Food Control xxx (2010) 1–7

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Biomass production and small-scale testing of freeze-dried lactic acid bacteria starter strains for cassava fermentations

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ARTICLE INFO

Article history:
Received 1 June 2010
Received in revised form
4 August 2010
Accepted 3 September 2010

Keywords:
Lactic acid bacteria
Gari
Starter cultures
Cassava
Fermentation
Linamarin

ABSTRACT

Based on their predominance in *Gari* fermentations, as well as suitable technological properties, *Lactobacillus plantarum, Lactobacillus fermentum, Weissella paramesenteroides* and *Leuconostoc mesenteroides* strains were investigated for their suitability for development as starter strains for this African traditional fermented cassava product. The strains were grown in optimized growth media in 2 L fermenters, harvested and freeze dried, and then tested in lab-scale cassava mash fermentation trials for their ability to ferment the cassava. The strains performed well and rapidly increased the titratable acidity from 1.1 to 1.3% at 24 h to 1.3–1.6% at 48 h. The benefit of including starter cultures was that it lowered the pH of the product much faster and to lower levels than in the uninoculated control fermentation. The results furthermore indicated that especially the *L. plantarum*-group strains could be produced as starter strains at low cost. Overall, the results of this study showed that starter strains could be easily and economically produced, and thus represent a feasible possibility for further development for application in the field.

1. Introduction

Lactic acid bacteria (LAB) occupy a central role in many vegetable, meat, dairy and cereal fermentations (Leroy & De Vuyst, 2004). There has been a recent trend to select wild-type strains from traditional products in order for them to be used as starter cultures in fermentation processes (Beukes, Bester, & Mostert, 2001; De Vuyst et al., 2002). A reason for this could be that pure cultures isolated from traditional fermented foods diverge strongly from comparable strains used as bulk starters in terms of their diversity of metabolic activities, and that the strains isolated from the traditional fermentation as predominant isolates are well adapted for growth in the fermentation substrate (Klijn, Weerkamp, & de Vos, 1995).

When LAB are isolated for development as starter cultures, they need to demonstrate an ability to be produced on a large scale, to withstand the freeze drying process and to maintain their functional activity, before they are considered practicable for industrial applications (Carvalho et al., 2002). Such industrial starter preparations furthermore require the use of cheap raw materials in order to be economically feasible. Freeze drying is currently the most suitable and widely used technique for LAB preservation (Carvalho et al., 2002; Zayed & Roos, 2003). The freeze-drying process imposes environmental stress on the bacterial cells, such as freezing, drying, long-term exposure to low water activities and rehydration. Intrinsic resistance of strains, initial concentration of the microorganisms, growth conditions, drying medium, protective agents used, freezing rate, storage conditions (temperature, atmosphere, relative humidity) and rehydration are all important factors that determine microbial survival (Andersen, Fog-Petersen, Larsen, & Skibsted, 1999; Carvalho et al., 2002; Morgan, Herman, White & Vesey, 2006).

Fermentation processes in Africa, such as the fermentation of cassava for *Gari* production, usually take place at a household level. Little use, if any, is made of starter cultures and most of these fermentations rely on back-slopping techniques to start the fermentation. Back-slopping involves the use of a residue ('starter

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doi:10.1016/j.foodcont.2010.09.008

dough') from a previous fermentation batch of acceptable quality for inoculation of a fresh batch (Holzapfel, 1997). However, retention of product characteristics over time may prove difficult due to changes in microbial types. Quality, safety and acceptability of traditional fermented foods may be significantly improved by using starter cultures that are selected on the basis of multifunctional properties which include technological properties, as well as possibly functional (probiotic) properties (Holzapfel, 2002). For the production of *Gari* it is also of utmost importance to develop a process that is not only industrially applicable, but also economically feasible.

This study focused on the assessment of selected predominant LAB strains isolated from *Gari* fermentations to be produced as starter cultures and their ability to withstand a freeze-drying process. Furthermore, the starters were evaluated for their suitability as starters by their capabilities for rapid substrate acidification in addition to linamarin degradation, thereby enhancing both product quality and safety.

2. Materials and methods

2.1. Strains and culture conditions

Seventeen predominant LAB strains (9 *Lactobacillus plantarum*, 3 *Lactobacillus pentosus*, 2 *Lactobacillus fermentum*, 2 *Weissella paramesenteroides* and 1 *Leuconostoc mesenteroides* ssp. *mesenteroides*, Table 1) isolated from cassava fermentations during the production of *Gari*, were previously identified (Kostinek et al., 2007) and preselected on the basis of technological characteristics as potential starter strains. These characteristics included production of β-glucosidase, which may aid in the detoxification of cyanogenic glucosides, in addition to showing antimicrobial activity i.e., presumptive bacteriocin activity (Table 1). All strains were routinely grown in de Man, Rogosa and Sharpe (MRS) (Merck, Darmstadt, Germany) broth at 30 °C for 18 h under aerobic conditions. Stock cultures of these were stored in MRS broth containing 20% glycerol (Merck, Darmstadt, Germany) at −80 °C. Stored vials for the selected strains were thawed as needed, and used as seed inoculum.

2.2. Biomass production in 2 L fermenters

Biomass production for all 17 strains was carried out in 2 L Biostat B (B. Braun Biotech International, Melsungen, Germany) fermenters. A working volume of 1.4 L was used, which comprised 1.3 L growth medium and 100 ml of inoculum. Inocula were prepared by adding 100 μl of the selected preserved strain to 100 ml of MRS broth (approx. 1×10^7 CFU/ml), in a 250 ml Erlenmeyer flask and were incubated aerobically at 30 °C for 16 h without agitation.

The raw material composition of the various media used in the fermentations is outlined in Table 2. MRS medium is referred to as medium 1. Medium 2 was MRS medium containing additional 20 g/L of glucose. Medium 3, which was specific for the biomass production of *L. plantarum* and *L. pentosus* strains, and which is often used at the Walloon Center for Industrial Biology, University of Liège for these purposes, contains 10 g/L corn steep liquor (CSL) (Roquette, Lestrem, France). Media 4 and 5 had minor variations to medium 3. Media 4 and 5 had no meat extract, but a higher yeast extract (1 g/L) content (Table 2).

To test starter culture growth conditions prior to the fermentations in the 2 L fermenters, the 17 selected strains were grown in static flask cultures. This was either done as a scale-up and also as a pilot experiment. All *L. plantarum* and *L. pentosus* strains were grown using media 3, 4 and 5 (Table 2), while all of the *L. fermentum*, *W. paramesenteroides* and *L. mesenteroides* ssp. *mesenteroides* were grown using all 5 media compositions (results not shown). The reason for this was that the media for *L. plantarum* and *L. pentosus* were already optimised from previous work on different strains of these species (result not shown). Parameters for these were thus only adjusted to the use of CSL products that were obtained locally in South Africa (African Products, Germiston, South Africa). For the *L. fermentum, W. paramesenteroides* and *L. mesenteroides* ssp. *mesenteroides* strains optimisation of the media was required. Individual fermentation medium compositions were chosen based on the cell counts, OD_{660} and biomass produced in these small-scale pilot experiments for each strain (results not shown). The fermentation medium selected for each strain is shown in Table 2.

The media used were sterilised in the fermenter at 121 °C for 15 min. Glucose monohydrate was autoclaved separately and added aseptically to the fermenter. The set point pH was 5.6, temperature 30 °C, stirrer speed 100 rpm and airflow 0.3 sLpm (standard litres per minute). The pH was adjusted with 1 N HCl or 1 N NaOH. The fermentation was run until glucose was depleted or for ~24 h. Samples (2 mL) were taken every 2–4 h for determinations of pH, glucose concentration, optical density at 660 nm and the viable cell count (CFU/ml). Biomass was estimated using the dry weight

Table 1
Morphological and biochemical properties of selected strains used for starter culture development.

Isolate BFE No.	Country of sample origin	Morphology	Presumptive bacteriocin activity[a]	β-glucosidase activity	Presumptive identification/group	Genotypic identification
BFE 6620	Benin	Rods	+	−	Obligatory heterofermentative rods	*L. fermentum*
BFE 6625	Benin	Rods	−	−	Obligatory heterofermentative rods	*L. fermentum*
BFE 7589	Benin	Rods	+	+	*L. plantarum*-group	*L. pentosus*
BFE 7596	Benin	Rods	+	+	*L. plantarum*-group	*L. pentosus*
BFE 6748	Benin	Rods	−	+	*L. plantarum*-group	*L. pentosus*
BFE 6793	Benin	Rods	+	+	*L. plantarum*-group	*L. plantarum*
BFE 6710	Benin	Rods	−	+	*L. plantarum*-group	*L. plantarum*
BFE 6739	Benin	Rods	−	+	*L. plantarum*-group	*L. plantarum*
BFE 6688	Benin	Rods	−	+	*L. plantarum*-group	*L. plantarum*
BFE 6711	Benin	Rods	+	+	*L. plantarum*-group	*L. plantarum*
BFE 6713	Benin	Rods	+	+	*L. plantarum*-group	*L. plantarum*
BFE 7685	Benin	Rods	+	+	*L. plantarum*-group	*L. plantarum*
BFE 7687	Benin	Rods	+	+	*L. plantarum*-group	*L. plantarum*
BFE 7688	Benin	Rods	+	+	*L. plantarum*-group	*L. plantarum*
BFE 7601	Benin	cocci	−	+	*Leuconostoc/Weissella*	*W. paramesenteroides*
BFE 7608	Benin	cocci	−	−	*Leuconostoc/Weissella*	*W. paramesenteroides*
BFE 7668	Kenya	cocci	−	+	*Leuconostoc/Weissella*	*L. mesenteroides* ssp. *mesenteroides*

[a] Positive results indicates a zone of inhibition of at least 2 mm against the indicator organism *W. paramesenteroides* DSM 20288 as detected by spot on lawn test.

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Table 2
Summary of the raw materials used for the biomass production of each selected lactic acid bacterium starter strain for *Gari* fermentation.

Strain number	BFE 6620	BFE 6625	BFE 7589	BFE 7596	BFE 6748	BFE 6793	BFE 6710	BFE 6739	BFE 6688	BFE 6711	BFE 6713	BFE 7685	BFE 7687	BFE 7688	BFE 7601	BFE 7608	BFE 7668
Identification	*L. fermentum*		*L. pentosus*			*L. plantarum*									*W. parames*[a]		*L. mes* ssp. *mes*[b]
Medium number	1	1	3	5	3	4	3	3	3	3	3	3	3	5	1	2	1
Fermentation raw materials	g/L																
Yeast extract	5	5	20	21	20	21	20	20	20	20	20	20	20	21	5	5	5
Peptone	10	10													10	10	10
Beef extract	5	5													5	5	5
Potassium phosphate	2	2													2	2	2
Tween 80	1	1													1	1	1
Tri-Ammonium citrate	2	2													2	2	2
Manganese sulphate	0.05	0.05	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.05	0.05	0.05
Magnesium sulphate	0.1	0.1	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.1	0.1	0.1
Sodium acetate	5	5													5	5	5
Glucose	20	20	50	50	50	50	50	50	50	50	50	50	50	50	20	40	20
CSL (Afprod)				50										50			
CSL (Roquette)			10		10	10	10	10	10	10	10	10	10				
Meat extract			1		1		1	1	1	1	1	1	1				
Iron sulphate			0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013			

[a] *W. parames.* = *Weissella paramesenteroides.*
[b] *L. mes.* ssp. *mes* = *Leuconostoc mesenteroides* ssp. *mesenteroides.*

method. Glucose concentration was determined using the Accutrend® *alpha* glucose meter (Boehringer Mannheim, Germany), pH was measured using a Jenway 3310 pH meter (Jenway Ltd, Essex, Cambridge, UK) and the optical density was determined using the Spectronic® 20 Genesys™ (Thermo Scientific, Johannesburg, South Africa) at 660 nm.

2.3. Production of starter cultures

The biomass produced from each organism after fermentation was collected and centrifuged at 10 000 rpm in a Beckman® Model J2 − 21 (Beckman Coulter, Halfway House, South Africa), Rotor JA 14 for 10 min. After centrifugation, the pellet was collected and weighed. Glycerol (2%) and maltodextrin (5%) were added to each pellet as cryoprotectants. The amount of glycerol and maltodextrin added was calculated as a percentage of the mass of the cell biomass (pellet) to which they were added. A sample was taken for plate counts on MRS agar to evaluate cell viability. Cell viability of the pellet was assessed by transferring 0.1 g of pellet to 0.9 g (900 μL) of sterile water and diluting this sample in a ten-fold dilution series, with subsequent spread plating on MRS agar and incubating plates at 30 °C for 48 h. The cell material with cryoprotectants added was then freeze dried.

The freeze-drying process was carried out in a Genesis 25 L freeze-dryer (VirTis, United Scientific, Gardiner, New York State, USA) at a vacuum set point of 80 mT. The freeze-dried material obtained varied from strain to strain, but generally ranged from ca. 1–5 g and was packaged into sterile aluminium bags and stored at −20 °C. The viability of the freeze-dried biomass was evaluated as above. Plate counts were done in triplicate on MRS agar and mean values were calculated.

2.4. Use of freeze-dried starter cultures in small-scale, laboratory fermentation trials of cassava fortified with palm oil and soybean

Fresh, bitter (>180 ppm HCN/kg) cassava roots used for this study were purchased from a local market in Tembisa, South Africa. The roots (approx. 50 kg) were washed with water to remove all dirt, peeled with a knife, then finely ground using a mechanical grinder and stored at −20 °C until use. Soybean and palm oil (obtained from Department of Nutrition and Food Science, University of Abomey-Calavi, Benin) were used to supplement the *Gari* with a protein and Vitamin A source, respectively. Soybeans were cooked at 94 °C for 15 min, dried (left in sunlight for 8 h), ground using a mechanical grinder and stored at ambient temperature before use. Palm oil was also stored at ambient temperature before use.

Ground cassava (500 g) was mixed with 30 g of soybean and 5 mL of palm oil in 1 L buckets. Each starter culture was inoculated in separate experiments at an initial concentration of approximately 1×10^8 CFU/g after rehydration of 1 g of freeze-dried starter culture in 10 mL of saline-peptone water (NaCl 5 g/L, casein peptone 1 g/L, Tween 80 1 g/L) for 10 min, and diluting in saline-peptone water using a ten-fold dilution series to obtain the appropriate concentration of 1×10^8 CFU/g. The uninoculated cassava mash served as a negative control. All buckets were sealed tight with lids and incubated at 30 °C for 48 h. Replicate fermentations were done for each starter culture. Samples were removed at 0 h, 8 h, 24 h and 48 h of fermentation. For pH and titratable acidity measurements, 10 g of samples were added to 20 ml of distilled water and homogenised. The pH was measured and 20 ml of distilled water was then added to the mixture and the titratable acidity was measured with 0.1 N NaOH using phenolphthalein as indicator. The titratable acidity was expressed as a percentage of lactic acid.

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For microbial analysis, samples were taken at 0, 24 and 48 h of fermentation. One gram of cassava was mixed with 9 mL of saline-peptone water. The samples were diluted in a ten-fold dilution series using saline-peptone water and aliquots of appropriate dilutions were spread plated onto MRS agar for LAB counts.

2.4.1. Cyanide determination of fermentation samples using a simple field kit

Total cyanide present in the cassava fermentation was determined using a picrate paper kit as described by Bradbury, Egan, and Bradbury (1999). This kit was a gift from Dr J. Howard Bradbury of the Australian National University. The kit was developed as a simple field kit that uses a colour chart to indicate between 0 and 800 ppm total cyanide.

3. Results

3.1. Biomass production in 2 L fermenters

These 17 fermentations in 2 L fermenters were stopped once the glucose was depleted as determined by glucose analysis. The majority of the fermentations were terminated after 10–14 h (Table 3), the exceptions were *L. fermentum* BFE 6625, *L. pentosus* BFE 7596 and *L. plantarum* BFE 7688, which took 18, 25 and 25 h, respectively, before glucose depletion. *L. mesenteroides* ssp. *mesenteroides* strain BFE 7668 and *W. paramesenteroides* strain BFE 7608 completed the fermentation in only 8 and 9.5 h, respectively. A summary of the cell concentrations, the fermentation times, the biomass produced and the cell biomass yield after freeze drying are shown in Table 3. Variations were evident in terms of cell concentration after fermentation and after freeze drying for all strains. *L. fermentum* strain BFE 6620 produced 2.8 g/L biomass compared to 6.7 g/L produced by *L. fermentum* strain BFE 6625. Strain BFE 6625 also grew to ca. 2 log CFU/ml higher in the fermentation, and it resulted in a 1 log CFU/g higher cell number in the freeze-dried powder, compared to strain BFE 6620. However, *L. fermentum* BFE 6620 had a yield of 43% after freeze drying compared to only 14% for strain BFE 6625. The biomass produced by the *L. pentosus* strains BFE 6748, BFE 7589 and BFE 7596 (Table 3) ranged from 6.1 g/L to 11.2 g/L. Their cell counts in freeze-dried powder ranged from 11.7 to 12.5 log CFU/g, and the yields after freeze drying ranged from 20 to 27%. The majority of strains studied were *L. plantarum*. The biomass produced by the *L. plantarum* strains ranged from 5.1 to 18.2 g/L. Cell counts in freeze-dried powder for these strains ranged from 11.1 to 13.0 log CFU/g. The yields after freeze drying ranged from 18 to 51%. Biomass produced by *W. paramesenteroides* strains BFE 7601 and BFE 7608 were only 1.4 and 3.7 g/L, respectively, and the counts in freeze-dried powder were between 11.3 and 12.2 log CFU/g, with yields after freeze drying between 20 and 34%. *L. mesenteroides* ssp. *mesenteroides* strain BFE 7668 produced a low amount of biomass (2.6 g/L), with a cell count of 11.2 log CFU/g in the freeze-dried form, and a yield of 24% after freeze drying.

A cost analysis was conducted on the raw materials to determine the cost per fermentation. This analysis excluded the costs associated with the freeze drying process and was based on material costs only. The raw materials costs associated with biomass production of the *L. plantarum* and *L. pentosus* strains were generally much lower than those for the *L. fermentum, Leuconostoc* and *Weissella* strains (Table 4). The costs/g biomass produced was between ZAR 1.64 and ZAR 3.95 for *L. fermentum*, between ZAR 0.59 and ZAR 0.99 for *L. pentosus*, between ZAR 0.36 and ZAR 1.30 for *L. plantarum*, between ZAR 2.98 and ZAR 8.13 for *W. paramesenteroides* and it was ZAR 4.22 for *L. mesenteroides* ssp. *mesenteroides*. However, the biomass production for *Leuconostoc/Weissella* strains was much lower due to their smaller cell size. This affected the costs/g analysis and made it appear as a more expensive fermentation process. Their ability to grow rapidly in a fermenter and maintain cell viability was still considered very high.

3.2. Cassava mash fermentation trials

To evaluate whether the freeze-dried cells had the ability to maintain their functional activity after freeze drying, fermentation tests were conducted using cassava fortified with soybean and

Table 3
Summary of the fermentation with the selected starter cultures in 2 L fermenters and yield after freeze drying.

Strain	Medium[a]	Initial counts (log CFU/mL)	Final counts (log CFU/mL)	Biomass (g/L)	Fermentation time (h)	Cell counts in freeze-dried powder (log CFU/g)	Yield after freeze drying (%)
L. fermentum							
BFE 6620	1	6.4	9.1	2.8	13.5	10.5	43
BFE 6625	1	8.1	11.6	6.7	18.0	11.5	14
L. pentosus							
BFE 7589	3	8.7	11.6	9.9	10.0	12.5	20
BFE 7596	5	7.9	10.2	6.1	25.0	11.7	27
BFE 6748	3	10.0	11.6	11.2	12.0	12.4	26
L. plantarum							
BFE 6793	4	6.5	9.8	6.0	13.0	11.1	33
BFE 6710	3	9.4	11.7	15.9	10.0	12.9	31
BFE 6739	3	9.5	11.7	5.9	13.5	12.6	51
BFE 6688	3	9.4	11.5	5.1	10.0	12.2	20
BFE 6711	3	9.8	11.7	12.2	12.0	12.4	20
BFE 6713	3	9.7	12.2	18.2	14.0	13.0	24
BFE 7685	3	10.0	11.5	18.2	14.0	12.3	18
BFE 7687	3	8.4	11.6	8.8	12.0	12.3	31
BFE 7688	5	9.0	11.3	6.0	25.0	12.4	47
W. paramesenteroides							
BFE 7601	1	6.2	9.4	1.4	14.5	11.3	34
BFE 7608	2	8.1	11.5	3.7	9.5	12.2	20
L. mesenteroides ssp. *mesenteroides*							
BFE 7668	1	9.3	10.6	2.6	8.0	11.2	24

[a] Refer Table 2.

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Table 4
Cost in South African Rands (ZAR) of raw materials and cost/g LAB starter strain biomass produced.

Strain number			BFE 6620	BFE 6625	BFE 7589	BFE 7596	BFE 6748	BFE 6793	BFE 6710	BFE 6739	BFE 6688	BFE 6711	BFE 6713	BFE 7685	BFE 7687	BFE 7688	BFE 7601	BFE 7608	BFE 7668
Genotypic identification			*L. fermentum*		*L. pentosus*			*L. plantarum*									*W. parames*[a]		*L. mes ssp. mes*[b]
Unit Costs	Unit	R/unit	R/L																
Yeast extract	g	0.26	1.3	1.3	5.2	5.46	5.2	5.46	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.46	1.3	1.3	1.3
Peptone	g	0.348	3.48	3.48	0	0	0	0	0	0	0	0	0	0	0	0	3.48	3.48	3.48
Beef extract	g	0.754	3.77	3.77	0	0	0	0	0	0	0	0	0	0	0	0	3.77	3.77	3.77
Potassium phosphate	g	7.82E-3	0.016	0.016	0	0	0	0	0	0	0	0	0	0	0	0	0.016	0.016	0.016
Tween 80	g	0.193	0.193	0.193	0	0	0	0	0	0	0	0	0	0	0	0	0.193	0.193	0.193
Tri-Ammonium citrate	g	0.352	0.704	0.704	0	0	0	0	0	0	0	0	0	0	0	0	0.704	0.704	0.704
Manganese sulphate	g	0.23	0.012	0.012	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.012	0.012	0.012
Magnesium sulphate	g	7.60E-2	0.008	0.008	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.008	0.008	0.008
Sodium acetate	g	0.272	1.36	1.36	0	0	0	0	0	0	0	0	0	0	0	0	1.36	1.36	1.36
Glucose	g	6.37E-3	0.127	0.127	0.319	0.319	0.319	0.319	0.319	0.319	0.319	0.319	0.319	0.319	0.319	0.319	0.127	0.255	0.127
CSL (Afprod)	g	0.004	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0.2	0	0	0
CSL (Roquette)	g	0.0125	0	0	0.125	0	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0	0	0	0
Meat extract	g	0.933	0	0	0.933	0	0.933	0	0.933	0.933	0.933	0.933	0.933	0.933	0.933	0	0	0	0
Iron sulphate	g	0.06	0	0	8E-04	8E-04	8E-04	8E-04	8E-04	8E-04	8E-04	8E-04	8E-04	8E-04	8E-04	8E-04	0	0	0
Total (ZAR/L)			10.97	10.97	6.585	5.987	6.585	5.912	6.585	6.585	6.585	6.585	6.585	6.585	6.585	5.987	10.97	11.1	10.97
Biomass (g/L), DWB			2.775	6.675	9.925	6.075	11.18	6.025	15.925	5.9	5.075	12.175	18.175	18.175	8.75	6	1.35	3.725	2.6
Cost (ZAR/g biomass)			3.953	1.643	0.663	0.986	0.589	0.981	0.414	1.116	1.298	0.541	0.362	0.362	0.753	0.998	8.125	2.979	4.219

[a] *W. parames.* = *Weissella paramesenteroides.*
[b] *L. mes.* ssp. *mes* = *Leuconostoc mesenteroides* ssp. *mesenteroides.*

palm oil, to determine the potential of the starter cultures for growth and acidification of the raw material. The changes in cell concentration, pH, titratable acidity and cyanide are shown in Table 5. The pH dropped from an initial of ca. pH 6.0 to pH 5.2 for both the uninoculated control fermentations after 24 h of fermentation (control 1 and control 2, Table 5). At the same time point, the pH for the fermentations inoculated with the selected starter strains was considerably lower (between pH 4.3 and 4.8). The titratable acidity at 24 h fermentation was 0.8 and 0.9% for control 1 and 2, respectively, while it ranged from 1.1 to 1.3% for the fermentations inoculated with the selected starter strains. Differences regarding pH and titratable acidity at 48 h were also evident between the controls and the selected strains. The titratable acidity for controls 1 and 2 were 1.0 and 1.1%, respectively, while it ranged from 1.3 to 1.6% for the fermentations with the selected starter strains. *L. plantarum* strain BFE 6688 had the highest titratable acidity at 1.6%. The pH for the two controls was between 4.8 and 4.9 at 48 h, while it ranged from 3.9 to 4.6 for the fermentations with the selected starter strains. *L. plantarum* strains BFE 6793 and BFE 6688 showed the greatest pH reduction at 48 h, i.e., down to pH 3.9 (Table 5).

In the controls fermented without addition of starters the cyanide concentration was reduced by 40 ppm, from 100 ppm at the beginning to 60 ppm after 48 h of fermentation (Table 5). The cyanide concentration after 48 h was between 10 and 50 ppm in the fermentations inoculated with the selected starter strains (Table 5). This suggested that for some β-glucosidase negative strains (e.g., BFE 6625 and BFE 7608) the cyanide concentration was up to 40 ppm lower than in the control (Tables 1 and 5), indicating a good reduction in cyanide. However, some starter strains which exhibited β-glucosidase activity (e.g., BFE 7687) reduced the cyanide content to only 10 ppm less than the control (Tables 1 and 5).

LAB counts varied between 9.1 and 10.6 log CFU/g for the fermentations inoculated with the starter strains after 48 h of

Table 5
Summary of cassava mash fermentations (values are a mean of two independent trials) using the selected lactic LAB starter strains.

Starter strain	Counts (log CFU/g)			Titratable acidity (%)		pH			Cyanide content (ppm)	
	0 h	24 h	48 h	24 h	48 h	0 h	24 h	48 h	0 h	48 h
Control 1	7.0	8.0	8.9	0.8	1.0	6.0	5.2	4.9	100	60
Control 2	6.9	8.1	8.9	0.9	1.1	6.0	5.2	4.8	100	60
L. fermentum										
BFE 6620	7.9	9.4	9.2	1.2	1.4	6.4	4.4	4.0	100	10
BFE 6625	8.6	10.2	10.9	1.1	1.4	5.6	4.6	4.3	100	20
L. pentosus										
BFE 7589	8.0	9.4	9.3	1.1	1.5	5.9	4.4	4.0	100	30
BFE 7596	8.1	9.1	9.2	1.1	1.5	6.0	4.4	4.0	100	30
BFE 6748	8.5	9.2	9.4	1.1	1.4	5.7	4.4	4.1	100	20
L. plantarum										
BFE 6793	8.6	10.2	10.5	1.1	1.4	6.1	4.3	3.9	100	10
BFE 6710	8.4	9.3	9.1	1.1	1.5	5.6	4.4	4.1	100	20
BFE 6739	8.5	9.4	9.1	1.2	1.5	5.6	4.4	4.2	100	30
BFE 6688	9.1	9.7	10.3	1.3	1.6	6.4	4.5	3.9	100	10
BFE 6711	8.3	10.2	9.9	1.2	1.4	5.4	4.4	4.1	100	10
BFE 6713	8.2	10.3	10.1	1.2	1.5	5.8	4.3	4.0	100	10
BFE 7685	8.4	10.0	10.6	1.1	1.4	5.9	4.4	4.0	100	10
BFE 7687	7.9	9.6	9.8	1.2	1.5	6.0	4.5	4.0	100	50
BFE 7688	7.8	9.6	9.4	1.1	1.4	5.9	4.4	4.0	100	10
W. paramesenteroides										
BFE 7601	8.3	10.2	10.1	1.1	1.4	6.4	4.7	4.4	100	20
BFE 7608	8.1	10.0	9.8	1.1	1.3	6.2	4.8	4.5	100	20
L. mesenteroides ssp. *mesenteroides*										
BFE 7668	8.3	9.7	9.9	1.1	1.3	6.0	4.8	4.6	100	40

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fermentation. At the same time, counts for the control 1 and 2 fermentations, which were left uninoculated, were both at a level of 8.9 log CFU/g. Thus, the LAB concentration in the inoculated fermentation was up to 1 log CFU/g higher. A more noticeable effect was at 24 fermentation, where the control fermentations showed a lactic acid bacterial count of ca. 8 log CFU/g, while the fermentations with starter culture added were generally more than 1 log unit higher (Table 5). Furthermore, the results indicated that most of the selected strains reached their maximum cell concentration after 24 h, and maintained at this level until 48 h of fermentation time. This differed from the uninoculated controls, in which the total count continued to increase up to 48 h fermentation time. A difference could also be observed with regard to the pH and titratable acidity. The titratable activity was generally approx. 0.5% higher, while pH was noticeably lower in the inoculated fermentations (Table 5).

4. Discussion and conclusions

LAB were selected on the basis of their predominance in the *Gari* fermentation and on the basis of their technological characteristics including production of β-glucosidase and antimicrobial activity as determined previously (Kostinek et al., 2007). It is well documented that the use of starter strains is dependent on variables such as optimal growth, cell concentration and preservation techniques used. These processes need to ensure that cultures are stable in terms of viability and function, as well as ease of use (Desmons, Krhours, Evrard, & Thonart, 1998; Carvalho et al., 2004; Morgan et al., 2006). Generally, all strains performed well in the medium that was selected for the individual strains biomass production in a pilot experiment with different growth media. The survival of strains after freeze drying, however, appeared to be strain dependent. There have been various suggestions related to the loss of viability during storage, and these include cell damage at the cell wall, damage to the cell membrane or damage as a result of membrane lipid oxidation. Different strains of the same species can also differ in their ability to withstand freeze drying and storage. This inter-strain variability could be due to differences in genetic constitution, in cell wall and membrane composition, or other mechanisms that are currently not completely understood (Gómez Zavaglia, Tymczyszyn, De Antoni, & Anibal Disalvo, 2003; Schoug, Olsson, Carlfors, Schnürer, & Hakansson, 2006).

The strains were all freeze-dried using maltodextrin (5%) and glycerol (2%) as cryoprotectants. These agents have been shown to be effective cryoprotectants for LAB (Oldenhof, Wolkers, Fonseca, Passot, & Marin, 2005; Schoug et al., 2006). The strains survived well as they maintained good cell counts after freeze drying (Table 3). *W. paramesenteroides* BFE 7608 showed very good survival among the *Leuconostoc* and *Weissella* strains, showing a final concentration of 12.2 log CFU/g. *L. fermentum* BFE 6625 showed the highest survival amongst the *L. fermentum* strains. For the *L. pentosus* strains, strain BFE 6748 and strain BFE 7589 showed the best survival, whereas *L. plantarum* strains BFE 6713, BFE 7688 and BFE 6710 yielded the highest viable counts among the *L. plantarum* strains after freeze drying. However, it must be noted that *L. plantarum* BFE 7688 took twice as long to complete the biomass production stage (Table 3), which may be a disadvantage in terms of the overall production costs as a consequence of higher energy requirements. From an economical point of view, clearly the *L. plantarum*-group strains (i.e., *L. pentosus* and *L. plantarum* strains) were the ideal candidates for utilisation as starter cultures, as they could generally be produced at the lowest cost of below 1 ZAR/g biomass (or approx. 0.13 USD/g) (Table 4).

An important technological property for all the selected strains is to their ability to ferment the cassava and to reduce the pH. In a previous study, the *L. plantarum* starter strain BFE 6710 was successfully used to quickly acidify and to predominate a cassava fermentation for *Gari* production (Huch et al., 2008). The success of different freeze-dried starter strains, including *L. plantarum* BFE 6710 to ferment fortified, bitter cassava was also investigated in this study. The *Gari* processing was replicated to match the production as documented in Benin under field conditions. The strains performed well in the small-scale bucket fermentations. For *L. plantarum* BFE 6710, this confirmed our previous results in which this strain was also shown to establish itself well in the fermentation (Huch et al., 2008). All strains in this study showed a rapid acidification, evidenced by an increase in titratable acidity, ranging from 1.1 to 1.3% at 24 h, and 1.3–1.6% at 48 h (Table 5). This correlated well with a rapid decrease in pH that was observed to range from 4.3 to 4.8 at 24 h, and 3.9 to 4.5 at 48 h. Quick acidification is important to prevent undesirable bacteria from growing during fermentation. The required pH to inhibit undesirable bacteria should be at least pH 4.2. This is because spoilage bacteria, as well as pathogens, notably those including members of the *Enterobacteriaceae,* do not grow below this pH level (Holzapfel, 2002). The effect of the starter was obvious in that it lowered the pH much faster and to lower levels than the control, which in traditional cassava fermentations is carried out by the autochthonous bacteria (Okafor & Ejiofor, 1985).

Starter culture addition provides important advantages when compared to spontaneous fermentations without starter cultures. Spontaneous fermentations generally take a long time (~96 h) to complete, i.e., to lower the pH to a sufficient level and to improve the product's structural and sensory characteristics. Initiation of the process can take a relatively long time (24–48 h) and there is also the risk that contaminating microorganisms compete with the desirable microorganisms (Holzapfel, 2002). Adding a starter culture reduced fermentation times notably, as demonstrated in this study, by a fast decrease in pH, and noticeable increase in acidity when compared to the controls. This helps reduce the risk of growth of contaminating microorganisms and also contributes to more control over aroma, texture and flavour of the final product (Holzapfel, 2002; Leroy & De Vuyst, 2004).

The determination of the cyanide content in the fermentations inoculated with starter strains compared to the control fermentation may lead to the assumption that the starter cultures contributed in varying degrees to the linamarin degradation. However, since some β-glucosidase negative strains also showed high reduction levels, and some β-glucosidase positive strains almost none, this result could have been a reflection of the inaccuracy of determining the actual levels with the picrate paper. The method for determining cyanide in this study depends on a visual evaluation of the picrate paper, which is quite difficult especially at similar concentrations. For this reason Bradbury et al. (1999) suggested to resuspend the picrate paper in diluent and subsequently determine concentrations spectrophotometrically for a more accurate determination, which was not possible to do in this study. Microorganisms were previously suggested to contribute to the degradation of linamarin due to the presence of β-glucosidase activities. This enzyme removes the glucose from the aglycone of the cyanogenic glucoside and it is then available for bacterial metabolism, especially when other sugars are limited (Brimer, 1994). Sequencing the genome of a *L. plantarum* WCFS1 strain showed that *L. plantarum* harbours ten β-glucosidase genes (Kleerebezem et al., 2003), which confirmed also our observations that the *L. plantarum* strains in this study were all positive for β-glucosidase activity (Table 1). In other investigations, we found that the 85% of the linamarin content in a model cassava fermentation was degraded in the first 5 h, probably by the plant enzymes themselves (results not shown). This concurs well with other publications (Agbor-Egbe & Lape Mbome, 2006). Westby and Choo (1994) described that microorganisms

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play little or no role in cyanogen reduction during the fermentation of grated cassava. Although many of the microorganisms present in the fermentation have the ability to hydrolyse linamarin, the major linamarin degrading activity appears to be the result of endogenous linamarase and 95% of linamarin appears to be hydrolysed by the plants endogenous enzyme within 3 h after grating.

Although this work was based on small-scale laboratory fermentations, it provided great insight into the benefit of using starter cultures for *Gari* production in the field, as the experiments were designed to mimic field conditions. It also served to illustrate that the chosen strains indeed had the potential to lower the pH of the fermentation quickly and reliably. Furthermore, the study determined quite well, that the survival of strains upon freeze drying was strain dependent and that therefore, a strains survival during freeze drying and storage must be studied before further development as starter culture. Finally, it showed that especially the *L. plantarum*-group strains could be produced as starter cultures at lower costs than compared to *L. fermentum*, *W. paramesenteroides* or *L. mesenteroides* strains. In addition, the *L. plantarum*-group strains in this study appeared to lower the pH in the model fermentations to lower levels than the *Weissella* or *Leuconostoc* strains, which was also considered an advantage. This, together with the lower costs involved in production, indeed seems to make *L. plantarum*-group strains particularly attractive for development as starter strains for the fermentation of cassava in the production of *Gari*.

Acknowledgements

Financial support from the EU Commission within the framework of the INCO RTD program is gratefully acknowledged. This study was partly 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. Moreover, we would like to thank Naledzani D. Nesengani, Vetja M. Haakuria and Amenan A. Yao for their contribution.

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