989), as modified for Gram-positive bacteria according to Björkroth and Korkeala (1996). RAPD and rep-PCR-based typing methods used for presumptive identification of LAB strains were done using primers M13 (5′-GAG GGT GGC GGT TCT-3′) and GTG5 (5′-GTG GTG GTG GTG GTG-3′), respectively, and methods and amplification conditions described previously by Kostinek et al. (2005a). Gel electrophoresis and fingerprint analyses were also conducted as described before (Kostinek et al., 2005a). The digitised images were normalised and 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) (Sneath and Sokal, 1973).

The almost complete 16S rDNA of selected strains was amplified by PCR as described by Kostinek et al. (2005a). The PCR products were cleaned using quantum prep PCR clean spin columns (Biorad, München, Germany) and sequenced at GATC Biotech (Konstanz, Germany).

3. Results

3.1. Phenotypic characterisation

In total, 401 Gram-positive bacteria were isolated from fermented and unfermented cassava mash on MRS, Kanamycin Esculin Azide, Rogosa and M17 agar plates and stained Gram-positive. Four strains failed to grow upon further propagation. Of the remaining 397 strains, 22 were catalase-positive and thus not considered to be presumptive LAB. Thus, a total of 375 presumptive LAB strains were isolated from cassava and were used for further characterisation. 139 of these strains were isolated in Germany, 22 in Kenya and the remaining 214 LAB strains in Benin.

3.1.1. Obligately heterofermentative rods

Fifty-three strains (14.1%) from cassava exhibited rod-shaped morphology and produced gas from glucose. All these strains produced DL-lactate and thus were characterised as obligately heterofermentative rods belonging either to the genus *Lactobacillus* or *Weissella*.

3.1.2. Obligately homofermentative rods

Three strains (0.8%) showed rod-shaped morphology, produced no gas from glucose and did not ferment any of the three pentose sugars (arabinose, ribose and xylose) tested. Therefore, they were characterised as obligately homofermentative lactobacilli. They produced D-lactate, L-lactate or DL-lactate, respectively.

3.1.3. Facultatively heterofermentative rods

217 (57.9%) strains showed rod-shaped morphology, produced no gas from glucose and fermented at least one of the three pentose sugars tested. 204 strains (54.4%) of this group produced DL-lactate and possessed *m*DAP in the cell wall. Therefore, these were considered to belong to the *L. plantarum*-group which consists of the closely related species *L. plantarum* (subsp. *plantarum* and subsp. *argentoratensis*), *L. pentosus* and *L. paraplantarum* (Kostinek et al., 2005a,b). Two (0.5%) of the facultatively heterofermentative strains produced DL-lactate, but did not possess *m*DAP in the cell wall, and eleven (3.0%) of these strains produced L-lactate.

3.1.4. Heterofermentative cocci

Fifty-two strains (13.9%) showed coccoid morphology, produced gas from glucose metabolism and D-lactate, indicating that they belong either to the genus *Leuconostoc* or *Weissella*.

3.1.5. Homofermentative cocci

Fifty coccus-shaped strains (13.3%) produced no gas from glucose and the L-lactate enantiomer, and thus were characterised as belonging either to the genera *Enterococcus*, *Lactococcus* or *Streptococcus*.

3.2. Succession study

In the first 12 h of fermentation, the facultatively heterofermentative rods, which produced DL-lactate (most of them belonging to the *L. plantarum*-group) were the dominant microorganisms constituting 50% of the isolates. These were followed by the facultatively heterofermentative rods, which produced L-lactate, which made up 35% of the isolates. The *Leuconostoc*/*Weissella*-group made up 10% of the isolates, while the entero-, lacto- or streptococci occurred at a frequency of 5%. In the second half (18–48 h) of the fermentation, the facultatively heterofermentative rods with DL-lactate production still dominated but at an even larger proportion, comprising 67.5% of the isolates. In comparison to the first half of the fermentation (0–12 h), the facultatively heterofermentative rods which produce L-lactate, were strongly reduced to 2.5% of the isolates. *Leuconostoc*/*Weissella* strains were isolated at an incidence of 10% in the first half of the fermentation and their frequency of isolation decreased to 7.5% of isolates in the second half of fermentation. The proportion of the presumptive entero-, lacto- or streptococci doubled in the second half of fermentation to 12.5% of the isolates. Also in the second half of

the fermentation, obligately heterofermentative rods were isolated (10% of the isolates), while these were not isolated in the first half of the fermentation.

3.3. Biochemical properties of LAB strains from fermenting cassava

The biochemical properties of the isolates were compared among the five major groups of LAB isolated in this study, i. e. the obligately heterofermentative rods, the obligately homofermentative rods, the facultatively heterofermentative rods, the obligately heterofermentative cocci and the obligately homofermentative cocci (Fig. 1).

3.3.1. Enzyme activities

Tannase and α-amylase activities were rare traits for the LAB strains isolated in this study, and only nine strains (18%) of the obligately homofermentative cocci were able to degrade starch. Tannase was produced by a few strains of the facultatively heterofermentative rods (11.8%) and by 2% of the obligately heterofermentative rods. On the other hand, β-glucosidase activity could be demonstrated for various strains belonging to all five LAB groups. The facultatively heterofermentative rods (most of which belong to the *L. plantarum*-group) showed the highest incidence of β-glucosidase activity (95.4%), followed by the presumptive entero-, lacto- or streptococci (72%). The presence of heme-dependent catalase was most common amongst strains belonging to the facultatively heterofermentative rods group (42.4% of strains).

3.3.2. Degradation of α-galactoside sugars

Raffinose was generally fermented by strains of all five groups, with the exception of the strains belonging to the group of the entero-, lacto- and streptococci, amongst which only 4% of strains were positive for this trait. The fermentation of stachyose was rare in all the LAB groups when compared to raffinose utilisation. Only strains from the *Leuconostoc*/*Weissella* group fermented stachyose at notable numbers (38.5% of strains).

3.3.3. Antimicrobial activity

H_2O_2-production was detected in all five LAB groups. A considerable number of *Leuconostoc*/*Weissella* strains and obligately heterofermentative rods exhibited H_2O_2-production at 65.4% and 54.7% of the strains, respectively. Presumptive bacteriocin activity (indicated by a clear zone of inhibition of >1 mm against at least one of the indicator bacteria tested) was produced by strains of four groups, with the exception of the obligately homofermentative rod group, which consisted of three strains which did not produce presumptive bacteriocin. From experience, zones of inhibition larger than 1 mm measured from the edge of the colony indicate a bacteriocin effect, while smaller zones usually indicate an acid effect. However, since the proteinaceous nature of the inhibitory compound was not tested with proteinase we could only presume a bacteriocin activity to be involved.

3.3.4. Acid production

Many of the facultatively heterofermentative strains were considered fast acid producers, as 55% and 50.6% of strains were capable of lowering the pH in MRS broth to below 5.3 and 3.9, after 6 and 48 h, respectively. A notable number of strains from the obligately heterofermentative rod group (29.4%) and the entero-, lacto- and streptococci group (27.3%) were also capable of rapid acid production, thereby lowering the pH of MRS broth to 5.3 within 6 h.

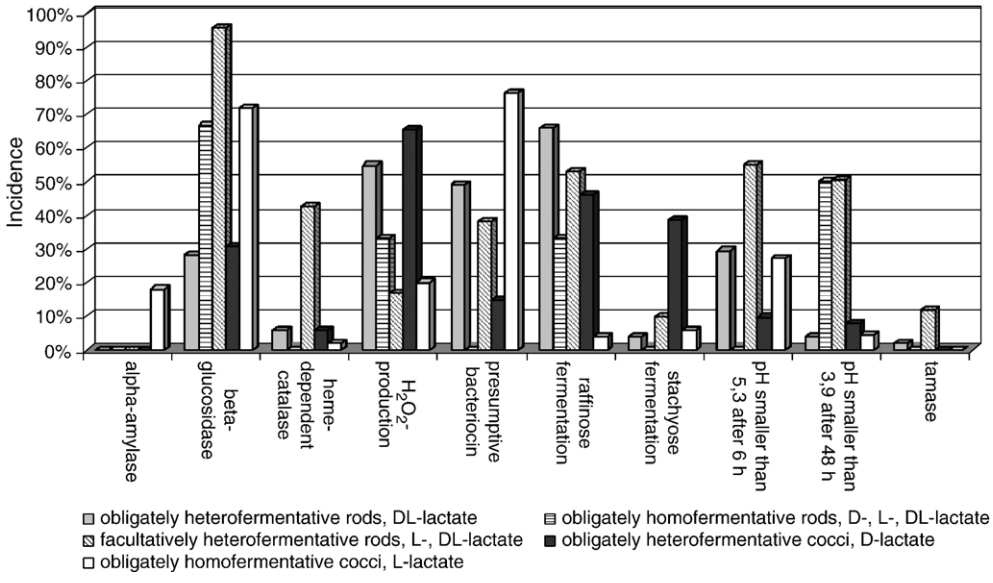

Fig. 1. Percentage of biochemical properties of all LAB strains belonging to the obligately and facultatively heterofermentative rods, obligately homofermentative rods and cocci and obligately heterofermentative cocci from fermenting cassava.

3.3.5. Selection of potential starter strains and further biochemical testing

On the basis of their biochemical properties, thirty-two of the 375 LAB strains of the 'Gari' culture collection were pre-selected as possible starter cultures for Gari production. Desirable biochemical characteristics were considered to be α-amylase activity, β-glucosidase activity, presence of heme-dependent catalase, production of hydrogen peroxide and presumptive bacteriocin, rapid acid production, fermentation of raffinose and/or stachyose, and presence of tannase. The results of all biochemical characteristics of these 32 pre-selected strains are shown in Table 1. The thirty-two strains were further investigated with the APIZYM test. In this test, all *L. plantarum* strains and all three *Leuconostoc* strains produced β-glucosidase (results not shown). Most selected strains from cassava were weakly lipoloytic, as indicated by alkaline phosphatase, esterase, esterase lipase and lipase activity. In addition, most selected strains also showed peptidase activity such as leucine arylamidase, valine arylamidase and cystine arylamidase activities. However, the strains showed nearly no protease activity, i.e., trypsin or α-chymotrypsin activity (results not shown).

3.4. Genotypic characterisation of 32 pre-selected strains as possible starter cultures for cassava fermentation

The thirty-two potential starter strains, pre-selected on the basis of biochemical properties, were characterised to species level in a polyphasic taxonomy approach involving both phenotypic and genotypic methods. Of these 32 strains, strains BFE 6620, BFE 6625 and BFE 7670 could be classified as obligately heterofermentative rods by phenotypic methods. In the rep-PCR analysis, strains BFE 6620 and BFE 6625 grouped together at $r=42.2\%$ with two different *L. fermentum* reference strains, i.e., *L. fermentum* LMG 8900 and the *L. fermentum* type strain DSM 20052^T (Fig. 2a), indicating that these strains could be presumptively identified as *L. fermentum.* To confirm the rep-PCR results, the 16S rDNA of strains BFE 6620 and BFE 6625 was sequenced and confirmed the identification as *L. fermentum*, as they showed 99.2% (BFE 6625) and 99.5% (BFE 6620) similarity to the sequence of the 16S rRNA gene of the *L. fermentum* type strain DSM 20052^T (results not shown). In the rep-PCR analysis, strain BFE 7670 grouped together with two *W. cibaria* reference strains

Table 1
Biochemical properties as pertaining to production of α-amylase, β-glucosidase, heme-dependent catalase, tannase, antimicrobial compounds (H_2O_2 and presumptive bacteriocin activity), acidification and fermentation of α-galactoside sugars of the 32 pre-selected strains determined *in vitro*

Strain	β-glucosidase	Heme-dependent catalase	H_2O_2-production	Presumptive bacteriocin production[a]	Raffinose fermentation	Stachyose fermentation	pH<5.3 after 6 h	pH<3.9 after 48 h	Tannase production
BFE 6620	−	−	+	+	+	−	−	−	−
BFE 6625	−	−	+	−	+	−	−	−	−
BFE 6633	+	−	−	−	+	−	−	−	+
BFE 6645	+	−	−	−	+	+	+	+	−
BFE 6661	+	−	−	−	+	+	+	−	−
BFE 6675	+	−	−	−	−	−	+	+	−
BFE 6678	+	−	−	−	+	+	+	+	−
BFE 6688	+	+	−	−	+	−	+	+	−
BFE 6690	+	−	−	−	+	+	+	+	−
BFE 6692	+	−	−	+	−	−	+	n.d.	−
BFE 6701	+	+	−	+	−	−	+	+	−
BFE 6708	+	−	−	−	+	+	+	+	−
BFE 6710	+	−	−	−	+	−	+	+	−
BFE 6711	+	−	−	+	−	−	−	−	−
BFE 6713	+	+	+	+	+	−	−	−	−
BFE 6739	+	+	−	−	−	−	+	+	−
BFE 6748	+	−	−	−	+	−	+	+	+
BFE 6757	+	+	+	+	+	−	−	−	−
BFE 6768	+	+	−	+	+	−	−	−	−
BFE 6781	+	−	−	+	+	−	−	−	−
BFE 6793	+	+	+	+	+	−	+	−	+
BFE 7589	+	+	+	+	+	−	+	−	+
BFE 7593	+	+	−	+	+	−	+	+	−
BFE 7596	+	+	+	+	−	−	+	−	+
BFE 7601	+	−	−	−	−	−	−	−	−
BFE 7608	−	−	−	−	+	+	−	−	−
BFE 7668	+	−	−	−	+	+	−	−	+
BFE 7670	+	−	+	n.d.	−	−	+	−	+
BFE 7685	+	+	−	+	+	−	+	−	−
BFE 7687	+	+	+	+	−	−	−	−	−
BFE 7688	+	−	−	+	+	−	−	−	+
BFE 7694	+	−	−	+	+	−	−	−	−

All strains were α-amylase negative.
n.d.: not done.
[a] Presumptive bacteriocin activity as indicated by a zone of inhibition surrounding the producer colony of at least 1 mm.

(*W. cibaria* LTH 18408 and the type strain *W. cibaria* LMG 17699^T). The sequencing of the 16S rDNA of strain BFE 7670 showed that this strain belonged to the species *W. cibaria* with 99.9% similarity to the 16S nucleotide sequence of the type strain (result not shown).

Five obligately heterofermentative cocci (BFE 6645, BFE 6692, BFE 7601, BFE 7608 and BFE 7668) were also chosen as possible starter cultures. On the basis of phenotypic tests, these belonged either to the genus *Leuconostoc* or *Weissella*, as they produced D-lactate and gas from glucose. Based on rep-PCR, the strains BFE 6645 and BFE 6692 were characterised as *L. fallax*, as they grouped closely together with the type strain *L. fallax* DSM 20189^T at r=84.9% (Fig. 2b). This identification was confirmed by of 16S rDNA sequencing, in which the strains BFE 6692 and BFE 6645 showed 99.5% and 99.6% homology to the type strain, respectively. Strain BFE 7668 grouped in the analysis of the rep-PCR patterns together with the type strain *L. mesenteroides* ssp. *mesenteroides* DSM 20343^T (r=63.8%). This characterisation was also confirmed by 16S rDNA sequencing (99.9% homology to the type strain). The two strains BFE 7601 and BFE 7608 were characterised as belonging to the species *W. paramesenteroides* in the rep-PCR, as they grouped together with the *W. paramesenteroides* type strain DSM 20288^T at r=67.2%. The 16S rDNA sequence of both strains showed a homology of 100% to each other and a homology of 99.0% to the type strains *W. hellenica* DSM 7378^T and *W. paramesenteroides* DSM 20288^T. The 16S rDNA of the type strains of *W. hellenica* and *W. paramesenteroides* as obtained from the GenBank databank showed a homology of 100%, indicating that these two species cannot be distinguished on this basis. However, the rep-PCR results indicated that the two strains BFE 7601 and BFE 7608 are more closely related to *W. paramesenteroides* as they grouped together in one cluster (Fig. 2b).

Twenty-four of the thirty-two pre-selected strains were phenotypically characterised as belonging to the *L. plantarum*-group. In the rep-PCR analysis, four strains (BFE 7589, BFE 6633, BFE 6748 and BFE 7596) grouped together with the *L. pentosus* type strain DSM 20314^T and three *L. pentosus* reference strains LTH 2067, LTH 482 and LTH 2792 at r=30.2% (Fig. 3), indicating that these may be characterised as *L. pentosus* strains. Eighteen strains grouped together with the type

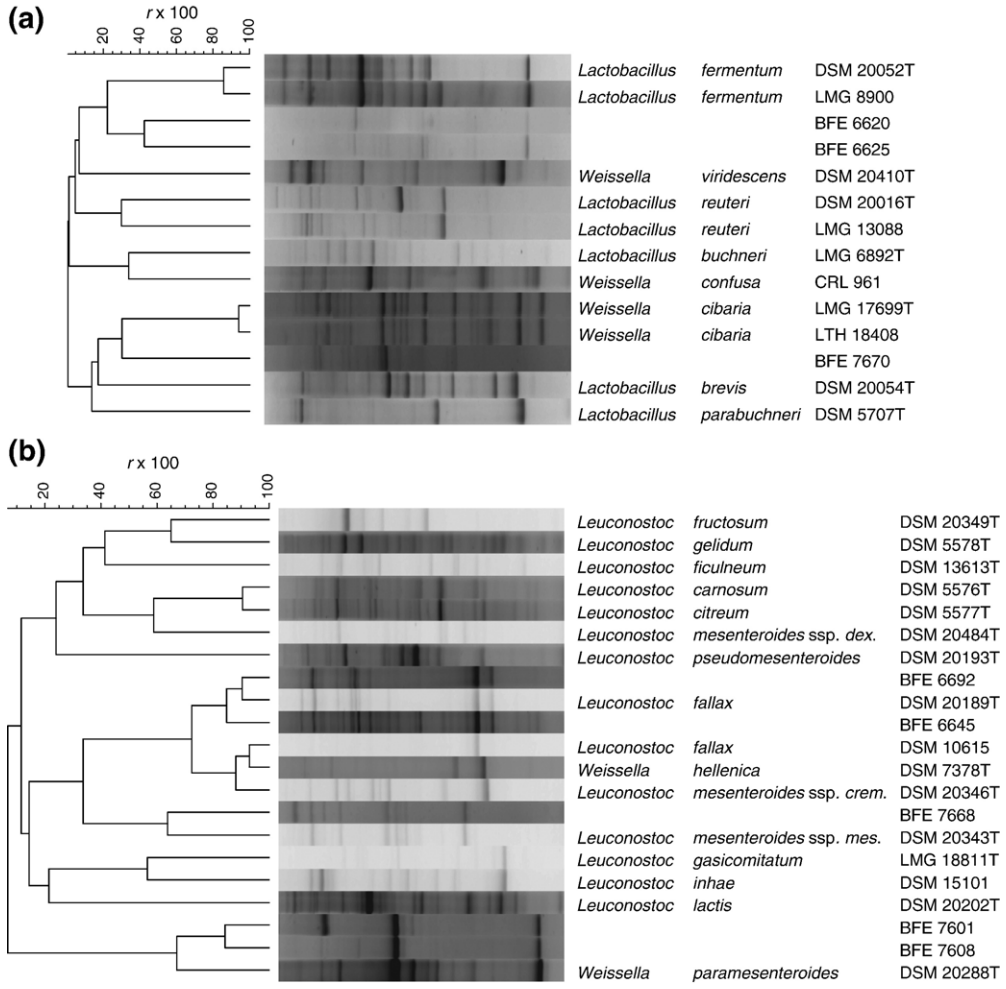

Fig. 2. Dendrogram obtained by UPGMA of correlation value r of rep-PCR fingerprint patterns of three heterofermentative rod-shaped *Lactobacillus* and *Weissella* isolates (a) and five heterofermentative, coccoid *Leuconostoc* and *Weissella* isolates (b) from fermenting cassava and reference strains obtained with the primer $(GTG)_5$.

strain *L. plantarum* DSM 20174^T at r=40.0%, indicating that these can be characterised as *L. plantarum* strains. The identity of the two strains BFE 6710 and BFE 6739 could not be unequivocally established as they grouped separately (Fig. 3). They could belong either to the species *L. plantarum* (subsp. *plantarum* and subsp. *argentoratensis*) or *L. paraplantarum*, or could constitute a new subspecies. This will be investigated in further studies. 16S sequencing was not used as a further identification test, as the 16S sequences of *L. plantarum* and *L. pentosus* are highly similar and do not allow a ready distinction between these species (Kostinek et al., 2005b).

RAPD-PCR was used to determine possible clonal relationships among the strains in order not to choose multiple isolates as starter cultures. The analysis is based on three fingerprints obtained with three different primers. The strain BFE 6713 was analysed in duplicate to determine the reproducibility of RAPD-PCR analysis and running conditions. These fingerprints obtained from the duplicate samples clustered at r=91.0% (data not shown), indicating this to be the cut-off value for determination of clonal relationships. The four strains BFE 6711, BFE 6739, BFE 7687 and BFE 6710 clustered together at r=94.5%, and therefore could be clonally related. The strains BFE 6768 and BFE 7593, strains BFE 6701 and BFE 7688 and strains BFE 7601 and BFE 7608 could also be multiple isolates of clonal origin, as they clustered at r=97.8%, r=94.0% and r=99.8%, respectively (data not shown).

4. Discussion

The most predominant LAB strains from fermenting cassava in this study were facultatively heterofermentative strains (57.9% of isolates), most of them belonging to the *L. plantarum*-group. This confirms former investigations, in which the presence of *L. plantarum* as a dominant species in cassava fermentation was reported (Ngaba and Lee, 1979; Ben Omar et al., 2000; Amoa-Awua et al., 1996). Other predominant strains belonged to *Weissella* or *Leuconostoc* and to the heterofermentative *Lactobacillus* species, as well as to the homofermentative cocci. This also agrees with former studies on the microbial populations associated with cassava fermentations. Amoa-Awua et al. (1996) for example reported the occurrence of *L. brevis*, *L. mesenteroides* and *Streptococcus* spp. in cassava fermentations. In the first phase of the fermentation, the facultatively heterofermentative lactobacilli which produce DL-lactate (mostly *L. plantarum*-group strains) dominated (50%) and even increased (67.5%) towards the end of the fermentation. *L. plantarum* strains are well-known to develop in vegetable fermentations to dominate in the later phases of

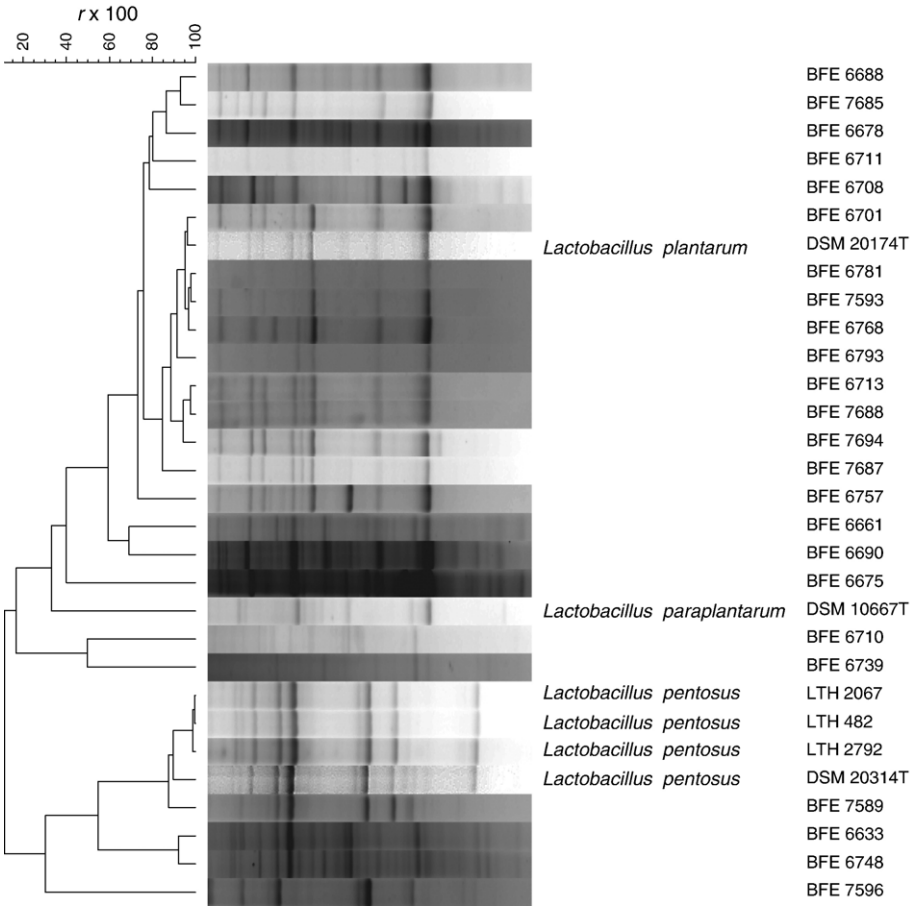

Fig. 3. Dendrogram obtained by UPGMA of correlation value r of rep-PCR fingerprint patterns of twenty-four strains belonging to the *L. plantarum*-group from fermenting cassava and reference strains obtained with the primer $(GTG)_5$.

fermentation (Ben Omar et al., 2000) and terminate many of the spontaneous lactic fermentations such as silage and vegetable fermentations (Daeschel et al., 1987). This agrees well also with the results on the succession in this study.

In our study, production of α-amylase was a rare trait among all strains, except a few obligately homofermentative cocci. This was quite surprising, as cassava contains about 84% of the carbohydrates as starch (Ketiku and Oyenuga, 1972) representing an important potential energy source also for the LAB. Only a few amylolytic LAB have been isolated from starchy fermented foods in Africa (Sanni et al., 2002).

Bacteria of all five groups produced β-glucosidase, which may be a reflection on the relative abundance of linamarin and other β-glucoside sugars in cassava. The enzyme β-glucosidase hydrolyses various compounds with β-D-glucosidic linkages (Shewale, 1982) including cyanogenic glucosides. As cassava contains various amounts of cyanogenic glucosides, bacteria with β-glucosidase activity are probably able to hydrolyse linamarin to glucose and acetone cyanohydrin and use the glucose for their metabolism. However, the cassava tuber itself contains an endogenous linamarase, which hydrolyses linamarin when the plant cells are disrupted (Kimaryo et al., 2000). The highest incidence of β-glucosidase activity was shown among the *L. plantarum* strains. These strains are well-known to hydrolyse various β-glucosides of vegetable origin (Ciarfardini et al., 1994). The results of the APIZYM test (which includes a test for β-glucosidase) were compared with the results of the test according to Weagant et al. (2001) for β-glucosidase production among the 32 pre-selected strains. In the APIZYM test, all strains belonging to the *L. plantarum*-group and three *Leuconostoc* strains produced β-glucosidase. The same result was obtained with the β-glucosidase test with *p*-nitrophenol-β-D-glucopyranoside of Weagant et al. (2001). The test for the presence of β-glucosidase according to Weagant et al. (2001), therefore, was considered a reliable test and an inexpensive alternative to the APIZYM for determining β-glucosidase activity.

All bacteria were tested for the ability to break down the oligosaccharides raffinose and stachyose, as these α-galactoside sugars are not susceptible to digestion by pancreatic and brush border enzymes (Quigley et al., 1999) of humans. In humans, these sugars are metabolised by microorganisms in the large intestine, liberating huge amounts of gas, which can then cause gastrointestinal disorders (LeBlanc et al., 2004). In general, the ability of the LAB to ferment raffinose was wider distributed as that for stachyose, but especially many of the *Leuconostoc*/*Weissella* strains were able to ferment both raffinose and stachyose.

Another biochemical property investigated concerned the production of H_2O_2, as it is well-known that this antimicrobial compound inhibits the growth and metabolic activities of many other microorganisms (Lindgren and Dobrogosz, 1990). A further important characteristic for potential starter strains is their ability to acidify their environment rapidly, as the acid production and the accompanying pH decrease are well-known to extend the lag phase of sensitive organisms including foodborne pathogens (Smulders et al., 1986). Lactic acid production during fermentation resulting in acidification to pH levels lower than 4.2 constitutes a major food safety factor (Holzapfel, 1997). The best acid producers were the facultatively heterofermentative rods mostly *L. plantarum* strains (204 out of 217 strains). Half of the strains of this group reduced the pH to lower than 3.9 after 48 h.

Cassava contains tannic acid in the peel and in the tuber itself (Hahn, 1989). The production of tannase among the LAB strains was investigated because tannins can affect the nutritive value of food products by forming a complex with protein, thereby inhibiting digestion and absorption (Osuntogun et al., 1987). They also bind iron and make it unavailable (Brune et al., 1991). Only a few of the LAB strains in this study exhibited tannase activity.

Concerning the genotypic characterisation of the 32 pre-selected strains, rep-PCR was shown in our study to be a suitable method for characterisation of the obligately heterofermentative, rod-shaped *Lactobacillus* and *Weissella*, as well as the heterofermentative, coccoid-shaped *Leuconostoc* and *Weissella* strains. This was based mainly on the fact that the rep-PCR-results could, in most cases, be confirmed by 16S rDNA sequencing. Rep-PCR was also a good method for distinguishing *L. pentosus* strains from *L. plantarum* strains. Strains of these two species are almost impossible to distinguish on the basis of phentoypic characteristics alone. The combination of rep-PCR and 16S rDNA sequencing of strains representative of specific rep-PCR clusters was thus shown to be effective in most cases for accurate determination.

One aim of this study was the selection of possible starter cultures for the production of Gari. Thirty-two potential starter strains were pre-selected on the basis of beneficial biochemical characteristics. The 32 pre-selected strains were also weakly lipolytic and proteolytic as detected with the APIZYM test. These latter properties could become important if Gari is fortified with soybean, palm oil or coconut milk, as strongly lipolytic or proteolytic strains might degrade added protein or lipids, which might affect product characteristics, nutritional value, and aroma. The starter cultures selected were also representative of the predominant LAB associated with cassava fermentation as determined in this study. They consisted mostly of strains belonging to the *L. plantarum*-group (24 strains), three obligately heterofermentative rods (two *L. fermentum* and one *W. cibaria*) and five obligately heterofermentative cocci (two *L. fallax*, one *L. mesenteroides* ssp. *mesenteroides* and two *W. paramesenteroides*). More than half of these were capable of fast acid production. Sixteen of these strains showed presumptive bacteriocin activity and seven of these presumptive bacteriocin-producing strains also were capable of hydrogen peroxide production. An additional two strains which did not produce bacteriocin were capable of producing hydrogen peroxide. Seven of the pre-selected strains fermented both raffinose and stachyose, while 16 fermented only raffinose and none fermented only stachyose (Table 1).

From the 32 strains pre-selected as starter cultures, 16 strains (*L. fermentum* BFE 6620 and BFE 6625, *W. paramesenteroides*, BFE 7601 and 7608, *L. mesenteroides* ssp. *mesenteroides* BFE 7668, *L. plantarum* BFE 6688, BFE 6710, BFE 6711, BFE

6713, BFE 6739, BFE 6793, BFE 7685 and BFE 7687, and *L. pentosus* BFE 6748, BFE 7596 and BFE 7589) were finally selected. Based on RAPD-analysis, we noticed that strains BFE 7601 and BFE 7608 were probably clonally related because their fingerprints clustered closely together at $r > 91.0\%$, which was the cut-off value for determination of clonal relationships. However, these strains had slightly different sugar fermentation patterns. BFE 7601 fermented both methyl-α-D-mannopyranoside and D-xylose, while strain BFE 7608 did not. For this reason we decided to select both these strains as possible starter cultures, as the different sugar fermentation suggest a difference at least at the phenotypic level.

In this study, therefore, representative isolates of predominant organisms associated with the fermentation of cassava for the production of Gari were selected as starter strains. The biochemical traits of these selected strains, however, were mostly investigated *in vitro*, except for the acidification of cassava, which was also determined in laboratory-scale investigation. Therefore, to assess the performance of the strains in establishing themselves as dominant starter cultures in actual fermentations, and to validate whether these biochemical traits are indeed expressed and have a positive impact on product quality, the strains are currently being developed as starter cultures on an industrial scale. These strains will then be used in pilot plant fermentations, where their performance will be evaluated.

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