Development of a Starter Culture for the Production of *Gari*, a Traditional African Fermented Food

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Declaration

I hereby declare that this thesis is my own, unaided work. It is being submitted for the Degree, Doctor of Technology, to the Durban University of Technology, Durban, South Africa. It has not been submitted before for any degree or examination to any other institution.

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May 2010

Dedication

To

My dad and mum, who sacrificed so much so I could get an education,

My wife, for standing by my side and encouraging me every step of the way,

My son, who makes this all worthwhile,

This is for you… with all my love.

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Summary

Cassava, (*Manihot esculenta* Crantz), is used for the production of a variety of West African foods and ranks fourth in the list of major crops in developing countries after rice, wheat and maize. *Gari* is one of the most popular foods produced from cassava. Cassava may contain high levels of linamarin, a cyanogenic glucoside, which in its natural state is toxic to man. Therefore, some processing methods that can enhance the detoxification of cassava and lead to the improvement of the quality and hygienic safety of the food are vitally important for less toxic products to be obtained. Quality, safety and acceptability of traditional fermented foods may be improved through the use of starter cultures. There has been a trend recently to isolate wild-type strains from traditional products for use as starter cultures in food fermentation. A total of 74 bacterial strains and 21 yeast strains were isolated from a cassava mash fermentation process in a rural village in Benin, West Africa. These strains were assessed, together with 26 strains isolated at the CSIR from cassava samples sent from Benin previously, for phenotypic and technological properties. Twenty four presumptive lactic acid bacteria (LAB) were selected for further phenotypic, genotypic and technological characterization during a research visit to the BFE (now Max Rubner Institute of Nutrition and Food). After assessment, the strains VE 20, VE 36, VE 65b, VE 77 and VE 82 were chosen for further study as starter cultures. These *L. plantarum* strains were chosen on the basis of predominance and possession of suitable technological properties. The investigation of this study was complemented by further, similar studies on further *Gari* isolates in Germany by the BFE. That study was done independently from this study, but both studies served to select potential starter cultures for cassava fermentation for the production of *Gari*, as this was the common goal of the project. Thus, a wider final selection of potential starter cultures was decided on at the project level and this selection was further tested in fermentation experiments. A total of 17 strains were grown in optimized media in 2 L fermenters. These strains were freeze-dried and thereafter tested in lab-scale cassava mash fermentation trials.

The strains performed well in the small scale bucket fermentations. There was a rapid acidification evidenced by the increase in titratable acidity, ranging from 1.1 to 1.3 % at 24 hours, and 1.3 to 1.6 % at 48 hours. The effect of the starter was obvious in that it lowered the pH much faster and to lower levels than the control. It appeared that both the processing and starter culture addition played a role in the removal of cyanide during processing of the cassava into *Gari*. This was evident from the lower cyanide values obtained for fermentations that included starter cultures. The study also 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. Overall the results of this study were crucial for the project in showing that a starter culture which is easy and economical to produce and which has the desired attributes is a feasible possibility for application in the field.

Chapter 1

Introduction and literature review

Cassava, (*Manihot esculenta* Crantz), is used for the production of a variety of West African foods and ranks fourth in the list of major crops in developing countries after rice, wheat and maize (Mlingi *et al*., 1992). Despite the useful application of cassava roots, there are four major drawbacks that limit the utilisation of cassava as a food; i.e., it has low energy density, low protein content, rapid post harvest deterioration and potential cyanide toxicity (Gidamis, 1988, Howlett *et al*., 1990; Mlingi *et al*., 1991; Oyewole and Aibor, 1992; Mlingi, 1995).

Cassava may contain high levels of linamarin, a cyanogenic glucoside, which in its natural state is toxic to man (Wood, 1965). Cyanide inhibits the enzyme called cytochrome C oxidase involved in ATP production in animal cells. This inhibition results in an upper motor neuron disease known as “Konzo” (Howlett *et al*., 1990; Howlett, 1994), which is characterised by abrupt onset of spastic paralysis (Tyllesker *et al*., 1992), nausea and vomiting (Mlingi *et al*., 1992).

The availability of these cynogenic glucosides leads to the limiting factor for the direct utilization of cassava (Rosling, 1990). Therefore, some processing methods that can enhance the detoxification and lead to the improvement of the quality and hygienic safety of the food (Ogunsua, 1980) are vitally important for less toxic products to be obtained.

The microbial stability of cassava is also of utmost importance because it is often consumed as processed food (Sanni, 1996). The fermentation of the cassava into various foods leads to a general improvement in the shelf life, texture, taste, aroma as well as nutritional value (Odunfa and Adeyele, 1985; Uzogara *et al*., 1990). *Gari* is a fermented, roasted, granular, shelf-stable food and is the most important staple food produced from cassava. The traditional processing methods for *Gari* production are, however, time consuming and labour intensive. For example, it takes 96 h to obtain a good quality *Gari* from cassava, of which most of the time is required for the fermentation process. For fast growing populations like those in Nigeria and other Eastern and Western African countries, whose demands for *Gari* far outstrips the supply,

the processing methods must be improved and the processes should not compromise the safety of the products (Kimaryo *et al*., 2000).

Quality, safety and acceptability of traditional fermented foods may be improved through the use of starter cultures (Holzapfel, 2002). A typical starter culture is adapted to the substrate and facilitates improved control of a fermentation process (Holzapfel, 1997).

Characteristics of the starter culture should include degradation of antinutritive factors and detoxification (Holzapfel, 1997, 2002; Hou *et al*., 2000; Wouters *et al*., 2002). Ikediobi and Onyike (1982) demonstrated that it was possible to reduce *Gari* toxicity by adding exogenous linamarase during the fermentation. It has also been reported that inoculation of cassava with linamarase producing microorganisms could reduce the toxicity and that various lactic acid bacteria (LAB) had the ability to hydrolyse linamarin (Giraud *et al.,* 1992).

1.1. Fermentation

Fermentation is considered one of the oldest methods of food processing. There has been archaeological evidence of ground grains in the Nile and Sahara regions between 12 000 and 10 000 B. C., which suggests that early man was already accustomed to acid fermented cereal foods (Wendrof and Schild, 1976). Excavations in Switzerland established that sourdough bread was part of a typical diet over 5000 years ago (Währen, 1990). Reference to fermented dairy products (cheese, yogurt, butter) is documented in archaic texts from Uruk/Warka (a large archeological site in present day Iraq), dated around 3200 B. C. (Nissen *et al*., 1991).

According to Steinkraus (1995), food fermentation serves five main purposes:

i. Enrichment of the diet through development of a diversity of flavours, aromas, and textures in food substrates
ii. Preservation of substantial amounts of food through lactic acid, acetic acid, alcoholic and alkaline fermentations

iii. Enrichment of food substrates biologically with protein, essential amino acids, essential fatty acids and vitamins
iv. Detoxification during food fermentation processing
v. A decrease in cooking times and fuel requirements

1.1.1 Fermentation methods

There are typically three ways that food can be fermented. These are natural (spontaneous) fermentation, back slopping and controlled fermentation. Many fermentation processes are also carried out on solid substrates.

1.1.1.1 Natural (spontaneous) fermentation

Spontaneous fermentations, i.e. processes started without the use of starter inoculum, have been applied in food preservation for thousands of years, by trial and error (Holzapfel, 2002). The strains that are best adapted to the substrate and with the highest growth rate will dominate during particular stages of the process. Bacteria typically dominate the early stages of the fermentation, due to their high growth rate. They are then followed by yeasts in substrates that are rich in fermentable sugars (Holzapfel, 2002).

It is difficult to produce a product with consistent characteristics over a long period of time, using spontaneous fermentation, as the natural microbial flora in the raw materials are not always the same. Spontaneous fermentation also increases the chances of product failure due to the growth of undesirable micropopulations and the possible transmission of foodborne pathogens is high (Ray, 2001).

1.1.1.2 Back-slopping

In many traditional fermentation processes, material from a previous successful batch is added to initiate the new process (Holzapfel, 2002). This process, called back-slopping, involves the use of residue (‘starter dough’) from a previous batch of acceptable quality for inoculation (Holzapfel, 1997).

Back-slopping shortens the initial phase of the fermentation process and there is also a reduced risk of fermentation failure (Holzapfel, 2002). Backslopping is still used to produce numerous fermented foods such as Sauerkraut, cucumbers and sourdough (Stiles and Holzapfel, 1997; Leroy and De Vuyst, 2004), or for products for which the microbial ecology and the role of succession in the microbial population are not well known (Mogensen *et al*., 2002; Leroy and De Vuyst, 2004). However, it is difficult to retain product characteristics over a long period of time due to changes in microbial types (Ray, 2001).

Backslopping and spontaneous fermentation still represent a cheap and reliable preservation technology in less developed countries. On the other hand in western countries, many fermentations are carried out on an industrial scale with defined LAB starter cultures (Leroy and De Vuyst, 2004).

1.1.1.3 Controlled fermentations using starter cultures

Production of fermented foods is based on the use of starter cultures. An example of this is lactic acid bacteria that initiate rapid acidification of the raw material (Leroy and De Vuyst, 2004). A starter culture may be described as a microbial preparation containing high numbers of viable cells of at least one microorganism, which may be added to raw materials to produce a fermented food by accelerating and steering the fermentation process (Holzapfel, 1997; Leroy and De Vuyst, 2004). Starter cultures that possess at least one inherent functional property are known as functional starter cultures. This functional property can contribute to food safety and/or offer one or more organoleptic, technological, nutritional or health advantages (Leroy and De Vuyst, 2004).

Early starter culture developments focused mainly on LAB known to be associated with well accepted fermented foods, which were mostly fermented dairy products (Franz *et al*., 2005). Soon, defined cultures (at least technically) were also introduced for other products, e.g. for sourdough fermentation around 1910. Later, starter cultures were also introduced for fermented meat products (Franz, 2008). Presently, a large variety of lactic

fermentations are employed at levels ranging from household to industrial scale, involving raw products such as milk, vegetables, meat and cereals (Stiles and Holzapfel, 1997; Nout and Sarkar, 1999; Holzapfel, 2002).

The important role of LAB in fermentation is illustrated by their contribution to rapid acidification of the raw material by production mainly of lactic acid. In addition, some strains produce acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides and other important enzymes such as proteases and thereby enhance the shelf life and microbial safety of the fermented product, in addition to improving the product texture and sensory characteristics (Leroy and De Vuyst, 2004; Franz *et al*., 2005).

1.1.1.4 Solid state fermentation

Solid-state fermentation (SSF) has been defined as the fermentation process which involves a solid matrix and is carried out in absence or near absence of free water. The substrate must possess enough moisture however to support growth and metabolism of the microorganism (Pandey, 2003; Singhania *et al*., 2009).

Fungi and yeasts have been considered ideal microorganisms for SSF because of the theoretical concept of water activity, while bacteria have been considered less suitable (Singhania *et al*., 2009). However, there is sufficient literature that shows that bacteria can also be used for successful SSF (Oyewole, 1997; Steinkraus, 1997; Holzapfel, 1997; 2002).

1.2 Lactic acid bacteria (LAB)

1.2.1 Taxonomy of LAB

Gram-positive bacteria group into two of the twenty three major eubacterial phyla, i.e. the *Firmicutes* and *Actinobacteria*. The *Firmicutes* include the classes *Clostridia* (class I), *Mollicutes* (class II) and *Bacilli* (Class III) and the lactic acid bacteria (LAB) are grouped in class III, order II i.e. the

'*Lactobacillales*' (Garrity and Holt, 2001). In one of the earliest definitions, Orla-Jensen (1919) described LAB as Gram-positive, non-motile, non-sporeforming, rod- or coccus-shaped organisms that ferment carbohydrates and higher alcohols to form mainly lactic acid. The classical approach to LAB taxonomy was based on morphological and physiological features (Stiles and Holzapfel, 1997). In contrast, a modern approach for classification of LAB is based on the premise that an unequivocal definition does not exist for this group of bacteria (Axelsson, 2004). Therefore, it was deemed more appropriate to describe the 'typical' lactic acid bacterium, which is Gram-positive, non-sporeforming, catalase-negative, devoid of cytochromes, non-aerobic but aerotolerant, fastidious, acid-tolerant and strictly fermentative, with lactic acid as the major end-product of sugar fermentation (Axelsson, 2004). Variations of this general description are common, and it is only the Gram-positive character really that cannot be argued with (Axelsson, 2004).

The common ancestor of the order *Lactobacillales* (to which the present day LAB belong) developed from a common ancestor of all *Bacilli* (Makarova *et al*., 2006). The origin of the *Lactobacillales* involved extensive loss of ancestral genes. The common ancestor of *Lactobacillales* had at least 2100-2200 genes, losing 600-1200 genes compared with the ancestor of all *Bacilli*, for which the genome size of 2700-3700 genes was predicted from reconstructing the events that occurred during evolution (Makarova *et al*., 2006). The heme/copper type cytochromes/quinol oxidase-related genes and catalase are characteristic for aerobic bacteria. *Lactobacillales* lost these genes during evolution as a transition to life in a nutritionally rich and anaerobic medium (Franz, 2008). Beyond gene loss, *Lactobacillales* exhibit clear ancestral adaptations for nutritionally rich and microaerophilic environments, which include acquisition of genes via horizontal gene transfer and duplication of genes for various enzymes and transporters of sugar and amino acid metabolism (Makarova *et al*., 2006).

Before the advent of bacterial genomics, the taxonomy of the LAB was a little unclear (Franz, 2008). Being based mostly on 16S rRNA gene sequences, however, three closely related lineages of the LAB were initially described by

Woese (1987), i.e. the *Leuconostoc* group, the *Lactobacillus casei/Pediococcus* group and the *Lactobacillus delbrueckii* group. *Carnobacterium, Enterococcus, Vagococcus, Aerococcus* and *Tetragenococcus* were considered more closely related to each other than to any other LAB, while *Lactococcus* and *Streptococcus* appeared to be very closely related to each other and were described to form a separate branch (Schleifer and Ludwig, 1995). The genus *Lactobacillus* alone, which contains more than 130 species, is unusually diverse and its taxonomy has long been considered unsatisfactory because of the highly heterogenous nature of its members (Schleifer and Ludwig, 1995). A recent investigation based on 16S rRNA gene diversity resulted in renaming of the *L. delbrueckii* group to the *L. acidophilus* group, while the *Lactobacillus casei*/*Pediococcus* group was split into further subgroups and a new genus (Dellaglio and Felis, 2005).

The recent availability of complete genomes of representative LAB strains of all major families of the *Lactobacillales* allows for a more definitive analysis of their evolutionary relationships, as it is based on sequences of multiple and suitable marker genes (Makarova *et al*., 2006). Therefore, it gives a clearer picture of the evolutionary relationships of LAB genera than those which were previously based mostly on 16S rRNA genes (Franz, 2008). Now the streptococci-lactococci branch is considered to be basal in the *Lactobacillales* tree (Fig. 1.1), and the *Pediococcus* group is a sister to the *Leuconostoc* group within the *Lactobacillus* clade. Thus, the *Lactobacillus* genus appears to be paraphyletic with respect to the *Pediococccus-Leuconostoc* group (Makarova *et al*., 2006). *L. casei* now is confidently placed at the base of the *L. delbrueckii* group (Makarova *et al*., 2006), which contradicts the previous classification of forming a group with the pediococci (Wood and Holzapfel, 1995; Holzapfel *et al*., 2001).

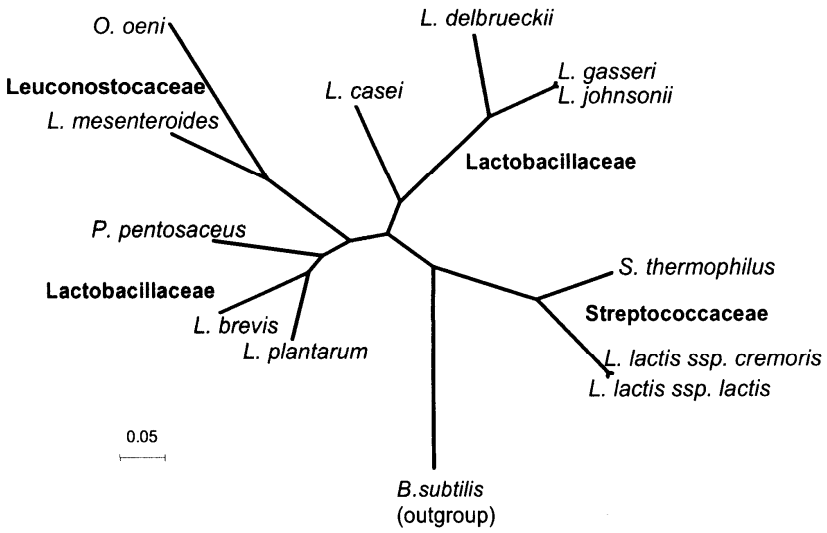

Fig. 1.1 Phylogenetic tree of *Lactobacillales* constructed on the basis of concatenated alignments of ribosomal proteins, adapted from (Makarova *et al*., 2006).

Whole genome comparisons of five *Lactobacillus* species (*L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. johnsonii* and *L. sakei*) that were completely sequenced showed that there is no extensive synteny of the genome sequences of these five species (Canchaya *et al*., 2006), and the observed extreme divergence of the *Lactobacillus* genomes supports the recognition of new subdivisions as proposed by Dellaglio and Felis (2005). Whole genome alignments showed that the sequences with the best alignments were *L. johnsonii* and *L. acidophilus*, but alignments of these two species with the other three species showed much lower degrees of synteny at the interspecies level than observed in other species genome comparisons with high and low-G+C content Gram-positive bacteria (Canchaya *et al*., 2006). These stepwise-decreasing degrees of similarity observed after genome alignments of members of the *L. delbrueckii*/*L. acidophilus* group were considered as a 'hallmark of Darwinian evolution' (Berger *et al*., 2007).

1.2.2 LAB classification at genus level

To date, 12 genera of LAB which are associated with foods have been described, *viz*., *Lactococcus, Enterococcus, Streptococcus, Aerococcus, Pediococcus, Vagococcus, Tetragenococcus, Leuconostoc, Oenococcus, Weissella, Lactobacillus* and *Carnobacterium* (Wood and Holzapfel, 1995; Vandamme *et al*., 1996; Stiles and Holzapfel, 1997) (Table 1.1). The LAB clearly are a heterogenous group when considering their morphologies, type of lactic acid produced as well as growth range capabilities (Table 1.1). Some of these genera were described only recently, i.e. the genera *Enterococcus* and *Lactococcus* were known as group D and group N streptococci, respectively, even until the 1980's (Franz, 2008). The streptococci were eventually divided into the three genera *Streptococcus*, *Enterococcus* and *Lactococcus*, mainly on the basis of 16S rRNA gene phylogenetic studies (Schleifer and Kilpper-Bälz, 1984). The genus *Tetragenococcus* contains only two species, one of which*, Tetragenococcus halophilus*, was classified as *Pediococcus halophillus* only a few years ago. Here also, the reclassification was possible mainly because of 16S sequence data analyses. Similarly, the lactic acid bacterial species formerly known as *Lactobacillus viridescens* and *Leuconostoc paramesenteroides* were re-classified into the new genus *Weissella* as *Weissella viridescens* and *Weissella paramesenteroides*, respectively, again based on 16S rRNA gene sequence comparisons (Franz, 2008). In addition, the wine-*Leuconostoc*, *Leuconostoc oenos*, could also be reclassified as *Oenococcus oenos* based on 16S rRNA sequence analysis (Dicks *et al*., 1995).

Table 1.1 Phenotypic characteristics of lactic acid bacteria used for classification at the genus level. Adapted from Axelsson (2004).

Morphology	Rods		Cocci							
Genus	*Carnobacterium*	*Lactobacillus*	*Aerococcus*	*Enterococcus*	*Lactococcus/ Vagococcus*	*Leuconostoc/ Oenococcus*	*Pediococcus*	*Streptococcus*	*Tetragenococcus*	*Weissella*[a]
Tetrad formation	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose	-[b]	+/-	-	-	-	+	-	-	-	+
Growth at 10°C	+	+/-	+	+	+	+	+/-	-	+	+
Growth at 45°C	-	+/-	-	+	-	-	+/-	+/-	-	-
Growth at 6.5% NaCl	ND	+/-	+	+	-	+/-	+/-	-	+	+/-
Growth in 18% NaCl	-	-	-	-	-	-	-	-	+	-
Growth at pH 4.4	ND	+/-	-	+	+/-	+/-	+	-	-	+/-
Growth at pH 9.6	-	-	+	+	-	-	-	-	+	-
Lactate enantiomer	l	d, l, dl[c]	l	l	l	d	l, dl[c]	l	l	d, dl[c]

+ : positive; - : negative; +/- : response varies between species; ND : not determined
[a] *Weissella* strains may also be rod-shaped
[b] small amounts of CO_2 can be produced, depending on the media
[c] production of d-, l- or dl-lactate varies among the species

1.2.3 Metabolism of LAB

Lactic acid bacteria are all chemo-organotrophs, i.e. they require carbohydrates for their metabolism and growth (Franz, 2008). These bacteria all produce lactic acid as the major end product of carbohydrate metabolism (Holzapfel *et al*., 2001; Axelsson, 2004). Although they ferment simple sugars anaerobically and are not capable of respiration, they are aerotolerant and capable of growth in the presence of oxygen. As LAB are unable to synthesize porphyrin groups (e.g., haeme), this makes them devoid of 'true' catalase activity when grown in laboratory medium which lack haematin or related compounds. Lactic acid bacteria have complex nutritional requirements and in addition to carbohydrate, they require vitamins, amino acids as well as nucleotides for growth (Franz, 2008). The occurrence and association of LAB over the course of evolution with ecosystems which are rich in nutrients (e.g., gastrointestinal tract, milk or fermenting plants, meats or vegetables) can explain their dependency on relatively complex nutrients (Axelsson, 2004).

The inability to respire, based on an absence of functional electron transport chains, ultimately dictates that LAB are relatively inflexible and limited in their metabolic abilities (de Vos, 1996). The fermentation of simple sugars, which serve both as electron donors and acceptors in substrate level phosphorylation reactions, leads to the formation of relatively few end products of metabolism, i.e. mainly lactic acid, carbon dioxide, and acetic acid or ethanol (Fig. 1.2). These end products are formed via the glycolytic pathway (homofermentative) or the phosphogluconate/phosphoketolase pathway (heterofermentative) (de Vos, 1996; Axelsson, 2004). Obligately homofermentative LAB ferment hexoses via the glycolytic pathway by splitting fructose-1,6,-diphosphate in two triose sugar moieties which are further converted to pyruvate and finally to lactate (Kandler, 1983), with a net gain of 2 ATP/mol glucose (Fig. 1.2). The heterolactic fermentation of obligate heterofermentative LAB is initiated by the oxidation of glucose-6-phosphate to gluconate-6-phosphate. This is followed by a decarboxylation reaction and splitting of the resulting pentose-5-phosphate to glyceraldehyde-3-phosphate

and acetyl-phosphate (Fig. 1.2), which are further metabolised to lactate and ethanol with a net gain of 1 ATP/mol glucose (Franz, 2008). In the case that another, alternative electron acceptor such as fructose is available, acetic acid is produced rather than ethanol from acetyl-phosphate, and either oxygen is reduced to H_2O, or H_2O and fructose are reduced to manitol (Kandler, 1983; Cogan, 1995;). The facultatively heterofermentative LAB can utilise pentose sugars and these are split by an inducible phophoketolase, similar as in the case of obligately heterofermentative LAB, into glyceraldehyde-3-phosphate or acetyl-phosphate, which are further converted to lactate and ethanol (Franz, 2008). However, the facultatively heterofermentative lactic acid bacteria do not produce CO_2 as end product of metabolism (Kandler, 1983; Cogan, 1995).

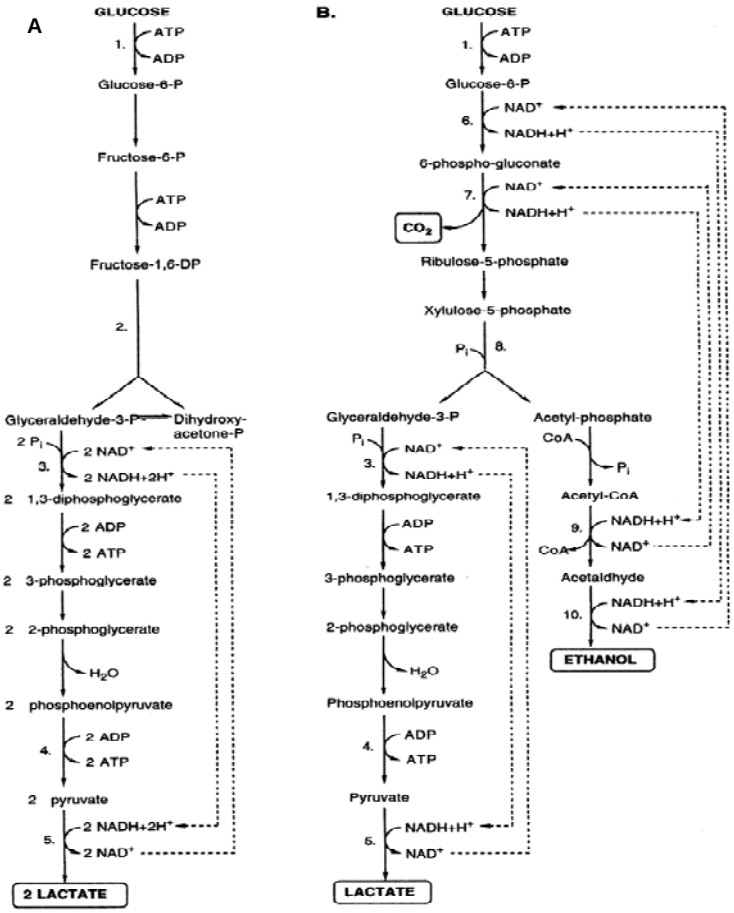

Fig. 1.2 Major fermentation pathways of glucose: A homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway); B heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway) (adapted from Franz, 2008).

1.2.4 LAB in foods – their role, impact and importance

LAB have been utilised either intentionally or unintentionally for the production of fermented milk, meat, vegetable or cereal fermentations for centuries (Franz, 2008). Their association with the human environment and their beneficial interactions, both in food and in the human intestinal tract, combined with the long tradition of production and consumption of lactic fermented foods in many cultures, have led to the general conclusion that this group may be 'generally recognised as safe' ('GRAS') (Holzapfel, 1997). From a historical perspective many of the milk, vegetable, meat and cereal fermentations were empirical processes which relied on the autochthonous microbial populations of the raw materials, simple processing techniques (mechanical size reduction of raw materials, backslopping, i.e. inoculation of raw materials with a residue of a previous batch, mixing and packaging), as well as addition of additives such as salt and/or sugar (Lindgren and Dobrogosz, 1990; Holzapfel *et al*., 1995; Stiles and Holzapfel, 1997; Leroy and De Vuyst, 2004). The fermentation of food with LAB is well known to lead to an improvement of taste, aroma and texture (Lindgren and Dobrogosz, 1990; Holzapfel *et al*., 1995; Stiles, 1996; Leroy and De Vuyst, 2004). An overview of lactic fermented foods and the LAB associated with these products is shown in Table 1.2. An additional effect of fermentation, that relies on the production of organic acids and possibly other antimicrobial compounds such as hydrogen peroxide and/or bacteriocins, is that lactic fermented foods have a longer shelf-life and are also safer with respect to foodborne pathogenic bacteria, when compared to unfermented foods (Lindgren and Dobrogosz, 1990; Holzapfel *et al*., 1995; Leroy and De Vuyst, 2004). A further important advantage of fermentation is that antinutritive factors, which occur especially in plant raw materials (e.g. polyphenols, tannin and phytic acid), as well as indigestible plant polysaccharides are degraded as a result of LAB enzyme activity, thus increasing bioavailability of nutrients and hence the nutritional value of the product (Holzapfel, 1997).

LAB are consumed in enormous quantities, mainly through consumption of fermented foods (Franz *et al*., 2005). According to the bulletin No. 355 of the

International Dairy Federation (IDF), the average annual consumption of fermented milk products is 22 kg per capita in Europe (Gasser, 1994). In total, this amounts to about 8.5 billion kg fermented milk per year. With an average microbial content in these fermented products of 10^8 bacteria per gram (or ml), this amounts to a total of 8.5 x 10^{20} LAB. If you assume that one bacterial cell weighs 4 x 10^{-12} gram, this means that 3400 tons of pure lactic acid bacterial cells are consumed every year in Europe (Franz *et al*., 2005). This figure does not take into account the LAB used in other food fermentations such as vegetable and meat fermentations, or especially the probiotic bacteria consumed as supplements or as pharmaceutical preparations. It can thus be expected to be far greater (Franz *et al*., 2005).

In addition, species from all LAB genera are known to be involved in fermentation of some or other lactic fermented food (Table 1.2). LAB species belonging to the genus *Enterococcus* and *Carnobacterium*, however, are seldom used as starter cultures. This is probably as a result of the association of enterococci and carnobacteria with human disease, and the classification of certain species of these genera into risk group II (Franz, 2008). Most LAB which have caused infections in humans belong to the species *E. faecalis* and *E. faecium* (Murray, 1990; Johnson, 1994; Saxelin *et al*., 1996; Morrison *et al*., 1997), but other LAB species such as *L. rhamnosus*, *L. acidophilus*, *L. jensenii, L. paracasei*, *L. casei*, *L. curvatus, W. confusa, P. acidilactici* and *P. pentosaceus* have also been noted to be associated with human disease (Franz *et al*., 2005). Nevertheless, enterococci and carnobacteria are important components of the autochthonous micropopulations in meat fermentations. *Enterococci* are also considered important especially in ripening and for aroma production of cheeses from Mediterranean countries (Franz, 2008). Thus, these 'non-starter lactic acid bacteria (NSLAB)' often are ascribed a tremendously important role in flavour and aroma production, even though they occur adventitiously and are not intentionally added as starter cultures (Leroy and De Vuyst, 2004).

Table 1.2 Fermented foods and beverages with which lactic acid bacteria are typically associated (adapted from Leroy and De Vuyst, 2004).

Fermented product	Associated lactic acid bacteria
Milk products	
Firm cheese without eyes	*L. lactis* ssp. *lactis*; *L. lactis* ssp. *cremoris*
Cheese with small eyes	*L. lactis* ssp. *lactis*; *L. lactis* ssp. *lactis* var. *diacetylactis* *L. lactis* ssp. *cremoris, Leuc. mesenteroides* ssp. *cremoris*
Swiss and Italian type cheese	*Lb. delbrückii* ssp. *lactis*; *Lb. helverticus, Lb. casei, Lb. delbrückii* ssp. *bulgaricus*, *S. thermophilus*
Butter and buttermilk	*L. lactis* ssp. *lactis*; *L. lactis* ssp. *lactis* var. *diacetylactis* *L. lactis* ssp. *cremoris, Leuc. mesenteroides* ssp. *cremoris*
Yogurt	*Lb. delbrückii* ssp. *bulgaricus*, *S. thermophilus*
Fermented probiotic milk	*Lb. casei, Lb. acidophilus, Lb. rhamnosus, Lb. johnsonii*
Kefir	*Lb. kefir, Lb. kefiranofaciens, Lb. brevis*
Meat products	
Fermented sausages (Europe)	*Lb. sakei, Lb. curvatus, E. faecium*
Fermented sausages (North America)	*P. acidilactici, P. pentosaceus*
Fish products	*Lb. alimentarius, C. piscicola*
Vegetable products	
Sauerkraut	*Leuc. mesenteroides, Lb. plantarum, P. acidilactici*
Fermented vegetables	*P. acidilactici, P. pentosaceus, Lb. plantarum, Lb. fermentum*
Olives	*Leuc. mesenteroides, Lb. pentosus, Lb. plantarum,*
Pickles	*Leuc. mesenteroides, Lb. plantarum, P. cerevisiae, Lb. brevis*
Soy sauce	*T. halophilus*
Cereals	
Sour dough	*Lb. sanfranciscensis, Lb. pontis, Lb. farciminis, Lb. fermentum, Lb. brevis, Lb. plantarum, Lb. amylovorus, Lb. reuteri, Lb. panis, Lb. alimentarius, W. cibaria*
Alcoholic beverages	
Wine	*O. oeni*
Rice wine	*Lb. sakei*

C. = Carnobacterium; L. = Lactococcus, Lb. = Lactobacillus, *Leuc*. *= Leuconostoc*, *O*. *= Oenococcus*, *P*. *= Pediococcus*, *S*. *= Streptococcus*, *T*. *= Tetragenococcus*, *W*. *= Weissella*

1.2.5 LAB as protective cultures

The preservative action of LAB in foods is a result of formation of fermentation end-products with antimicrobial activity (Lindgren and Dobrogosz, 1990; Holzapfel *et al*., 1995). Such antimicrobial compounds include: organic acids (e.g., lactic, acetic and formic); carbon dioxide; hydrogen peroxide (in the presence of oxygen); diacetyl, aldehydes (e.g., β-hydroxy- propionaldehyde) and bacteriocins (Lindgren and Dobrogosz, 1990; Holzapfel *et al*., 1995; Nes *et al*., 1996; Drider *et al*., 2006). The bacteriocins have especially received great interest in recent years for potential application as food 'biopreservatives' (Franz, 2008). Consumers today demand food of high quality, which is less severely processed (less intensive heating, minimal freezing damage), less heavily preserved, more natural (free of artificial additives) and safer (Gould, 1992). These demands are addressed by food processors by the emergence of a new generation of chill stored minimally processed foods (Ohlsson, 1994; Stiles, 1996). However, minimal processing may lead to a loss of intrinsic preservation (e.g., less sugar, salt, preservatives) and to a loss in protection from processing (e.g., less severe heating) and, as a result, new preservation technologies are sought which employ natural antimicrobials and which prolong shelf life or safeguard foods from foodborne pathogens (Ohlsson, 1994; Holzapfel *et al*., 1995). Such a 'natural' preservation possibility is offered by biopreservation technology, which makes use of either 'natural' LAB microflora as so-called 'protective cultures', or their antimicrobial metabolites, notably bacteriocins (Holzapfel *et al*., 1995; Stiles, 1996).

1.2.5.1 Organic acids and pH reduction

LAB fermentation products are characterised by the accumulation of organic acids, primarily lactic and acetic acid, and the accompanying reduction in pH (Lindgren and Dobrogosz, 1990). The acid production and the accompanying pH decrease extend the lag phase of sensitive organisms (Smulders *et al*., 1986). According to Holzapfel (2002), organic acids, which show strong antagonistic effects in the undissociated form at lower pH values, are

particularly effective in inhibiting Gram-negative bacteria, such as pathogens. Relatively large numbers of LAB need to be present to produce sufficient acid to achieve a useful inhibition of bacterial pathogens. Studies have shown that bacterial pathogens are inhibited when added to a pre-fermented food where LAB have grown to large numbers and the pH is already low (Nout *et al*., 1989; Mensah *et al*., 1990; Svanberg *et al*., 1992).

1.2.5.2 Carbon dioxide

CO_2 production by heterofermentative LAB may also contribute to food preservation. The CO_2 accumulation in fermented plant products is the result of an endogenous respiration of the plant cells combined with microbial activities (McDonald, 1981). The protective role of CO_2 is especially important in the fermentation of silages and vegetables to prevent growth of moulds (Lindgren and Dobrogosz, 1990). CO_2 inhibition of microorganisms is widely exploited on a commercial scale in modified and controlled atmosphere packing of foods. The role of CO_2 in lactic acid fermentations has not yet been defined, though its greatest contribution is likely to be at the start of fermentations, when the less acid-tolerant heterofermenters can grow and when CO_2 can inhibit the large numbers of aerobes that are present and favour the growth of LAB as the dominant microorganisms in the fermentation (Adams and Nicolaides, 1997).

1.2.5.3 Hydrogen peroxide

LAB produce hydrogen peroxide (H_2O_2) as a result of the activity of NADH oxidase, in the presence of oxygen (Axelsson, 2004). Furthermore, H_2O_2 can be synthesised from pyruvate, α-glycerophosphate and even from lactate. Accumulation of H_2O_2 can also result from dismutation of superoxide anions (O_2^-) by the action of a superoxide dismutase that is present in most LAB, or by manganese-ions that are present in the cytoplasm of bacteria lacking superoxide dismutase (De Vuyst and Vandamme, 1994). Since LAB are generally catalase-negative, H_2O_2 can accumulate in the medium and its

inhibitory effect against organisms such as *S. aureus* and *Pseudomonas* spp. has been demonstrated (Adams and Nicolaides, 1997). The bactericidal effect of H_2O_2 has been attributed to its strong oxidising effect on the bacterial cell (Fooster *et al*., 1957), and to the destruction of basic molecular structures of cell proteins (Sykes, 1965). However, LAB can also possess NADH peroxides, which use H_2O_2 as an electron acceptor with the formation of H_2O (Axelsson, 2004).

1.2.5.4 Diacetyl

Diacetyl, which is an important flavour volatile in a number of fermented dairy products (Varman and Sutherland, 1994), is produced by strains of several species of LAB from pyruvate, generated from carbohydrate metabolism (Ray and Sandine, 1992; Adams and Nicolaides, 1997). The antibacterial activity of diacetyl has been described for a number of organisms including *Aeromonas hydrophila*, *Bacillus* spp., *Staphylococcus aureus* and *Yersinia enterocolitica* (Jay, 1982; Motlagh *et al*., 1991). Although diacetyl levels necessary to produce appreciable inhibition are usually regarded as too high (about 200 mg/kg) when considering the acceptable sensory levels (2-7 mg/kg) for use in dairy products (Luecke and Earnshaw, 1991), recent studies have suggested that lower diacetyl concentrations can be effective as an antimicrobial, particularly at lower temperatures (Archer *et al*., 1996).

1.2.5.5 Ethanol

Ethanol is a well established anti-microbial and is produced by the heterofermentative LAB under anaerobic conditions (Adams and Nicolaides, 1997). The amounts of ethanol and acetic acid produced are inversely related, since these compounds represent alternative fates of acetyl phosphate in the heterofermentative pathway. As with acetic acid, ethanol may make a significant contribution in the early stages of natural fermentations when heterofermenters are most active (Adams and Nicolaides, 1997).

1.2.5.6 Bacteriocins

Bacteriocins are extracellularly released peptide antimicrobials produced by bacteria which are inhibitory to other, normally very closely related bacteria (Adams and Nicolaides, 1997). Since LAB are widely regarded as food-grade microorganisms, any antimicrobials they produce could be considered as 'natural' food additives and are therefore more acceptable than many other food preservatives (Adams and Nicolaides, 1997). Currently, only one bacteriocin, i.e. nisin, which is produced by strains of *Lactococcus lactis*, is permitted for use as a food preservative in some countries (Delves-Broughton, 1990). It has a broader spectrum of activity than most other bacteriocins, being inhibitory to most Gram-positive organisms (Adams and Nicolaides, 1997). Nisin inhibits *S. aureus* and *Listeria monocytogenes*. However, the application of LAB bacteriocins for the preservation of fermented food is generally not very useful, because the bacteriocins are not active against Gram-negative bacteria (the majority of diarrhoeagenic bacterial pathogens) under normal circumstances (Adams and Nicolaides, 1997). Furthermore, *L. monocytogenes* has been shown to acquire nisin resistance quite readily (Harris *et al*., 1991; Ming and Daeschel, 1993). Also, it is known that the antimicrobial activity of bacteriocins is dependent on the food matrix and is greatly affected by fat, temperature and ionic strength (Blom *et al*., 1997; Leroy and De Vuyst, 2005).

1.3 Cassava

Cassava is a staple food for more than 500 million people in the developing world (Cock, 1985). Cassava is the second most important staple, after maize in terms of calories consumed for approximately 40% of the African population (Nweke, 1992).

Cassava was first cultivated in Brazil more than 4000 years ago. It was introduced in Africa during the 16th century by Portuguese navigators who took it from Brazil to the west coast of Africa and later to east Africa through Madagascar (Jennings, 1976). It only reached Asia about 150 years ago.

Today, about 40% of global cassava output occurs in Africa, the rest being produced by Asia and Latin America almost equally (Nestel, 1980).

Cassava is a typical tropical plant that grows in a variety of conditions. It is a perennial that grows under cultivation to a height of approximately 2.4m (Grace, 1977). It has the ability to grow in poor and acidic soils, which are not suitable for other crops. It yields a harvest in times of drought when all other crops have failed because of a lack of water (Mlingi, 1995). Cassava is often described as a famine killer because it can be harvested any time from nine months after planting and can be left growing for up to two years (Otto, 1988; Nofal, 1999).

1.3.1 Cassava uses

Cassava has various uses, both at a household level and at industrial scale. It is used as a raw material for starch extraction, alcohol production, as sun dried chips for animal feed and incorporated into bread making (Kimaryo, 2000). Cassava is also used to produce *Gari*, a fermented food, which is a staple diet for much of the population in West Africa (Caplice and Fitzgerald, 1999).

1.3.1.1 Ethanol production

Cassava pulp which is a by-product of starch manufacturing is a major biomass resource in Southeast Asian countries. Kosugi *et al*. (2009) successfully used the yeast strain *S. cerevisiae* K7G to produce ethanol from cassava pulp. Ethanol is increasingly used as an alternative fuel in the transportation sector. It may not be practical to use cassava directly for the production of ethanol because it is also an important source of food and feed. The use of agricultural wastes, in this case cassava pulp, as a source for ethanol production is therefore an effective alternative because it contains large amounts of starch and cellulosic substances that can be hydrolyzed and fermented to make ethanol (Kosugi *et al.*, 2009).

1.3.1.2 *Gari*

Gari is one of the most popular foods derived from fermented cassava and is consumed by more than 200 million people in West Africa (Okafor and Ejiofor, 1990). *Gari* is a granular, starchy food which is produced from cassava by fermenting grated pulp, followed by dewatering, sieving and roasting (Okafor, 1977a; Vasconcelos *et al*., 1990). *Gari* is consumed in different forms. It is primarily consumed in the form of Eba. This is prepared by soaking *Gari* in boiling water to swell the starch to get a semi-solid, plastic dough. The dough is rolled into a ball of approximately 15 g with the fingers, and is dipped into a stew containing vegetables, palm oil, and meat or fish (Okafor, 1977a; Onyekwere and Akinrele, 1977; Nissen *et al*., 1991). *Gari* is also eaten without stew, especially in hot weather, by merely soaking it in cold water with added sugar or salt, or it may be eaten with coconut. *Gari* is made into a gelled product, in some West African countries, by sprinkling with cold water and blending in fried palm oil containing corned beef or fresh fish (Okafor, 1977a; Onyekwere and Akinrele, 1977; Nissen *et al*., 1991).

1.3.1.2.1 Fortifying *Gari*

A study to determine the effect of soy supplementation on *Gari* was conducted by Edem *et al.* (2001). Their findings suggested that the consumption of soy *Gari* at 10% soy protein supplementation would help to reduce the problems of protein deficiency disorders in *Gari*-eating areas.

Table 1.3 shows the difference in nutritional value between *Gari* produced from cassava (traditionally) and *Gari* produced from this substrate combination, during a natural fermentation process (without using starter inocula) (Egounlety *et al*, 2001). The results show a significant increase in nutritional value after supplementation with soy bean and palm oil during a natural fermentation process.

Table 1.3 Composition of *Gari* and fortified *Gari* (g/kg) (Egounlety *et al.*, 2001)

Composition	Moisture (%)	Kj	Kcal	Prot. (%)	Fat (%)	Carb. (%)	Ash (%)	Vit A (ug)
Ordinary *Gari*	4.4	1673	400	1.9	0.7	96.7	0.7	0
Soy-palm oil *Gari*	4.9	1757	420	7.0	4.9	87.0	1.0	755.4

1.3.1.3 Other fermented foods derived from cassava

'Abacha' or 'Akpu-mmiri' refers to wet cassava chips that are consumed as a popular snack in southeastern Nigeria. 'Abacha' is prepared by washing, peeling and boiling the cassava root tuber in water for about 1 hour. It is then cut into long slices or chips. The chips are steeped in water for 1-2 days left to ferment. The chips are then washed a few times in fresh cold water (Iwuoha and Eke, 1996). An alternative long term storage method is to dry the chips under the sun for several days (Uzogara *et al*., 1990).

'Elubo' or 'lafun' is a fermented cassava dry flour commonly consumed in the western states of Nigeria (Uzogara *et al*., 1990; Iwuoha and Eke, 1996). It is produced by peeling the cassava roots, washing, cutting into chunks, soaking in pots or at edges of a stream and left for 3-4 days to ferment and soften. After the fermentation process, the softened chunks are dried under the sun for 2 days, ground and sieved to produce 'lafun' or 'elubo' (Uzogara *et al*., 1990).

'Fufu' or 'utara akpu' is a meal of soaked fermented cassava roots which is consumed in eastern Nigeria (Iwuoha and Eke, 1996). To produce 'fufu' the cassava tubers are peeled, washed, cut into thick chunks and steeped in earthenware pots or in a slow-flowing stream for 4-5 days to ferment, soften and produce a characteristic of retted cassava meal (Iwuoha and Eke, 1996). The tubers are then disintegrated in clean water, sieved and allowed to settle

for the water to be decanted. The sediment is the 'fufu' (raw) which can be consumed by hydrothermal gelatinization to form stiff dough (Hahn, 1989; Uzogara *et al*., 1990).

1.3.2 Cyanogenic glucosides in cassava

Cyanogenic glucosides are secondary plant products present in many plants consumed by humans or animals (Conn, 1979). Depending on the amount of cyanide present, cassava roots can be classified into toxic (bitter) and non-toxic (sweet) varieties. The sweet varieties also contain cyanogenic glucosides, but in a lower percentage (Kostinek, 2007). Bitter cassava accumulates the cyanogenic glucosides linamarin (alpha-hydroxyisobutyronitrile-*β*-D-glucopyranoside) and lotaustralin (2(R)-hydroxy-2-methylbutyronitrile-*β*-D-glucopyranoside) in roots and leaves in ratios of about 93:7 (Nartey, 1968). The cyanogenic glucosides are largely localised within the vacuoles of the plant cells, while their hydrolytic enzymes (*β*-glucosidases) are cytoplasmic (Nartey, 1978). Upon cell disruption, they give rise to the release of hydrogen cyanide (HCN) through the action of the *β*-glucosidases (linamarases) (Conn, 1980; Hughes *et al*., 1992). This enzyme is an endogenous *β*-glucosidase, i.e. produced by the plant itself (Giraud *et al*., 1992). Generally, fermentation has been assumed to contribute to the reduction in cyanogenic content (Kostinek, 2007). This is supported by the fact that LAB and yeasts, which are responsible for the fermentation process, are also thought to contribute to linamarin degradation by β-glucosidase activity (Ikediobi and Onyike, 1982; Padmaja and Balagopal, 1985; Okafor and Ejiofor, 1990; Giraud *et al*., 1992, 1993; Okafor *et al*., 1998a; Okafor *et al*., 1998b). Okafor and Ejiofor (1985, 1990) described the ability of a *Leuconostoc* sp. to break down linamarin by the activity of 'linamarase'. Amoa-Awua *et al*. (1996) showed that many strains of *L. plantarum* possess β-glucosidase activity. The presence of β-glucosidase in *L. plantarum* and *L. mesenteroides* correlates well with the ability to break down cyanogenic glucosides (Lei *et al*., 1999). Genomic data available currently has indicated that both of the sequenced *L. plantarum* and *L. mesenteroides* strains contain

genes associated with *β*-glucoside sugar metabolism (Kleerebezem *et al*., 2003; Makarova *et al*., 2006).

The ability to produce HCN from cyanogenic compounds is called cyanogenesis (Kostinek, 2007). Cyanogenesis of linamarin is a two-step process (Fig 1.3). The enzyme linamarase (EC 3.2.1.21) hydrolyses linamarin to glucose and acetone cyanohydrin (Mpong *et al*., 1990; Egan *et al*., 1998). Acetone cyanohydrin can be degraded enzymatically (hydroxynitrile lyase, EC 4.1.2.39), but also decomposes spontaneously at pH $>$ 5.0 into acetone and hydrogen cyanide (Nartey, 1968; Cooke, 1978).

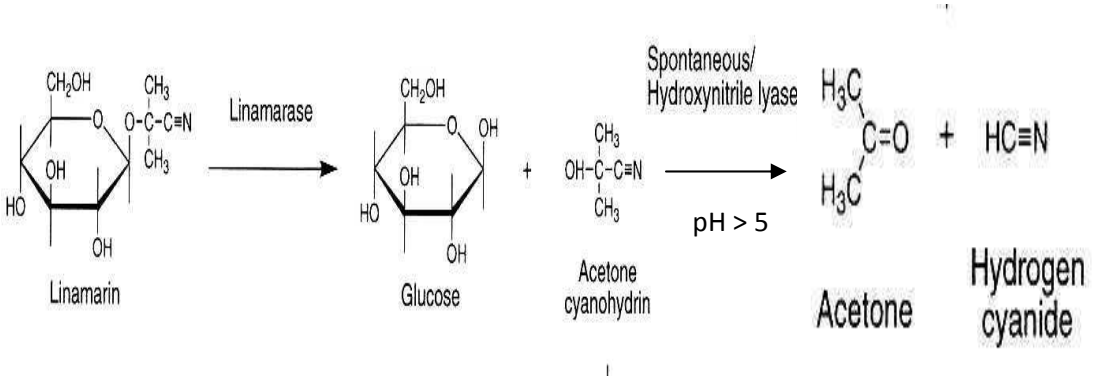

Fig. 1.3 Biochemical degradation of linamarin showing the two-step process

HCN has a boiling point of 25.7°C, which makes it gaseous at tropical temperatures and thus poses problems in cassava fermentation. The maximum allowable concentration for humans is 10 ppm or 11 mg/m^3 of air. It can become a toxic hazard through ingestion, inhalation and skin absorption (Kostinek, 2007). HCN is a true protoplasmic poison, combining in the tissues with the enzymes associated with cellular oxidation (i.e. the cytochrome oxidases). The altered enzymes thereby render oxygen unavailable to the tissues, causing death through asphyxia (Kostinek, 2007). It was observed that the peeling, grating and garifying stages were particularly dangerous to process workers in view of HCN intoxication (Okafor, 1977a; Onyekwere and Akinrele, 1977; Nissen *et al*., 1991). In order to prevent further outbreaks of diseases associated with cassava consumption, it is necessary to determine the cyanogenic potential of cassava roots and cassava products such as flour

or *Gari* (Kostinek, 2007). Egan *et al*. (1998) developed a simple picrate paper kit method for the semi-quantitative determination of the cyanogenic potential for cassava flour. This simple method is targeted for the use in the field by relatively unskilled persons in the developing countries (Egan *et al*., 1998).

1.3.2.1 Exposure to cyanide from cassava

The average lethal dose of cyanide in humans is approximately 1-3 mg/kg body weight (Emsley, 2008). Although hydrocyanic concentrations of 15 – 400 mg/kg of fresh weight in cassava varieties are reported in the literature, more frequent values fall within the interval of 15 – 150 mg/kg (Cereda and Mattos, 1996). Some cassava varieties, however, contain concentrations above 1000 mg/kg of cyanide (Cereda and Mattos, 1996).

A study has shown that there is exposure to cyanide after consumption of *Gari*, but the amount absorbed from a single meal is small and unlikely to cause acute intoxication (Oluwule *et al*., 2002). The toxicity of ingested glucosides in man is not well understood (Essers *et al*., 1993). Whilst there are few reports of poisoning and death due to cyanide intake from cassava consumption (Mlingi *et al*., 1992; Akintonwa *et al*., 1994; Cliff, 1994), there are several disorders which have been associated with regular intake of sub-lethal quantities of cyanogens (Egan *et al*., 1998). These include goitre and cretinism, which are due to iodine deficiency, but may be exacerbated by intake of cyanide (Delange and Ahluwalia, 1983; Delange *et al*., 1994).

There have been occurrences of tropical ataxic neuropathy (TAN), which causes unsteady walking, produces loss of sensation in hands, deafness, loss of vision and weakness amongst mainly older people in Nigeria who have consumed *Gari* and other cyanide containing cassava products over many years (Howlett, 1994; Osuntokun, 1994 – cited by Bradbury, 2009; Bradbury, 2009). Ingested cyanohydrins are presumed to decompose at the alkaline pH level in the small intestine to yield equal molar amounts of cyanide. The long transit time of absorbed cyanide in the plasma suggests that frequent intake

of *Gari* could cause cyanide to accumulate in the plasma (Oluwule *et al*., 2002).

There is a much lower total cyanide content in *Gari* compared to cassava flour and this could account for the apparent absence in West Africa of konzo (Cardoso *et al*., 2005), an irreversible paralysis of the legs that occurs particularly in children and in women of child bearing age in Eastern, Southern and Central Africa due to high cyanide intake associated with bitter cassava (Howlett *et al*, 1990; Banea *et al*., 1992; Cliff, 1994; Howlett, 1994; Ernesto *et al*., 2002; Ministry of Health Mozambique, 1984 – cited by Bradbury, 2009).

1.3.3 The role of LAB populations in cassava fermentation

Two organisms, a *Corynebacterium* sp. and *Geotrichum candidum*, were reported to be responsible for acid production and flavour development in early *Gari* fermentation studies (Collard and Levi, 1959; Collard, 1963). Among the microorganisms isolated from native *Gari*, *L. plantarum* produced the most *Gari*-like flavour according to Ngaba and Lee (1979). Okafor (1977b) was able to isolate and enumerate substantial numbers of organisms from five different genera: *Leuconostoc*, *Alcaligenes*, *Corynebacterium*, *Lactobacillus* and *Candida*, using a variety of media and conditions for incubation.

Meraz *et al*. (1992) reported the presence of species belonging to the genera *Lactobacillus*, *Corynebacterium*, *Streptococcus* and *Leuconostoc* in cassava fermentations. *Lactobacillus, Pediococcus, Clostridium, Propionibacterium* and *Bacillus* were identified by Miambi *et al.* (2003) as representative genera in fermented cassava. Figueroa *et al.* (1995) suggested that a sour cassava starch fermentation proceeds by a succession of two microbial groups: heterofermentative LAB, mainly *Leuconostoc*, start the acidification, followed by a second population of homofermentative strains, dominated by *L. plantarum*. Figueroa *et al*. (1995) also reported the presence of the *Lactobacillus* species *L. brevis, L. paracasei, L. curvatus* and two *Lactococcus* species (*L. lactis* and *L. raffinolactis*).

The majority of studies on the microbiology of indigenous cassava fermentations have been performed by analysing isolates cultured on plates (Ngaba and Lee, 1979; Okafor *et al*., 1984; Oyewole and Odunfa, 1988). None of these studies, however, approached the microbial diversity investigations or the identification of LAB species involved in the fermentation from a polyphasic taxonomical point of view (Kostinek, 2007). Thus, these studies did not rely on a combination of physiological and molecular biological (fingerprinting) identification techniques, which would have allowed an exact LAB diversity investigation, or which would have led to accurate species determination (Kostinek, 2007). In addition, most studies on traditional fermentations did not include quantitative data of the strains that were isolated (Miambi *et al*., 2003).

When advanced, molecular characterisation methods became more readily available and popular, a few studies were done to investigate the microbial community responsible for the fermentation of cassava. Morlon-Guyot *et al*. (1998) isolated a new amylolytic species, *Lactobacillus manihotivorans* from cassava sour starch fermentation, and proved its status as a new species with 16S rRNA sequencing, the protein pattern profiles and the determination of the mol% G+C content of the DNA. Miambi *et al*. (2003) investigated representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods and, as mentioned above, identified *Lactobacillus, Pediococcus, Clostridium, Propionibacterium* and *Bacillus* as representative genera. However, such a complex flora was not described to be associated in fermentation of cassava for the production of *Gari* in any other studies. The fact that the flavour of *Gari* is produced by the fermentative activities of LAB and yeasts is now well accepted (Ngaba and Lee, 1979; Okafor and Uzuegbu, 1987; Okafor and Ejiofor, 1990). *Gari* with a total acidity of 0.58 to 1.2% as lactic acid has been reported to exhibit satisfactory flavour (Okafor, 1977a; Onyekwere *et al*., 1989; Nissen *et al*., 1991).

1.4 Scope of the study

The GARISECURE project was initiated in September 2002 and this study formed part of the EU INCO-DEV Contract No.: ICA4-CT-2002-10058. The project titled 'Improving the quality and nutritional status of *Gari* through the use of starter cultures and fortification with soybean, palm oil and coconut milk' was conducted by a consortium consisting of six partners:

i. Council for Scientific and Industrial Research (CSIR), Biosciences, South Africa
ii. World Association of Industrial and Technological Research Organisations, Denmark
iii. Department of Nutrition and Food Science, National University of Benin
iv. The Walloon Centre of Industrial Biology, University of Liege, Belgium
v. Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition (now Max-Rubner Institute of Nutrition and Food), Germany
vi. Department of Food Science and Technology, University of Nairobi, Kenya

The purpose of the project was to improve the nutritional value and safety of *Gari*, an African traditional food derived from fermented cassava mash. The researchers also aimed to reduce the duration of the production process through the development of a starter culture and to empower rural women to consistently produce at large scale. By using a starter culture, the project aimed to improve the product's nutritional quality and product standardisation, prolonging shelf life and improving distribution and marketing possibilities.

Increasing the protein and vitamin content of *Gari* using a substrate combination of cassava, soybeans and palm oil were aimed to therefore contribute to the health and well-being of consumers, and would also offer opportunities for employment creation in several communities.

The CSIR's role in the project was to contribute to:

i. the isolation, screening and selection of appropriate strains
ii. starter culture development
iii. small scale cassava fermentations.

1.4.1 Aim

The aim of this study was to contribute to the development of a starter culture for the production of *Gari*, fortified with soybean and palm oil.

1.4.2 Objectives

i. To isolate and screen lactic acid bacteria (LAB) and yeast from fermenting cassava mash and other cassava samples.
ii. To select LAB on the basis of technological traits such rapid lactic acid production, detoxification of cyanogenic glucosides, and the production of α-amylase.
iii. To identify LAB by the application of classical biochemical and molecular biological methods.
iv. To grow the LAB in small scale fermenters for use as inoculum.
v. To conduct a preliminary cost analysis for biomass production.
vi. To test the selected starter cultures in small scale, fortified *Gari* fermentations.

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Chapter 2

Isolation and screening of microorganisms from a *Gari* fermentation process for starter culture development

2.1 Introduction

Cassava is a staple food for more than 500 million people in the developing world (Cock, 1982, 1985). It ranks fourth after rice, wheat and maize on the list of major food crops in developing countries (Mlingi *et al*., 1992). Cassava has the ability to grow 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 (Mlingi, 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 (Gidamis, 1988; Howlett *et al*., 1990; Mlingi *et al*., 1991; Oyewole and Aibor, 1992; Mlingi, 1995).

In cassava, cyanide occurs as cyanogenic glucosides, mostly linamarin (>80%) and to a lesser extent lotaustralin (Cereda and Mattos, 1996; Kimaryo *et al*., 2000). 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) as well as 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). *Gari*, one of the most popular foods derived from cassava fermentation, is consumed by more than 200 million people across West Africa (Okafor and Ejiofor, 1990).

In order to gain a better understanding of the *Gari* production process, a visit was made to a rural village in Benin, West Africa. *Gari* is traditionally processed by women in these villages, where they produce enough to sustain their household usage. The purpose of the visit was to document the *Gari*

process, as well as to take samples during the fermentation process for isolation of predominant bacteria which are typically associated with the fermentation. Commercial starter cultures generally originate from food substrates or from the processes in which they are applied (Holzapfel, 2002). There has also been a trend recently to isolate wild-type strains from traditional products for use as starter cultures in food fermentation (Beukes *et al*., 2001; De Vuyst *et al*., 2002; Leroy and De Vuyst, 2004). It was envisaged that the microorganisms isolated from the samples taken would be ideal for the development of a starter culture, as they would be typical of the fermentation and well adapted to the ecological conditions as experienced during the *Gari* fermentation.

2.2 Materials and methods

2.2.1 Microbiological sampling during preparation and fermentation of cassava in a Benin village

Gari, fortified with soybean and palm oil, was prepared in a village near Cotonou, Benin. The process is illustrated in Figure 2.1 and Figure 2.2. Samples were taken initially (T_0) just before the buckets were sealed with lids (Fig. 2.1, step 6) and every six hours thereafter during the 48h fermentation process of cassava for the preparation of *Gari* in Benin. This was conducted in order to isolate the microbes involved in the fermentation. Ten gram samples were diluted 1:10 using 90 ml Ringer's solution (Merck, Darmstadt, Germany) and homogenised for 1 min using a blender. Samples were diluted further in a ten-fold dilution series (10^{-2}-10^{-9}) and spread plated onto different agar media: de Man, Rogosa and Sharpe (MRS) agar, M17 agar, Rogosa agar and Malt Extract Agar (MEA) agar (all from Merck, Darmstadt, Germany). MRS, M17 and Rogosa media were suited for isolating different LAB groups and were thus used to obtain the greatest diversity of LAB associated with the fermentation. MEA was used to isolate any yeast present during fermentation. The plates were incubated aerobically at 30°C for 48h. For isolation and identification of potential starter strains, colonies were

randomly picked from the plates with the highest dilutions and purified by streaking onto agar. Picking the bacteria from plates of the highest dilution ensured that the most predominant bacteria associated with the fermentation, i.e. those occurring in highest numbers, were isolated. For pH measurements during the fermentation, 10 g of samples were added to 20 ml of distilled water and homogenised. The pH was measured every 6 h using a portable pH meter (Jencons). For transport of the isolates to the CSIR in South Africa, the strains were streaked onto the selective media that they were isolated from (see above). Samples were transported to South Africa within 48 h and then purified by streaking out repeatedly onto the appropriate growth media.

The isolates were kept in 20 % (v/v) glycerol at -80°C and added to the culture collection already present at the CSIR. The microorganisms from Benin and LAB isolates from the CSIR culture collection, which were previously isolated from cassava (obtained from Benin), were revived on suitable agar media, and screened for the production of suitable compounds and enzymes as described below.

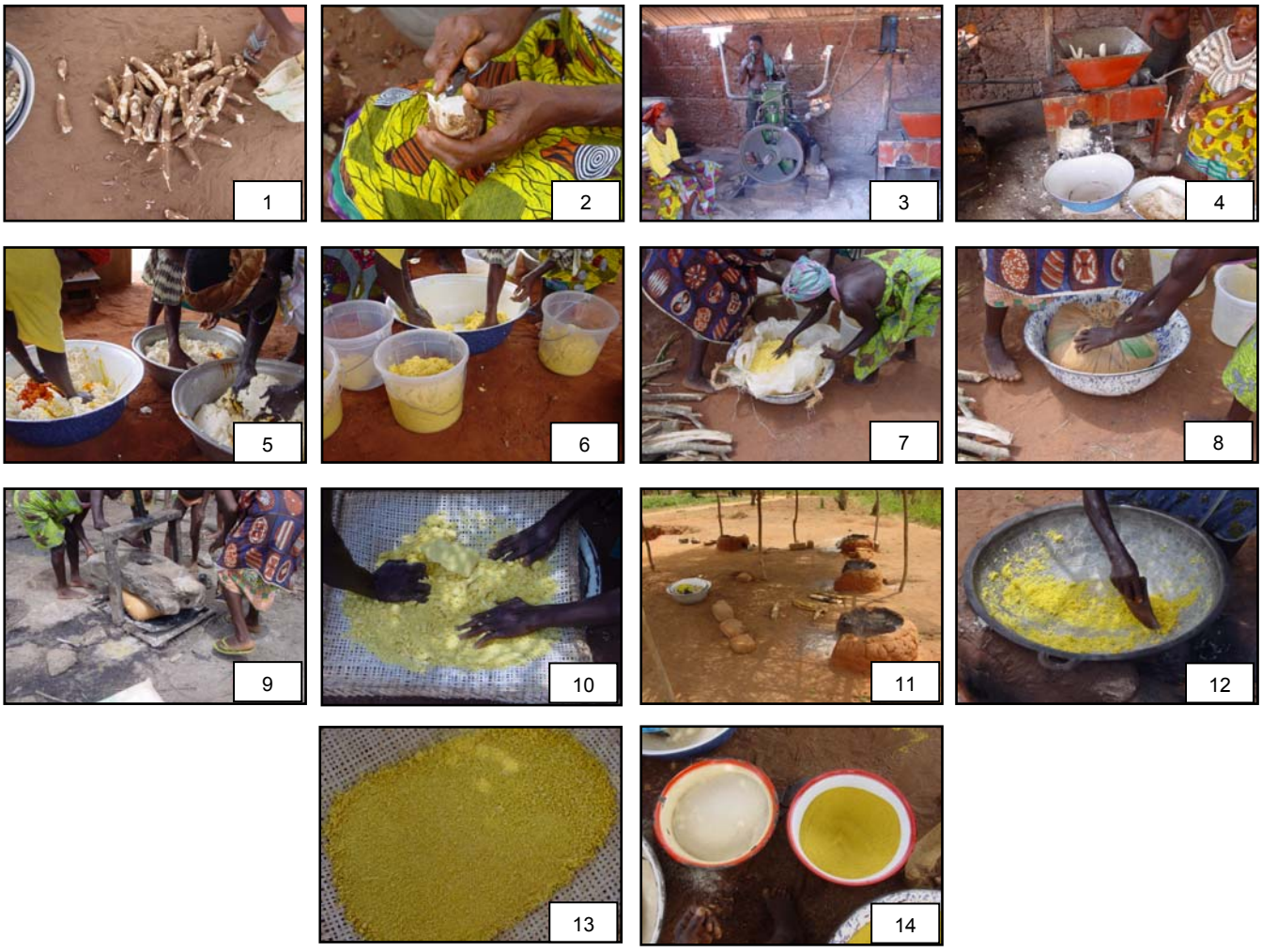

Fig. 2.1 A graphic representation of the *Gari* production process. (1) Cassava tubers after harvesting, (2) tubers are peeled and washed, (3) mechanical grinding of the tubers, (4) collection of the ground tubers, (5) palm oil and soy bean added, (6) placed in buckets, sealed with lids, and left to ferment for 48 h, (7) and (8) mixture is placed in a large bag, (9) the dewatering process, (10) sieving to remove large particles, (11) area where mixture is fried/garified, (12) frying/garification, (13) fine sieving (14) comparison between traditional *Gari* (left) and *Gari* fortified with soybean and palm oil

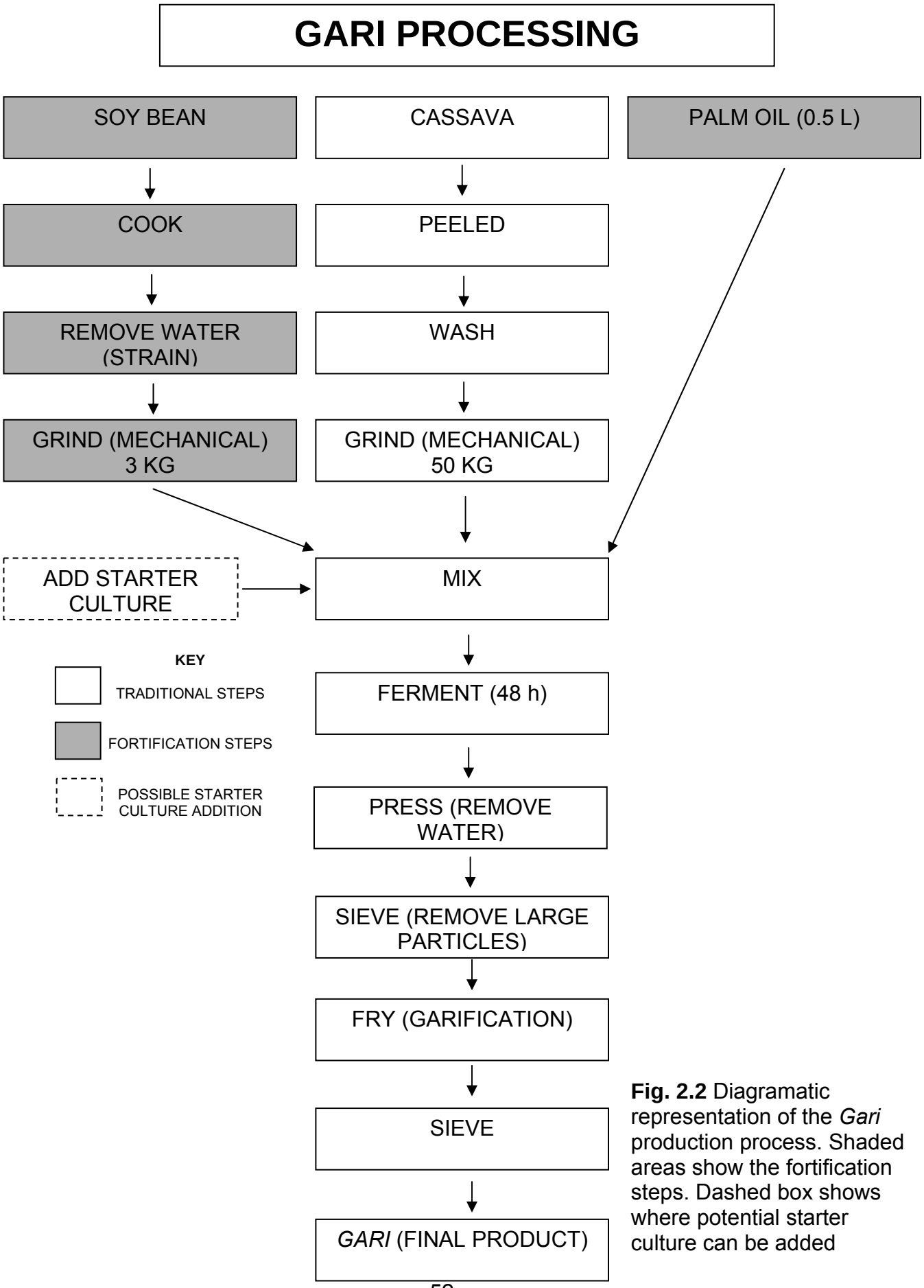

Fig. 2.2 Diagramatic representation of the *Gari* production process. Shaded areas show the fortification steps. Dashed box shows where potential starter culture can be added

2.2.2 Phenotypic characterisation

LAB strains were characterised by determination of cell morphology using phase contrast microscopy, Gram staining, catalase test and gas (CO_2) production from glucose using the methods as described by Schillinger and Lücke (1987). Sugar fermentations patterns of LAB isolates were determined using the API 50 CHL system (bioMérieux, France) according to the manufacturer's instructions and the identification of LAB strains was performed using the computer program APILAB PLUS (Version 3.2.2., BioMérieux, France).

Yeast strains were characterized by cell morphology. Sugar fermentation patterns were determined using the API 20 AUX system (bioMérieux, France) according to the manufacturer's instructions and the identification of yeast strains was also performed using the computer program APILAB PLUS (Version 3.2.2., BioMérieux, France).

2.2.3 Technological properties

2.2.3.1 Production of β-glucosidase

A medium for testing β-glucosidase was prepared by adding 0.1 g of 4-nitrophenyl-β-D-glucopyranoside (Merck, Darmstadt, Germany) to 100 ml 0.666 M NaH_2PO_4 (pH 6) (Merck, Darmstadt, Germany). The mixture was dissolved and filter-sterilized. The test culture was grown on MRS agar for 24 h at 30°C. Colonies were picked from the plates using a sterile loop and were emulsified in physiologic saline to McFarland Turbidity Standard No. 3. Thereafter, 0.75 ml of culture was added to 0.25 ml of the test medium. It was incubated at 30°C overnight. Positive isolates that produced β-glucosidase degraded the linamarin analogue and changed the colour of the mixture from colourless to a distinct yellow. For yeast isolates, the process above was repeated, except that MEA agar, containing 50 mg/l of the antibiotic kanamycin, was used instead of MRS agar.

2.2.3.2 Production of α-amylase

In order to detect α-amylase production, LAB strains were grown on modified MRS agar plates and yeast strains were grown on modified MEA plates (with 50 mg/l kanamycin) both containing 0.4% soluble starch as the sole carbon source. A cotton swab was used to streak out the LAB and yeast strains onto the starch MRS and MEA plates, respectively. The cultures were incubated at 30°C for 24 hours, after which the plates were flooded with iodine. Production of amylase was evident by a zone of clearing surrounding the streak, indicating starch utilisation. *Lactobacillus amylovorus* DSM 20531 was used as a positive control for the LAB cultures and *Saccharomyces cerevisiae* SC 3 (CSIR culture collection) was used as a positive control for the yeast cultures.

2.2.3.3 Acid production

The LAB test strains (1% of an overnight culture) were inoculated into MRS broth (pH 6.2 after autoclaving) and grown aerobically at 30°C. Acid production was determined by measuring the pH of the culture after 24 and 48 hours. MRS broth medium was prepared from a single batch which was pH adjusted and then dispensed into tubes of 10 ml each before autoclaving (Kostinek *et al*., 2005). Acid production was not assessed for the yeast strains.

2.3 Results

2.3.1 Microbiological sampling from fermenting cassava mash

The growth of microorganisms in fermenting cassava mash and development of pH during the fermentation is shown in Fig. 2.3.

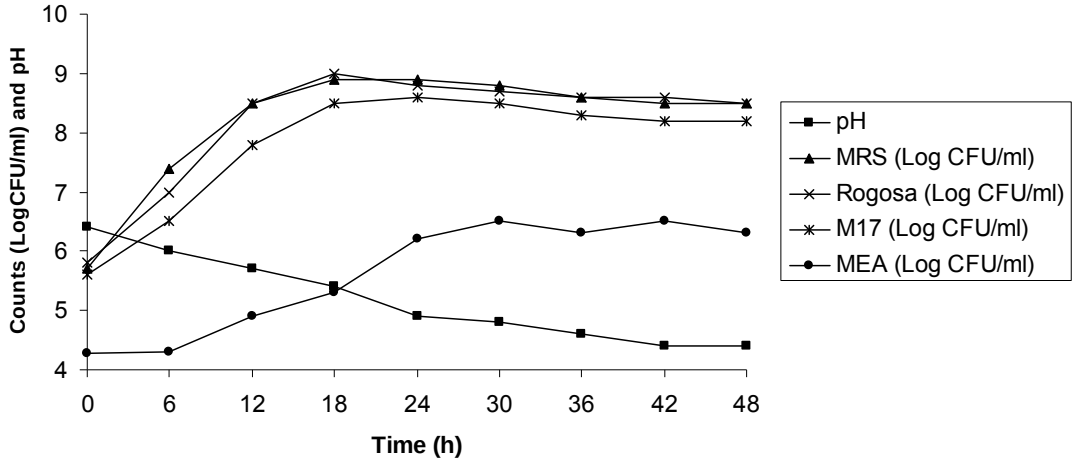

Fig. 2.3 Growth of microorganisms and pH reduction in fermenting cassava mash

The numbers of bacteria were assessed during 48 h of cassava fermentation for the production of *Gari* by plate counting. Bacterial strains were the most predominant microorganisms in the fermentation. Bacterial counts determined on Rogosa, MRS and M17 agar media started from an initial level of ca. 10^5 to 10^6 CFU/g and a rapid increase in their numbers could be observed during the first six hours of the fermentation (Fig. 2.3) and continued to increase to a maximum of ca 10^8 CFU/g after 12 hours. Yeasts were present at lower counts, their numbers increased little during the first 6 hours but did increase from ca. 10^4 CFU/g to 10^6 CFU/g over the 48 h fermentation period. During the 48 h of fermentation, pH was reduced from 6.4 to 4.4.

The majority of bacterial strains (36) were isolated from MRS agar plates, while 24 and 14 strains were isolated from Rogosa and M17 agar media,

respectively. Thus a total of 74 bacterial strains were isolated. A total of 21 yeast isolates were also isolated from MEA plates (Fig. 2.4). Fifty-six of the bacterial strains were isolated during the first 18 h of fermentation, while 14 of the yeast isolates were isolated during the second half of the fermentation process (24 – 48 h).

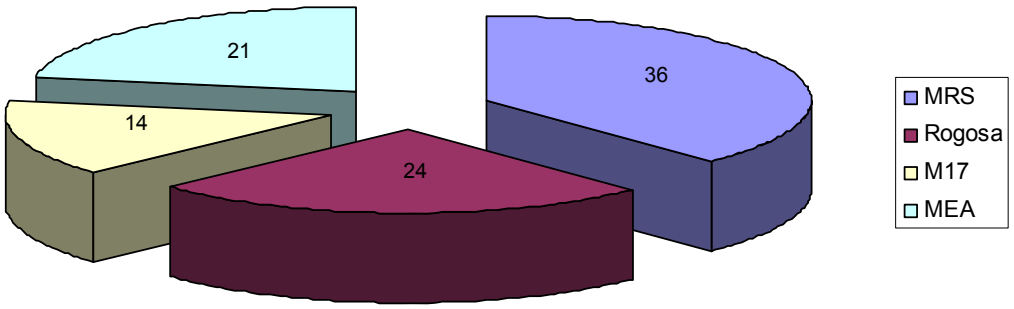

Fig. 2.4 Number of bacterial strains (MRS, Rogosa, M17) and yeast isolates (MEA) isolated from the different media used in microbial count determinations of fermenting cassava mash

Counts from all fermentations timepoints generally ranged from between $1X10^5$ to $1X10^9$ Log CFU/g (Fig. 2.3). The microbial counts on MRS and Rogosa agar media compared well (Fig. 2.3) showing that both these media are well suited for isolation of lactic acid bacteria from *Gari*. Counts from M17 media, which is better suited for isolation of lactococci and streptococci as it contains lactose as sole carbon source, were generally slightly lower during the 48 hour fermentation process.

2.3.2 Characterisation of phenotypic and technological properties

The 74 bacterial strains and 21 yeast strains isolated from the fermenting cassava mash in Benin, as well as 26 bacterial strains isolated from cassava samples sent to the CSIR from Benin, were examined as shown in Tables 2.1, 2.2 and 2.3.

2.3.2.1 Isolates obtained from cassava samples sent from Benin

There were 23 (88.5%) rods and 3 (11.5%) cocci among the 26 bacterial isolates obtained from the cassava samples sent from Benin (Table 2.1). One (3.8%) strain (VE 18) was Gram +ve and catalase +ve. This meant that is was not a LAB, as LAB are typically Gram +ve and catalase –ve. Three (11.5%) of the strains produced CO_2 from glucose, indicating that they are heterofermentative. All other strains were either facultatively heterofermentative or homofermentative. None of the isolates showed any α-amylase activity. Nine (34.6%) of the strains displayed *β*-glucosidase activity. The pH in MRS broth after growth for 24 h ranged from 3.91 and 5.23, with strain VE 26 demonstrating the best pH reduction. At 48 h, the pH ranged from 3.66 to 4.96, with strain VE 26 again demonstrating the best pH reduction. The API 50 CHL kit for identification of *Lactobacillus* and related species, was used to tentatively identify the isolates. The majority of strains (65.4%) were identified as *Lactobacillus plantarum*. Other rod-shaped strains identified from the fermentation were *L. acidophilus* (3.8%), *L. fermentum* (7.7%) and *L. buchneri* (7.7%). The coccus-shaped isolates were all tentatively identified as *Lactococcus lactis* ssp. cremoris (11.5%) using the API kit. One strain (3.8%) was not identified because it was catalase +ve.

Table 2.1 Phenotypic properties and tentative identification of strains isolated from cassava samples sent to CSIR from Benin

Strain number	Morphology	Gram reaction	Catalase	CO_2 from glucose	α-amylase activity	β-glucosidase activity	pH after 24h	pH after 48h	Tentative ID
VE 1	rods	+	-	-	-	+	4.12	3.98	*L. plantarum*
VE 2	rods	+	-	-	-	-	4.27	4.10	*L. plantarum*
VE 3	rods	+	-	-	-	+	4.21	4.05	*L. plantarum*
VE 4	rods	+	-	-	-	-	4.15	4.02	*L. plantarum*
VE 5	rods	+	-	+	-	-	4.08	3.96	*L. fermentum*
VE 6	cocci	+	-	-	-	-	4.36	4.13	*Lc. lactis* ssp. *cremoris*
VE 7	rods	+	-	-	-	+	4.27	4.05	*L. plantarum*
VE 8	rods	+	-	-	-	-	4.02	3.92	*L. plantarum*
VE 9	rods	+	-	-	-	+	4.19	4.07	*L. plantarum*
VE 10	rods	+	-	+	-	-	4.26	4.10	*L. buchneri*
VE 11	rods	+	-	-	-	-	4.38	4.09	*L. fermentum*
VE 12	rods	+	-	-	-	-	4.22	3.99	*L. plantarum*
VE 13	rods	+	-	-	-	+	4.11	3.96	*L.plantarum*
VE 14	**rods**	**+**	**-**	**-**	**-**	**+**	**3.92**	**3.78**	***L. plantarum***
VE 15	cocci	+	-	-	-	-	4.15	4.01	*Lc. lactis* ssp. *cremoris*
VE 16	rods	+	-	-	-	-	4.31	4.06	*L. plantarum*
VE 17	rods	+	-	-	-	-	4.08	3.97	*L. plantarum*
VE 18	rods	+	+	-	-	-	5.23	4.96	N/D
VE 19	cocci	+	-	-	-	-	4.00	3.91	*Lc. lactis* ssp. *cremoris*
VE 20	**rods**	**+**	**-**	**-**	**-**	**+**	**3.96**	**3.82**	***L. plantarum***
VE 21	rods	+	-	-	-	+	4.18	4.04	*L. plantarum*
VE 22	rods	+	-	-	-	-	4.21	4.00	*L. acidophilus*
VE 23	rods	+	-	-	-	-	4.07	3.96	*L. planatrum*
VE 24	rods	+	-	-	-	-	4.13	4.01	*L. plantarum*
VE 25	rods	+	-	-	-	-	4.04	3.95	*L.plantarum*
VE 26	**rods**	**+**	**-**	**+**	**-**	**+**	**3.91**	**3.66**	***L. buchneri***

N/D – not determined; strain catalase positive

2.3.2.2 Isolates obtained from fermenting cassava mash in Benin

There were 66 (89.2%) rods and 8 (10.8%) cocci among the 74 bacterial isolates obtained from fermenting cassava mash in Benin (Table 2.2). Ten (13.5%) strains were Gram +ve and catalase +ve. Three (4.1%) of the strains produced CO_2 from glucose, indicating that they are heterofermentative. All other strains were thus either facultatively heterofermentative or homofermentative. Three of the strains (4.1%) showed α-amylase activity. Thirty-eight (51.4%) of the strains displayed *β*-glucosidase activity. The pH of MRS broth after 24h growth ranged from 3.92 and 5.56, with strain VE 98 showing the best pH reduction at 24h. At 48h, the pH ranged from 3.53 to 5.37, with strain VE 82 demonstrating the best pH reduction. The API 50 CHL kit was again used to tentatively identify the isolates. The majority of strains (44.6%) were identified as *Lactobacillus plantarum*. Other rod-shaped strains identified from the fermentation were *L. acidophilus* (16.2%), *L. fermentum*

(9.5%), *L. buchneri* (4.1%) and *L. pentosus* (2.7%). *Lactococcus lactis* (4.1%), *Lactococcus lactis* ssp. *cremoris* (1.4%) and *Leuconostoc* (4.1%) were the cocci which could be identified with the aid of the kit. Ten (13.5%) strains were not identified using the API kit after they were found to be catalase +ve.

The majority of yeast strains (90.5%) were identified as *Candida* species (Table 2.3). Two (9.5%) yeast strains were identified as *Cryptococcus laurentii.* Fourteen strains (66.7%) showed α-amylase activity, and nine strains (42.9%) showed *β*-glucosidase activity. Seven strains (33.3%) showed both α-amylase and *β*-glucosidase activities.

Table 2.2 Phenotypic properties and tentative identification of strains isolated from fermenting cassava mash in Benin

Strain number	Morphology	Gram reaction	Catalase	CO_2 from glucose	α-amylase activity	β-glucosidase activity	pH after 24 h	pH after 48 h	Tentative ID
VE 36	**rods**	**+**	**-**	**-**	**-**	**+**	**4.01**	**3.70**	***L. plantarum***
VE 37	rods	+	-	-	-	-	4.42	4.15	*L. plantarum*
VE 38	rods	+	-	-	-	-	4.36	4.09	*L. acidophilus*
VE 39	rods	+	-	-	-	-	4.19	4.03	*L. plantarum*
VE 40	cocci	+	-	-	-	-	4.20	4.11	*Lactococcus lactis*
VE 41	rods	+	+	-	-	-	5.36	5.12	N/D
VE 42	rods	+	-	-	-	-	4.18	4.07	*L. plantarum*
VE 43	**rods**	**+**	**-**	**-**	**-**	**+**	**4.12**	**3.91**	***L. fermentum***
VE 44a	rods	+	-	+	-	-	4.42	4.20	*L. fermentum*
VE 44b	rods	+	-	-	-	-	4.56	4.39	*L. plantarum*
VE 45	rods	+	-	-	-	+	4.38	4.27	*L. acidophilus*
VE 46	rods	+	-	-	-	-	4.31	4.16	*L. acidophilus*
VE 47	cocci	+	+	-	-	+	5.56	5.32	N/D
VE 48	rods	+	+	-	-	-	5.37	5.30	N/D
VE 49	rods	+	+	-	-	-	5.45	5.37	N/D
VE 50	rods	+	-	-	-	+	4.62	4.43	*L. fermentum*
VE 51	rods	+	-	-	-	-	4.55	4.32	*L. fermentum*
VE 52	cocci	+	-	-	-	-	4.38	4.24	*Lc. lactis* ssp. *cremoris*
VE 53	rods	+	-	-	-	+	4.61	4.46	*L. plantarum*
VE 54	rods	+	-	+	-	-	4.33	4.24	*L. buchneri*
VE 55	rods	+	-	-	-	-	4.45	4.36	*L. plantarum*
VE 56	**rods**	**+**	**-**	**-**	**-**	**+**	**4.10**	**3.79**	***L. plantarum***
VE 57	rods	+	-	-	-	+	4.38	4.10	*L. plantarum*
VE 58	rods	+	-	-	-	+	4.29	4.17	*L. plantarum*
VE 59	**rods**	**+**	**-**	**-**	**-**	**+**	**4.05**	**3.89**	***L. pentosus***
VE 60a	**rods**	**+**	**-**	**-**	**-**	**+**	**4.28**	**3.90**	***L. fermentum***
VE 60b	**cocci**	**+**	**-**	**-**	**-**	**+**	**4.15**	**4.01**	***Lactococcus lactis***
VE 61	rods	+	-	-	-	+	4.38	4.19	*L. plantarum*
VE 62	rods	+	-	-	-	+	4.28	4.08	*L. plantarum*
VE 63	**rods**	**+**	**-**	**-**	**-**	**+**	**3.96**	**3.82**	***L. buchneri***
VE 64	rods	+	-	-	-	-	4.30	4.14	*L. fermentum*
VE 65a	**cocci**	**+**	**-**	**-**	**-**	**+**	**4.13**	**3.84**	***Lactococcus lactis***
VE 65b	**rods**	**+**	**-**	**-**	**-**	**+**	**4.02**	**3.76**	***L. acidophilus***
VE 66	rods	+	-	-	-	+	4.56	4.37	*L. plantarum*
VE 67	rods	+	-	-	-	+	4.47	4.28	*L. acidophilus*
VE 68	rods	+	-	-	-	-	4.29	4.10	*L. plantarum*
VE 69	rods	+	-	-	-	-	4.38	4.12	*L. plantarum*
VE 70	**rods**	**+**	**-**	**-**	**-**	**+**	**4.00**	**3.72**	***L. plantarum***
VE 71	rods	+	-	-	-	-	4.26	4.12	*L. plantarum*
VE 72	rods	+	-	-	-	-	4.20	4.11	*L. plantarum*
VE 73	rods	+	-	+	-	-	4.35	4.28	*L. buchneri*
VE 74	rods	+	-	-	-	+	4.19	3.98	*L. fermentum*
VE 75	rods	+	-	-	-	-	4.44	4.26	*L. plantarum*
VE 76	rods	+	-	-	-	+	4.37	4.18	*L. plantarum*
VE 77	**rods**	**+**	**-**	**-**	**-**	**+**	**3.99**	**3.67**	***L. plantarum***
VE 78	rods	+	-	-	-	-	4.33	4.11	*L. acidophilus*
VE 79	rods	+	-	-	-	-	4.29	3.98	*L. acidophilus*
VE 80	rods	+	-	-	-	+	4.39	4.09	*L. plantarum*
VE 81	rods	+	-	-	-	-	4.36	4.25	*L. plantarum*
VE 82	**rods**	**+**	**-**	**-**	**-**	**+**	**3.96**	**3.53**	***L. plantarum***
VE 83	rods	+	-	-	-	-	4.23	3.95	*L. plantarum*
VE 84	rods	+	-	-	-	+	4.30	3.89	*L. plantarum*
VE 85a	**rods**	**+**	**-**	**-**	**-**	**+**	**4.08**	**3.78**	***L. plantarum***
VE 85b	**rods**	**+**	**-**	**-**	**-**	**+**	**4.13**	**3.69**	***L. acidophilus***
VE 86	rods	+	-	-	-	-	4.31	4.06	*L. plantarum*
VE 87	rods	+	+	-	-	-	5.35	5.00	N/D
VE 88	rods	+	-	-	-	+	4.32	4.11	*L. plantarum*
VE 89a	rods	+	+	-	-	+	4.39	4.07	N/D
VE 90	**rods**	**+**	**-**	**-**	**-**	**+**	**4.22**	**3.95**	***L. pentosus***
VE 91	**cocci**	**+**	**-**	**-**	**+**	**-**	**4.26**	**3.97**	***Leuconostoc* spp.**
VE 93	rods	+	+	-	-	-	5.39	5.05	N/D
VE 95a	rods	+	+	-	-	+	5.28	5.02	N/D
VE 95b	rods	+	-	-	-	-	4.52	4.18	*L. acidophilus*
VE 97	**cocci**	**+**	**-**	**-**	**+**	**-**	**3.99**	**3.71**	***Leuconostoc* spp.**
VE 98	**rods**	**+**	**-**	**-**	**-**	**+**	**3.92**	**3.56**	***L. acidophilus***
VE 99	**rods**	**+**	**-**	**-**	**-**	**+**	**4.27**	**3.98**	***L. plantarum***
VE 100	**rods**	**+**	**-**	**-**	**-**	**+**	**4.19**	**3.90**	***L. plantarum***
VE 101	rods	+	+	-	-	-	5.36	4.99	N/D
VE 102	**rods**	**+**	**-**	**-**	**+**	**-**	**4.02**	**3.86**	***L. acidophilus***
VE 103	rods	+	+	-	-	-	5.42	5.01	N/D
VE 104	cocci	+	-	-	-	+	4.37	4.02	*Leuconostoc* spp.
VE 105	rods	+	-	-	-	+	4.43	4.16	*L. plantarum*
VE 106	rods	+	-	-	-	+	4.33	4.12	*L. plantarum*
VE 107	rods	+	-	-	-	-	4.36	4.09	*L. acidophilus*

N/D – not determined; strain catalase positive

Table 2.3 Yeast isolates obtained from fermenting cassava mash in Benin

Organism number	Description	α-amylase activity	β-glucosidase activity	Tentative ID
VE 89b	Yeast	+	-	*Candida tropicalis*
VE 92	Yeast	+	+	*Candida krusei*
VE 94	Yeast	+	+	*Cryptococcus laurentii*
VE 96	Yeast	-	-	*Candida inconspicua*
VE 108	Yeast	+	-	*Candida krusei*
VE 109	Yeast	+	+	*Candida famata*
VE 110	Yeast	-	-	*Candida rugopelliculosa*
VE 111	Yeast	+	-	*Candida maris*
VE 112	Yeast	+	-	*Candida inconspicua*
VE 113	Yeast	+	-	*Candida glabrata*
VE 114	Yeast	+	+	*Cryptococcus laurentii*
VE 115	Yeast	-	-	*Candida guilliermondii*
VE 116	Yeast	+	-	*Candida tropicalis*
VE 117	Yeast	+	-	*Candida famata*
VE 118	Yeast	-	+	*Candida rugopelliculosa*
VE 119	Yeast	-	-	*Candida tropicalis*
VE 120	Yeast	+	+	*Candida rugopelliculosa*
VE 121	Yeast	-	-	*Candida tropicalis*
VE 122	Yeast	-	+	*Candida rugopelliculosa*
VE 123	Yeast	+	+	*Candida tropicalis*
VE 124	Yeast	+	+	*Candida krusei*

A selection was made from the bacterial strains that were isolated from the cassava samples sent from Benin, as well as the strains isolated from a cassava mash fermentation process in Benin during a research visit there. A comparison of these strains can be seen in Fig. 2.5. Strains were selected for further characterisation and were further identified at the BFE in Germany during a research visit. The 24 strains selected for further characterisation (Table 2.4), were chosen on the basis of good acid production, ie. a good pH reduction, α-amylase activity or β-glucosidase activity. No yeast strains were selected for further characterisation.

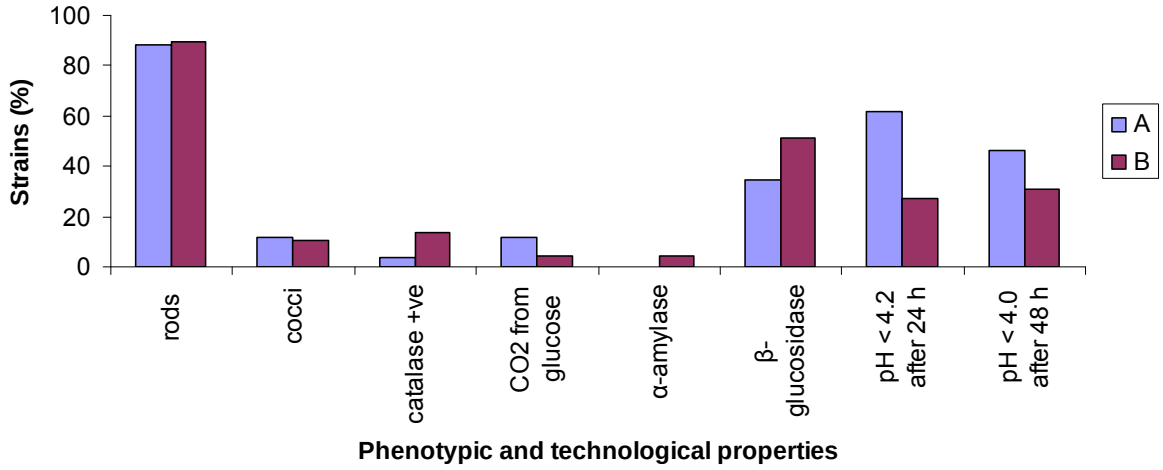

Fig. 2.5 Comparison of phenotypic and technological properties of (A) strains isolated from cassava samples sent to CSIR from Benin and (B) strains isolated from fermenting cassava mash during a research visit to Benin

Table 2.4 Strains selected for further phenotypic, genotypic and technological characterisation at the BFE, Germany

Organism number	Morphology	Gram reaction	Catalase	CO_2 from glucose	α-amylase activity	β-glucosidase activity	pH after 24 h	pH after 48 h	Tentative ID
VE 14	rods	+	-	-	-	+	3.92	3.78	*L. plantarum*
VE 20	rods	+	-	-	-	+	3.96	3.82	*L. plantarum*
VE 26	rods	+	-	+	-	-	3.91	3.66	*L. buchneri*
VE 36	rods	+	-	-	-	+	4.01	3.70	*L. plantarum*
VE 43	rods	+	-	-	-	+	4.12	3.91	*L. fermentum*
VE 56	rods	+	-	-	-	+	4.10	3.79	*L. plantarum*
VE 59	rods	+	-	-	-	+	4.05	3.89	*L. pentosus*
VE 60a	rods	+	-	-	-	+	4.28	3.90	*L. fermentum*
VE 60b	cocci	+	-	-	-	+	4.15	4.01	*Lactococcus lactis*
VE 63	rods	+	-	-	-	+	3.96	3.82	*L. buchneri*
VE 65a	cocci	+	-	-	-	+	4.13	3.84	*Lactococcus lactis*
VE 65b	rods	+	-	-	-	+	4.02	3.76	*L. acidophilus*
VE 70	rods	+	-	-	-	+	4.00	3.72	*L. plantarum*
VE 77	rods	+	-	-	-	+	3.99	3.67	*L. plantarum*
VE 82	rods	+	-	-	-	+	3.96	3.53	*L. plantarum*
VE 85a	rods	+	-	-	-	+	4.08	3.78	*L. plantarum*
VE 85b	rods	+	-	-	-	+	4.13	3.69	*L. acidophilus*
VE 90	rods	+	-	-	-	+	4.22	3.95	*L. pentosus*
VE 91	cocci	+	-	-	+	-	4.26	3.97	*Leuconostoc* spp.
VE 97	cocci	+	-	-	+	-	3.99	3.71	*Leuconostoc* spp.
VE 98	rods	+	-	-	-	+	3.92	3.56	*L. acidophilus*
VE 99	rods	+	-	-	-	+	4.27	3.98	*L. plantarum*
VE 100	rods	+	-	-	-	+	4.19	3.90	*L. plantarum*
VE 102	rods	+	-	-	+	-	4.02	3.86	*L. acidophilus*

2.4 Discussion

For development of starter cultures, it is important to isolate predominant strains from previous fermentation batches in order to achieve successful further fermentations. Screening of strains in this work showed that *L. plantarum* was predominant in the samples obtained from Benin, as well as the cassava mash fermentation process studied while in Benin. Oyewole and Odunfa (1990) showed that *L. plantarum* is a common occurrence in fermenting cassava. *L. plantarum* has been shown previously to be the predominant LAB species in sour cassava starch (Lacerda *et al*., 2005). This is discussed in more detail in Chapter 3 (section 3.4).

Bacterial counts compared well with MRS and Rogosa agar, and were lower on M17 agar. Lower counts on M17 agar, which is better suited for isolation of lactococci and streptococci, may be expected, as these bacteria would probably occur in numbers less than lactobacilli and leuconostocs. Alternatively, this could also be attributed to the fact that the bacteria were less well adapted to utilise lactose, which appeared to be the case as we determined at a later stage that we could not identify any lactococci from the M17 agar plates. Thus, this media was not very selective for the growth of lactococci from *Gari*, but instead allowed the growth of other LAB that were able to utilise lactose and which were associated with the *Gari* fermentation. Nevertheless, as counts were generally lower on M17 media, it was determined that this media was not well suited for isolation of the majority of LAB from *Gari*, probably a reflection of the fact that this is not a typical sugar present in the cassava root.

The majority of LAB strains isolated from both processes showed good pH reduction (Table 2.1 and 2.2). Good pH reduction (a fast lowering of the pH to low levels) is important to reduce the levels of contaminating microorganisms present on the raw materials, utensils and the environment which can compete with the starters for nutrients (Holzapfel, 2002). According to Holzapfel (1997), starter cultures introduced in traditional small-scale fermentations should have attributes for improving processing conditions and

product quality through: (i) rapid accelerated metabolic activities (acidification or alcohol production); (ii) improved and more predictable fermentation processes; (iii) desirable sensory attributes; and (iv) improved safety and reduced hygienic and toxicological risks.

Giraud *et al*. (1993) showed that *Lactobacillus plantarum* strain A6 isolated from cassava, cultured on cellobiose MRS medium, produced both an intracellular linamarase (76.4 U/g of biomass) and an extracellular amylase (36 U/ml) simultaneously. The use of this strain as a cassava fermentation starter for *Gari* production caused a change from a heterofermentative pattern observed in natural fermentation, to a homofermentative one, a lower final pH, a faster pH decline rate and a greater production of lactic acid (50 g/kg) (Giraud *et al*., 1993). The ability of several yeast strains to produce *β*-glucosidase was shown by Fia *et al*. (2005). In addition, Freer (1993) demonstrated that *Candida wickerhamii* produced an extracytoplasmic, cell-bound beta-1,4-glucosidase, which is important to the *Gari* fermentation. LAB and yeasts, which are responsible for the fermentation process, are also thought to contribute to linamarin degradation by β-glucosidase activity (Ikediobi and Onyike, 1982; Padmaja and Balagopal, 1985; Okafor and Ejiofor, 1990; Giraud *et al*., 1992; Okafor *et al*., 1998a; b).

Machado and Linardi (1990) studied a total of one hundred and five yeast strains, belonging to the genera *Aureobasidium*, *Candida*, *Cryptococcus*, *Debaromyces*, *Rhodotorula* and *Trichosporum* for their ability to produce amylase and beta-galactosidase. They showed that some strains showed high enzymatic activity for amylase. Linardi and Machado (1990) screened 228 yeasts, isolated from natural habitats, for their ability to produce amylases in semisolid medium of wheat bran. Strains of *Aureobasidium pullulans*, *Candida famata*, and *Candida kefyr* showed high enzymatic activity for alpha - amylase, glucoamylase, and debranching enzyme.

Azoulay *et al*., (1980) showed that *Candida tropicalis* possesses the enzyme needed to hydrolyze starch, namely, an α-amylase. *Candida tropicalis* grows on soluble starch, corn, and cassava powders without requiring that these

substrates be previously hydrolyzed. This property has been used to develop a fermentation process whereby *C. tropicalis* can be grown directly on corn or cassava powders so that the resultant mixture of biomass and residual corn or cassava contains about 20% protein, which represents a balanced diet for either animal fodder or human food (Azoulay *et al*., 1980).

API kits are known to be inaccurate when it comes to identification of Gram-positive bacteria. A study conducted by Boyd *et al*. (2005) showed that the API 50 CH system misidentified 59% of the *L. jensenii* and *L. gasseri* isolates as *L. acidophilus* and the system also misidentified 7 out of 20 (35%) *L. vaginalis* isolates as *L. fermentum*. Over half of the 97 isolates yielded an uninterpretable or doubtful API profile (Boyd *et al*., 2005).

For yeasts, similar problems with identification based on API profiles have been reported. Oguntoyinbo (2008), for example, showed that the results obtained using the API 20 AUX kit for *Candida* strains was is in agreement with that of the 18S rDNA gene sequence identification analysis at the genus level only, and often did not provide satisfactory identification. Strains of *C. krusei* have been consistently isolated from cassava and cereal fermentation in West Africa (Hayford and Jakobsen, 1999). Oguntoyinbo (2008) however stated that the inadequacy of the use of phenotypic methods to differentiate *C. inconspicua*, *C. krusei* and *C. rugopelliculosa* in the previous studies have been responsible for the inability to determine the roles (functional and virulence) of these strains during cassava fermentation.

Yeasts, especially *Candida* species, may be important because of their ability for co-metabolism with lactic acid bacteria, a parameter reported as desirable for adequate fermentation of cassava (Amoa Awua *et al*., 1996; Oyewole, 2001). On the other hand, the fermentation is rather quick and mainly dominated by lactic acid bacteria, especially in the latter parts of the fermentation, indicating that the yeasts may only play a minor role at the beginning of the fermentation. For purposes of this study, no further work was carried out with the yeast strains. Research on yeast strains would be conducted by other members of the project consortium. Our aims were to

more precisely identify the predominant lactic acid bacteria involved with the *Gari* fermentation. Results so far allowed a selection of a representative consortium of predominant lactic acid bacteria that also displayed useful technological characteristics, such as production of α-amylase or *β*-glucosidase. These preliminary characterisation results also allowed a coarse grouping of the strains and it was determined that the majority of strains belonged to the *L. plantarum* group, while presumptive *L. fermentum, L. buchneri and L. acidophilus strains*, as well as heterofermentative cocci, i.e., presumptive *Leuconostoc* spp., could also be identified. The further work thus aimed to more accurately identify these bacteria in a polyphasic taxonomical approach (Chapter 3).

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Chapter 3
Phenotypic, genotypic and technological properties of selected lactic acid bacteria

This chapter has contributed to the following publications:

Melanie Kostinek, Ingrid Specht, **Vinodh A. Edward**, Ulrich Schillinger, Christian Hertel, Wilhelm H. Holzapfel and Charles M. A. P. Franz. 2005. Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of *Gari*, a traditional African food. *Systematic and Applied Microbiology* 28: 527-540. (Appendix A)

M. Kostinek, I. Specht, **V.A. Edward**, C. Pinto, M. Egounlety, C. Sossa, S. Mbugua, C. Dortu, P. Thonart, L. Taljaard, M. Mengu, C.M.A.P. Franz and W.H. Holzapfel. 2007. Characterisation and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures. *International Journal of Food Microbiology* 114: 342-351. (Appendix B)

3.1 Introduction

Cassava may be processed by boiling, roasting, drying or by fermentation, depending on the variety (Holleman and Atten, 1956). The most popular processing method, however, especially for the varieties high in cyanogenic glucosides, is by fermentation. *Gari* is one of the most popular foods derived from fermented cassava, and is consumed by nearly 200 million people across West Africa (Okafor and Ejiofor, 1990). *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 (Vasconcelos *et al*., 1990). During grating, the endogenous linamarase in the root is released and degrades the toxic cyanogenic glucoside 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* (Okafor, 1977). 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 (Ngaba and Lee, 1979; Okafor and Ejiofor, 1990; Okafor and Uzuegbu, 1987). 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 (Collard, 1963; Collard and Levi, 1959). Later studies showed, that among the microorganisms isolated from fermenting cassava, *Lactobacillus plantarum* produced the most typical *Gari* flavour (Ngaba and Lee, 1979). 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 (Okafor, 1990).

There have been various investigations on the microbiology of cassava fermentations and *Gari* production. Most of the work done thus far identified microorganisms associated with fermentation by phenotypic means. In this study, selected LAB isolated from various stages of fermenting cassava mash were characterised and identified at both the phenotypic and genotypic levels

in a polyphasic taxonomical approach. Technological properties were also assessed in order to select potential starter strains which can be further developed for the industrialisation of *Gari* fermentation in African countries. This study thus served to establish which lactic acid bacteria were predominant in the *Gari* fermentation process, and which of the predominant strains possess suitable technological traits that would make them suitable for further development as starter culture.

3.2 Materials and methods

3.2.1 Phenotypic characterisation

Twenty four selected presumptive LAB strains isolated from various stages of fermenting cassava mash (described in Chapter 2; Fig. 2.1 and Table 2.4) were transported during a research visit to the Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition in Karlsruhe, Germany (now the Max-Rubner-Institute, Federal Research Institute of Nutrition and Food) where they were further characterised by determination of cell morphology using phase contrast microscopy, Gram staining, catalase test, growth in MRS broth at pH 9.6 and in MRS broth with 6.5% NaCl (cocci only as this is a typical characteristic for enterococci), growth at 10, 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 and Lücke (1987). All strains were tested for their sugar fermentation patterns using 20 key sugars (Jayne-Williams, 1976). Based on the results of the phenotypic characterisation, the strains were divided into three groups, *viz*., obligately heterofermentative rods (group I), facultatively heterofermentative and obligately homofermentative rods (group II) and obligately homofermentative cocci (group III). Each strain was assigned a BFE number in addition to the original VE strain designation, and was added to the BFE culture collection. All strains were kept in MRS broth with 15% glycerol (vol/vol) added at -80°C.

3.2.2 Genotypic characterisation

3.2.2.1 DNA isolation

The total genomic DNA from 24 LAB was isolated according to the method of Pitcher *et al*. (1989) as modified by Björkroth and Korkeala (1996) for Gram-positive bacteria.

3.2.2.2 RAPD-PCR strain typing

RAPD-PCR fingerprinting was done using the primer M13 (5'-GAG GGT GGC GGT TCT-3') which was previously shown to be useful for differentiation of lactic acid bacteria (Huey and Hall, 1989, Andrighetto *et al.,* 2001; Rossetti and Giraffa, 2005). DNA was amplified using methods and amplification conditions described by Andrighetto *et al*. (2001).

3.2.2.3 Rep-PCR

Rep-PCR was used for identification of representative strains using methods as previously described by Gevers *et al*. (2001). Strains representative of RAPD-PCR clusters were used for rep-PCR typing to confirm the RAPD-PCR identification. While RAPD fingerprinting with primer M13 is capable of distinguishing at both the strain as well as species level, rep-PCR is known to be well suited for discriminating LAB at the species level (Gevers *et al.,* 2001) The primer GTG5 (5′-GTG GTG GTG GTG GTG-3′), as described by Gevers *et al.* (2001) for the identification of LAB, was used. DNA was amplified in 50 µl volumes containing 100 ng template DNA, 1x*Taq* DNA polymerase buffer (Amersham Pharmacia, Freiburg, Germany), 200 mM dNTPs, 50 pM of each primer, 4% dimethyl sulphoxide (Sigma, Steinheim, Germany) and 1.5 U *Taq* DNA polymerase (Amersham Pharmacia).

3.2.2.4 Gel electrophoresis

PCR products were separated by electrophoresis on 1.8% (w/v) agarose gels using 1xTBE Buffer (Sambrook *et al*., 1989) and a GNA 200 electrophoresis chamber (Amersham Pharmacia). Rep-PCR and RAPD-PCR products were subjected to electrophoresis at 48V 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 digitized images were normalised and subsequently analyzed using the Bionumerics (version 2.5) software package (Applied Maths, Sint-Martens-Latem, Belgium) (Kostinek *et al*., 2005a). 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).

3.2.3 Technological properties

3.2.3.1 Bacteriocin activity

Bacteriocin activity was detected by the deferred inhibition assay (Ahn and Stiles, 1990) using LAB strains from fermented cassava as the producing organisms. *Weissella paramesenteroides* DSM 20288, *Lactobacillus sakei* DSM 20017, *Lactobacillus buchneri* LMG 11439 and *L. fermentum* DSM 20052 were used as indicator bacteria. Cultures used for testing bacteriocin production were spotted (5 µl) onto the MRS agar plates and were incubated overnight at 30°C. Indicator bacteria were inoculated (1%, ca. 1 x 10^6 CFU/ml) into soft (0.75%) MRS agar, which was used to overlay MRS agar plates (Franz *et al*., 1999b). Plates were again incubated at 30°C to allow growth of the indicator bacteria as a ‘lawn’ and then examined for zones of bacteriocin activity surrounding the producer colony. Bacteriocin producing cultures were further assessed by confirming the proteinaceous nature of the inhibiting compound in separate experiments. This was determined by transferring 5 µl of proteinase K solution (10 mg/ml in bidistilled water) next to the bacteriocin

producing colonies following overnight growth. Plates were thereafter incubated for 3 h at 37°C. Plates were then overlayered with indicator bacteria and incubated overnight as described above. The absence of a zone of inhibition next to the bacteriocin producer on the side of the colony where the proteinase K was spotted indicated the proteinaceous nature of the inhibiting compound.

In order to quantify the bacteriocin activity, supernatant of a bacteriocin producing isolate grown at 30°C overnight in MRS broth was adjusted to pH 6.5 (near neutral to avoid acid effect). It was then heated at 94°C for 10 min to inactivate any remaining cells. A double dilution series was made by adding 100 µl of the cell free, neutralised supernatant to 100 µl sterile MRS in microtiter plates, and thus continuing to obtain a dilution series of 1:2, 1:4, 1:8 and 1:16 of the cell free, neutralised supernatant in MRS broth. The bacteriocin activity was expressed in arbitrary activity units (AU), which is a reciprocal of the last (highest) dilution that shows a clear inhibition zone when 10 µl were spotted onto a plate that was overlayered with indicator bacteria and subsequently incubated at 30°C overnight.

3.2.3.2 Production of hydrogen peroxide

The production of hydrogen peroxide was tested according to the method of Marshall (1979) using MRS agar containing 0.5 mM of 2-20-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma, Deisenhofen, Germany) and 3 mg/l horseradish peroxidase (Sigma). Five µl of culture were spotted onto the plates and incubated at 37°C for 20 h. The horseradish peroxidase in the medium oxidizes ABTS in the presence of hydrogen peroxide to form a purple pigment in and surrounding the H_2O_2 producing colony.

3.2.3.3 Utilisation of non-digestible α-galactoside sugars

The fermentation of the sugars raffinose and stachyose was tested according to the method of Jayne- Williams (1976). The medium containing either of the sugars was inoculated with a 1% overnight culture grown in MRS broth and

incubated at 30°C for up to three days. Acid production as a result of sugar utilisation was indicated by a change in colour of the medium from red to yellow.

3.2.3.4 Production of *β*-glucosidase

β-Glucosidase activity was tested according to the method of Weagant *et al*. (2001) using 4-nitrophenol- *β*-D-glucopyranoside (Sigma) as substrate.

3.2.3.5 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 zone around the streak of 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 (1990).

3.2.3.6 Production of aminopeptidase

Production of aminopeptidase was assessed after spotting 10 µl of an overnight culture on MRS agar and incubating for 48 h. After incubation, the presence of aminopeptidase was determined by testing for Gly-Pro-ß-Naphthylamide breakdown using a solution of 0.5 ml of Tris (0.2M, pH 7.5) with 0.2 ml of Gly-Pro-ß-Naphthylamide (Sigma, 10 mg/ml in formamide) and 10 mg fast garnet GBC Salt (Sigma #F 8761) added. A positive reaction was indicated by the observation of a deep red coloration. *L. plantarum* DSM 20174 was used as a positive control.

3.2.3.7 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). 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 (Kostinek *et al*., 2005a). Strains were then incubated at 30°C for 48 h. Acid production was determined by measuring the pH of the culture after 6, 24 and 48 h.

3.3 Results

3.3.1 Phenotypic characterisation

Only one heterofermentative rod isolate occurred among the 24 strains. All other strains did not produce gas from glucose fermentation and thus were either facultatively heterofermentative (i.e. able to utilise pentoses and produce acetate and lactate without the production of gas) or homofermentative (Fig. 3.1). The majority of strains (15/24 = 62.5%) consisted of rods which did not produce gas from glucose, produced DL-lactate and also contained m-DAP in their cell walls (Fig. 3.3), indicating that they belong to the *L. plantarum*-group (Fig. 3.1). In addition, these strains generally fermented pentose sugars such as arabinose, ribose or xylose, indicating that they are facultatively heterofermentative, which is another characteristic of the *L. plantarum*-group (Fig. 3.2). One strain (VE 20) was facultatively heterofermentative as it fermented ribose, contained meso-diaminopimelic acid in the cell wall but produced L-lactic acid. This strain could thus be presumptively identified as either *L. agilis* or *L. algidus* (Figs. 3.1 and 3.2). Two strains (VE 85a and VE 63) did not produce gas from glucose fermentation but fermented ribose, indicating that they were also facultatively heterofermentative. The strains also produced L-lactate. According to the sugar fermentation tests and growth at 15 and 45°C these isolates could be presumptively identified as either *L. casei, L. paracasei*, or *L. perolens*, or as *L. rhamnosus* in the case of VE 63 as it ferments rhamnose (Fig. 3.2). One strain, VE 102 was obligately homofermentative as it did not ferment any pentose sugars. It produced L-lactate. According to these characteristics this strain could either be a *L. amylophilus*, *L. iners*, *L. mali, L. manihotivorans*, or *L. sharpeae* strain. Four isolates (VE 60b, 65a, 91 and 97) were

homofermentative, coccus shaped and produced L-lactate. According to these characteristics they belonged to either of the genera *Enterococcus, Lactococcus* or *Streptococcus.* Strains VE 60b and 65a grew at 45°C, which is typical for enterococci or streptococci (e.g. *S. thermophilus*). The strains did not grow in MRS broth containing 6.5% NaCl (results not shown). Further phenotypic and genotypic characterisation would thus be required to determine to which of the genera these strains belong.

The *Lactobacillus plantarum* strains are difficult to distinguish from *L. pentosus* and *L. paraplantarum* strains. Some *L. pentosus* strains, however, ferment xylose while *L. plantarum* strains generally do not (Swezey *et al.,* 2000; Kostinek *et al.,* 2005b).

Thus the strains VE 36 and VE 99, both of which showed all the characteristics of belonging to the *L. plantarum*-group fermented xylose, and thus could be presumptively identified as *L. pentosus,* while the other strains of this group were presumptively identified as *L. plantarum* (Fig. 3.2).

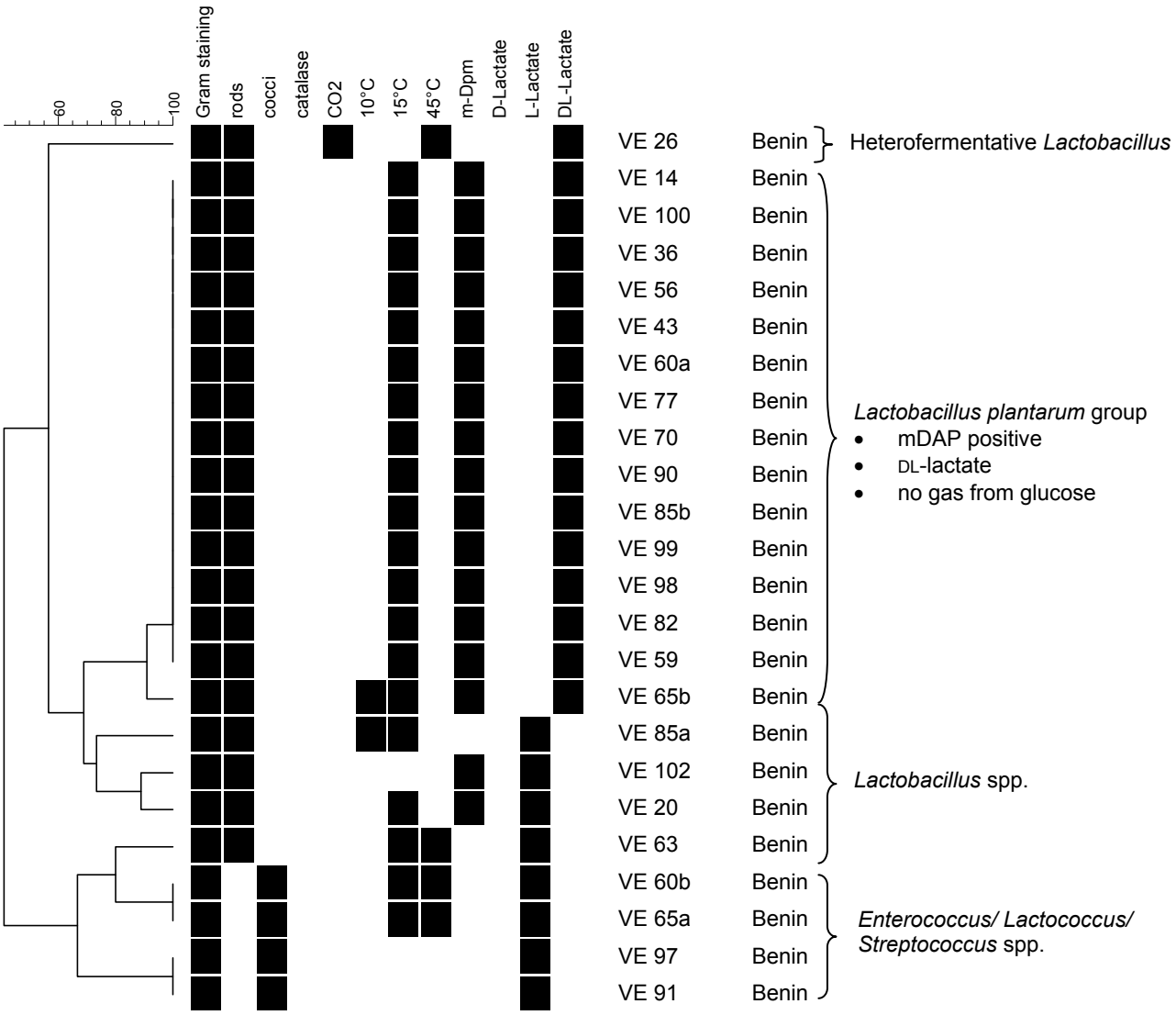

Fig. 3.1 Dendrogram obtained after clustering of the different LAB using various phenotypic methodologies, *viz*. Gram staining, morphology, catalase test, growth at 10, 15 and 45°C, gas (CO_2) production from glucose, determination of the presence of D-meso-diaminopimelic acid (mDAP) in the cell wall and determination of the type of lactic acid enantiomer produced

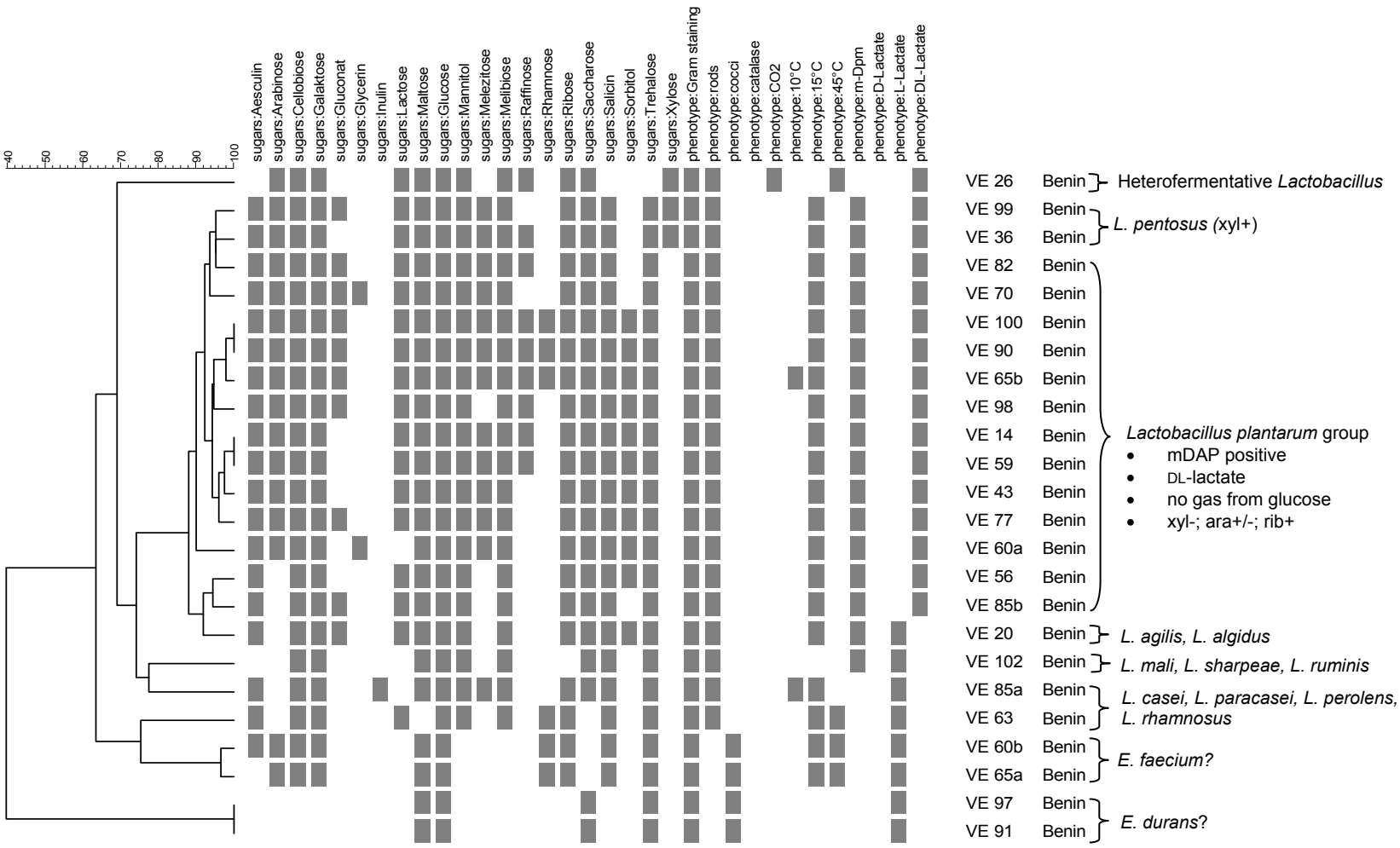

Fig 3.2 UPGMA clustering of combined phenotypic and sugar utilisation data for the selected LAB strains

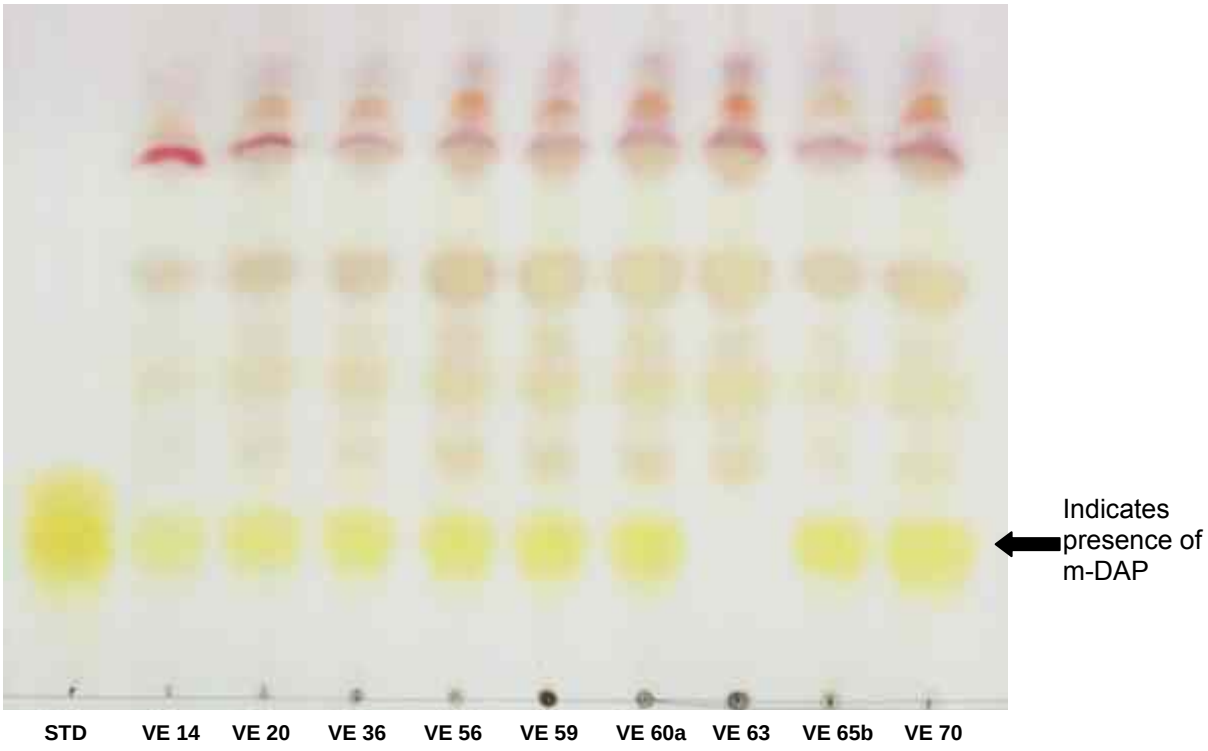

Fig. 3.3 Detection of presence of m-DAP in the cell wall (yellow coloured dot). VE 63 was negative for m-DAP

3.3.2 Genotypic characterisation

A genotypic investigation was done only for the one heterofermentative rod-shaped strain (VE 26) as confirmed in Fig. 3.4, as well as for selected rod-shaped isolates isolated from Benin. The coccus-shaped isolates were not included in the genotypic investigation as these isolates were identified as presumptive enterococci based on phenotypic results (see above). *Enterococcus* spp. have been associated with human infection and their use as starter cultures is thus controversial (Franz *et al.,* 1999a, Franz *et al.,* 2003). In addition to the rod-shaped strains isolated from Benin, further isolates from *Gari* isolated in Germany were also investigated.

RAPD strain typing was done for all heterofermentative strains isolated in Germany and in Benin. In this analysis, the strain VE 26 isolated from Benin in this study grouped together with *L. fermentum* LMG 11441 (Fig 3.4) as well as other *L. fermentum* reference strains, indicating that this isolate could be identified as *L. fermentum*.

RAPD-PCR strain typing was also done with facultatively heterofermentative, rod-shaped strains which belonged to the *L. plantarum*-group (Fig. 3.5). One of these strains grouped together with the *L. pentosus* DSM 20314 type strain, indicating that this strain could also be identified as *L. pentosus.* This strain also fermented xylose (Fig. 3.2) which is a characteristic of *L. pentosus* strains. Two strains (VE 98 and VE 59) clustered together with the *L. plantarum* BFE 617 strain, 9 strains with the *L. plantarum* CNRZZ 1228 reference strain and the DSM 20174^{T} type strain, while 5 strains clustered together with the *L. plantarum* ATCC 8014 and DSM 20246 reference strains. Thus these 16 strains (16/24 = 66.67%) could be identified as *L. plantarum* on the basis of RAPD-PCR strain typing.

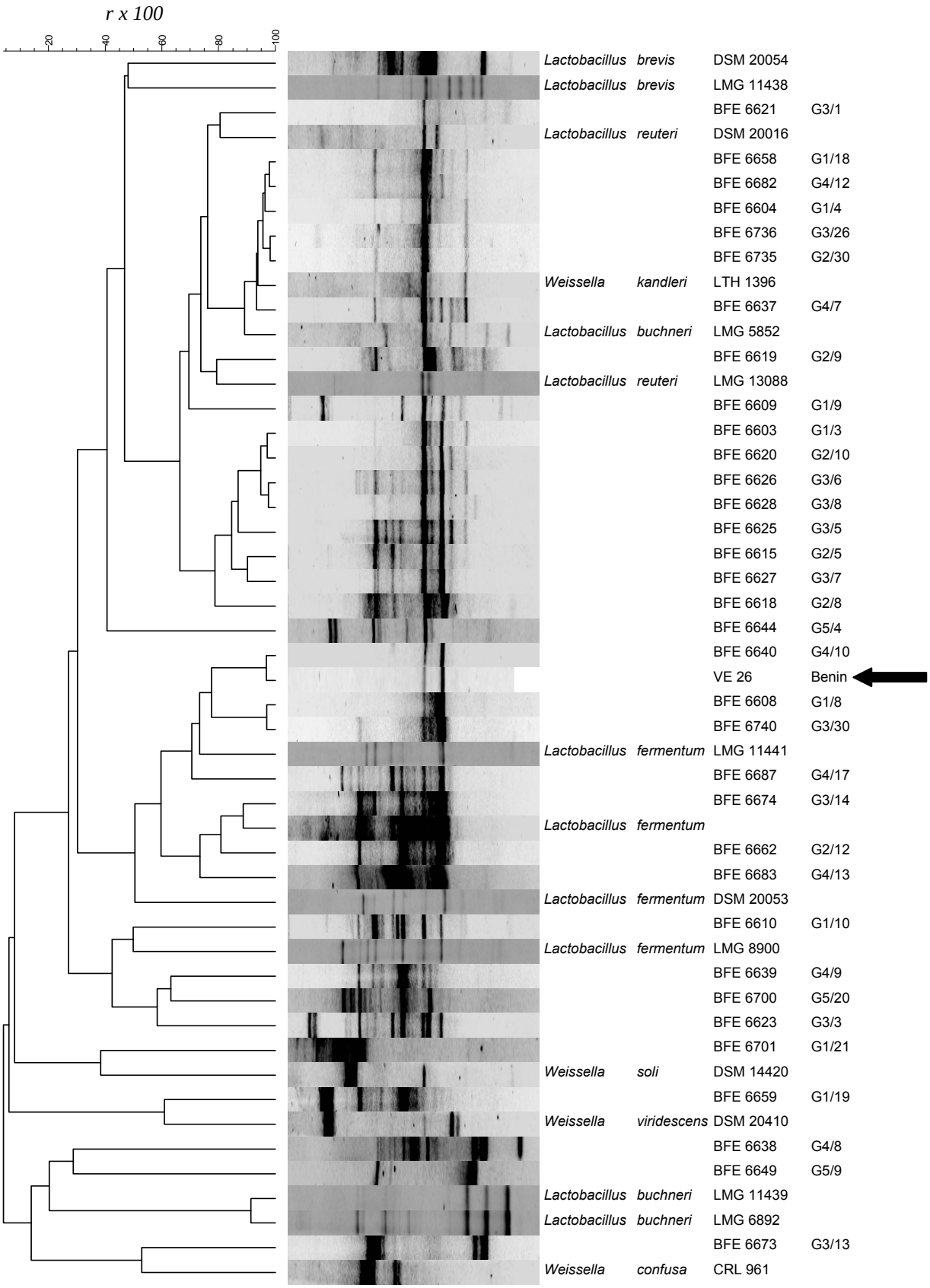

Fig 3.4 Dendrogram obtained by UPGMA of RAPD-PCR fingerprint patterns of obligately heterofermentative LAB rods (group I) using primer M13. Arrow indicates VE 26

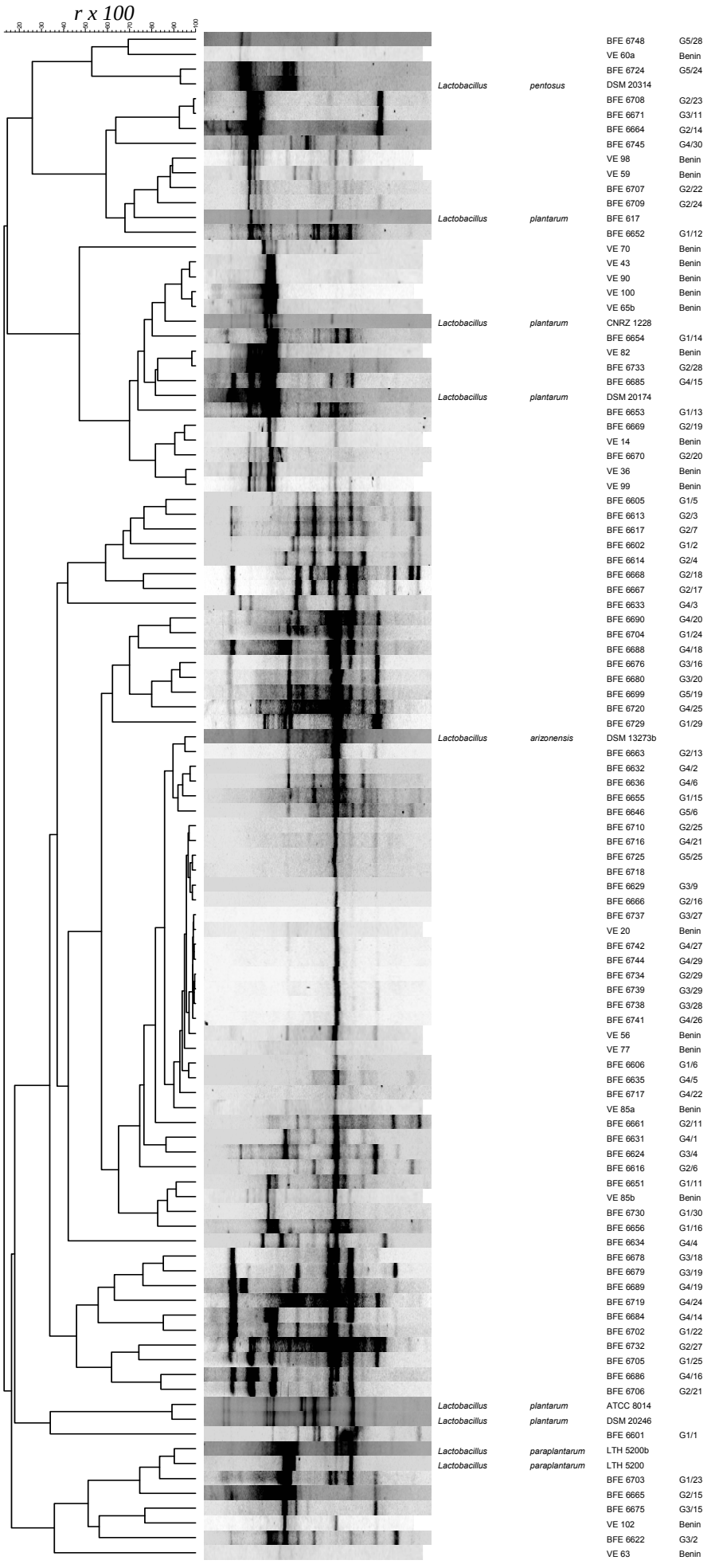

Fig. 3.5 Dendrogram obtained by UPGMA of RAPD-PCR fingerprint patterns of facultatively heterofermentative and obligately homofermentative rods (group II) using primer M13

Rep-PCR strain typing has been used successfully in *Lactobacillus* species identification (Gevers *et al*., 2001, Kostinek *et al*., 2005b, Kostinek *et al*., 2008). In this study, a few representative strains of each of the clearly delineated RAPD-PCR clusters were chosen for rep-PCR typing. Thus the isolates VE 77, VE 36, VE 82, VE 98, VE 20, VE 65b and VE 70 also clustered together with the *L. plantarum* DSM 20174 type strain and the DSM 20686 strain, confirming their identification as *L. plantarum* (Fig. 3.6). The strain VE 102 clustered outside in the RAPD-PCR as well as the rep-PCR analyses and thus the identification of this strain is not clear and this is the reason why this strain was not used as a potential starter culture in further studies.

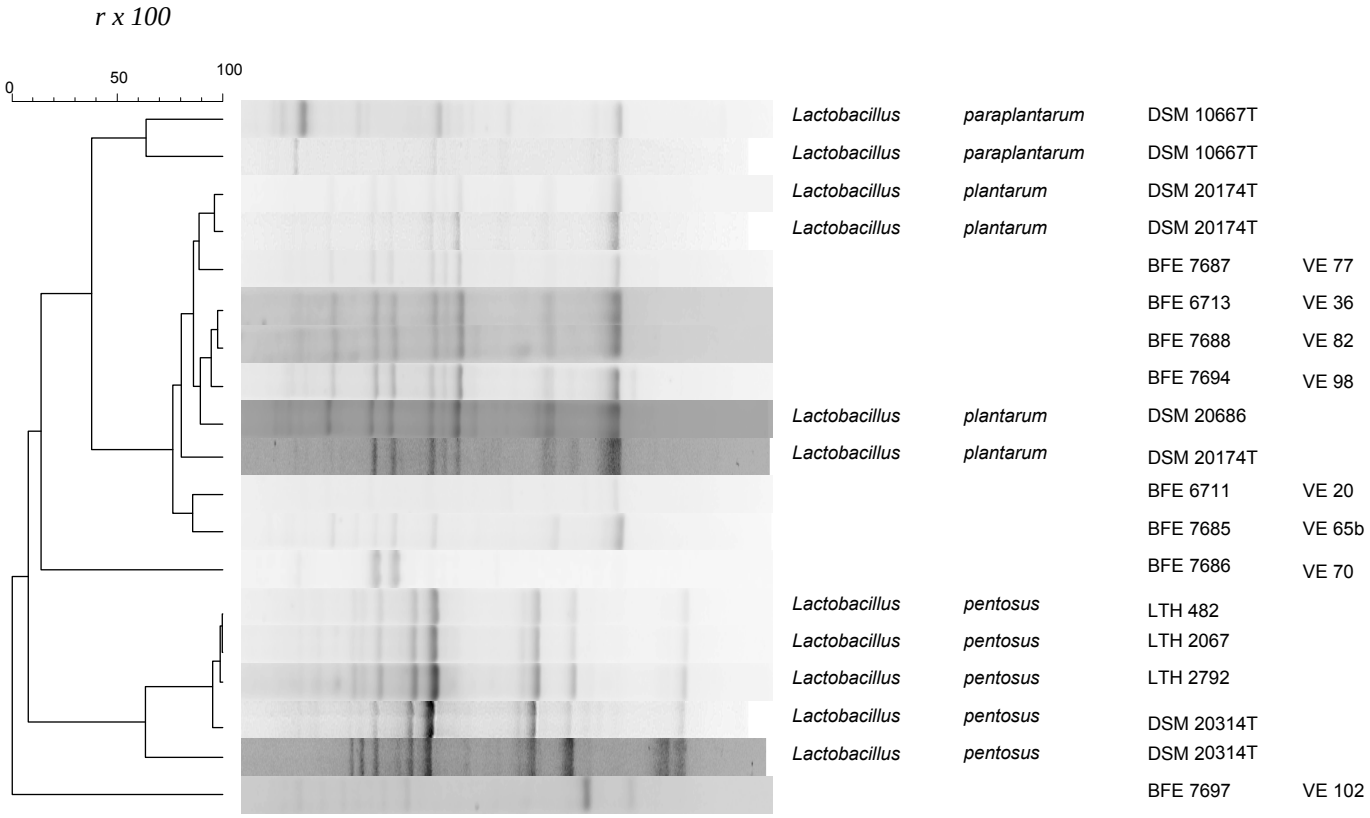

Fig. 3.6 Dendrogram obtained by UPGMA of rep-PCR fingerprint patterns of selected *Lactobacillus* strains

3.3.3 Technological properties

The technological traits of the 24 chosen strains are shown in Fig. 3.7. All of the 24 chosen strains were able to reduce the pH of MRS broth below 4.0 within 48 h of fermentation. Only 3 of 24 strains (12.5%), *viz*. the strain *L. fermentum* VE 26 and *L. plantarum* strains VE 82 and 98 were able to reduce the pH to below 3.5 after 48 hours. None of the strains were able to lower the pH below 3.0 after 48 h.

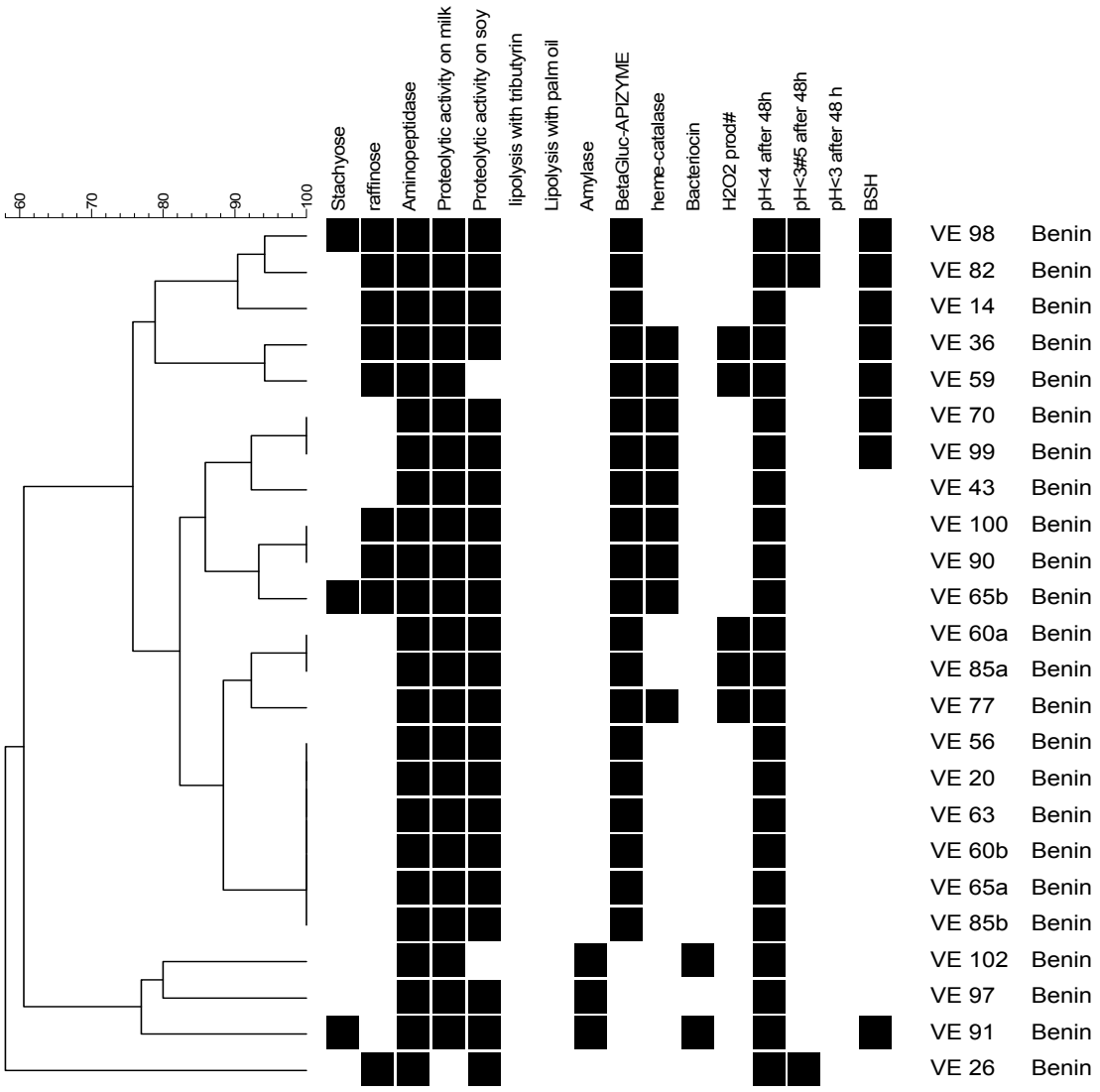

Fig. 3.7 Dendrogram of the technological properties of the 24 LAB strains

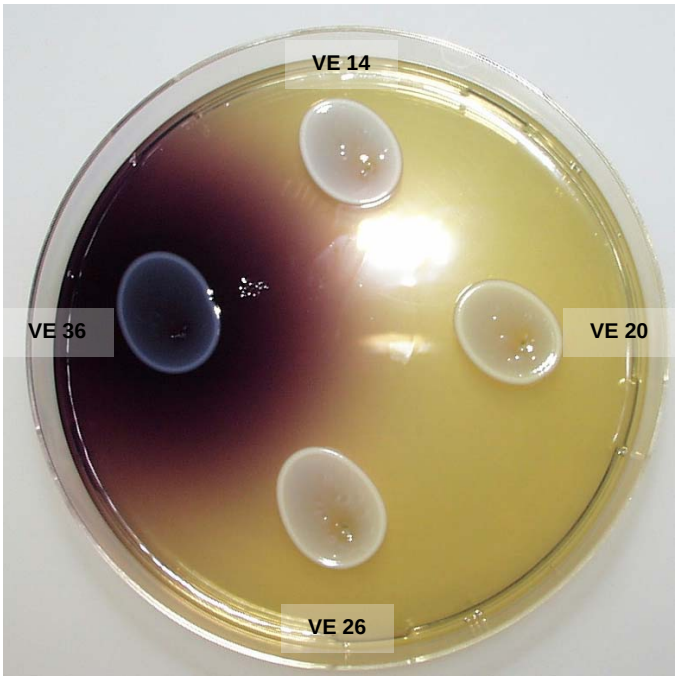

Fig. 3.8 Plate showing hydrogen peroxide producing (purple colouration) colony (VE 36) and non-producing strains VE 14, VE 20 and VE 26

Five of the 24 strains (20.8%) produced hydrogen peroxide (H_2O_2), which is shown as a deep purple colouration that forms when a strain (in this example VE 36) is positive for H_2O_2 (Fig. 3.8). The production of H_2O_2 may play a role in antagonistic activity (Lindgren and Dobrogosz, 1990) of lactic acid bacteria against other spoilage or food pathogenic bacteria, when oxygen is present. This could therefore be considered a useful property to prevent food borne pathogens from growing during cassava fermentation, as the fermentation is not an anaerobic process.

Two strains VE 91 and VE 102 showed bacteriocin activity. It is well known that bacteriocins are active against various spoilage and pathogenic bacteria (Nes *et al*., 1996; Franz *et al*., 2001; Drider *et al*., 2006). Thus bacteriocin production may also play a role in inhibiting food borne pathogens in cassava fermentations and may possibly help to establish the starter culture to predominate in the fermentation.

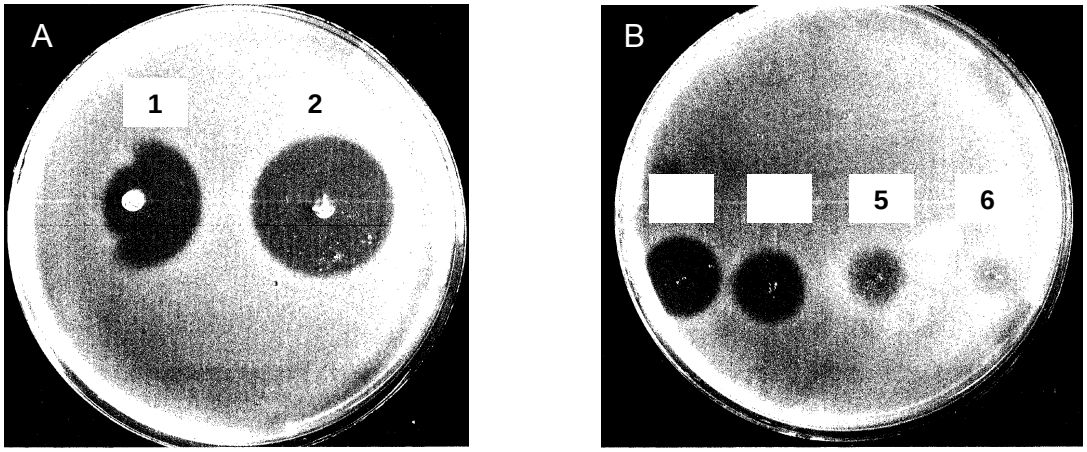

Fig. 3.9 (A) – VE 91 showing a zone of inhibition (1 and 2). The zone on the left (1) shows the half moon effect caused by proteinase K spotted next to the culture. The absence of a zone of inhibition next to the bacteriocin producer on the side of the colony where the proteinase K is spotted indicates the proteinaceous nature of the inhibiting compound

(B) – The arbitrary activity units – 200 (3), 400 (4), 800 (5), 1600 (6) AU/ml, are a reciprocal of the last (highest) dilution that shows a clear zone when 10 µl is spotted onto a plate overlayered with indicator bacteria and subsequently incubated at 30°C overnight

3.4 Discussion

A polyphasic taxonomical approach based on a combination of phenotypic and genotypic techniques was used in this study to identify the predominant isolates from fermented *Gari* from Benin. The phenotypic identification allowed a clear grouping of strains into obligately heterofermentative rods (group I), facultatively heterofermentative and obligately homofermentative rods (group II) and obligately homofermentative cocci (group III). The homofermentative cocci (group III) most probably consisted of *Enterococcus* or *Lactococcus* species. *Enterococcus* strains are often isolated from African fermented foods (Franz *et al*., 2003, Yousif *et al.,* 2005; Abriouel *et al*., 2006). As mentioned above, enterococci are often isolated from human infections and it is not yet clear whether there is a foodborne transmission (Franz *et al*, 2003). Therefore, enterococci are not usually chosen as starter cultures without an intensive safety investigation. This problem with enterococci and food safety is also the reason why these presumptive enterococci were not further investigated in this study. Based on both phenotypic and genotypic analysis, the strains VE 26 and VE 60a could be identified as *L. fermentum* (group I) and *L. pentosus* (group II) respectively.

The *Lactobacillus* species *L. plantarum*, *L. paraplantarum* and *L. pentosus* form a closely related group known as the *Lactobacillus plantarum*-group. All of these species are facultatively heterofermentative, produce DL-lactate, contain meso diaminopimelic acid in their cell wall (Curk *et al.,* 1996: Hammes and Hertel, 2003) and are difficult to distinguish from each other by phenotypic characteristics alone. Nevertheless, *L. pentosus* is generally positive for xylose fermentation, whereas *L. paraplantarum* is unable to ferment this carbohydrate (Kandler and Weiss, 1986: Sweyzey *et al*., 2000). Unlike *L. plantarum* and *L. paraplantarum*, *L. pentosus* ferments glycerol (Bringel *et al*., 1996; Curk *et al*., 1996). The majority of the strains (18 of 24, 75%) could, on the basis of the polyphasic taxonomical approach, be unequivocally identified as *L. plantarum* strains, especially by the combination of the two genotypic methods, i.e. RAPD-PCR and rep-PCR.

L. plantarum has been often shown to be the predominant species associated with traditional African food fermentations, especially those fermentations based on plant materials. For example, *L. plantarum* was shown to be the predominant lactic acid bacterium species isolated from cocoa (Ardhana and Fleet, 2003; Camu *et al*., 2008; Kostinek *et al*., 2008), fermented gruel from pearl millet (Ben Omar *et al*., 2006), sour cassava starch (Lacerda *et al*., 2005), caper berries (Pérez Pulido *et al*., 2005) and fermented maize (Ben Omar and Ampe, 2000; Abriouel *et al*., 2006). *L. plantarum* is known to be highly competitive in this environment, probably as a result of fast acid production and a coupled ability to tolerate low pH. In general, lactobacilli are known to be among the most aciduric of the lactic acid bacteria (Axelsson, 2004) and fast acid production and an associated pH drop to levels below pH 4.2 constitutes a major food safety factor (Holzapfel, 1997). Furthermore, analysis of the genome of *L. plantarum* has shown that this LAB species has one of the largest genomes of all LAB as a result of the presence of large set of genes involved in sugar uptake and utilisation. These genes are arranged in the region of the origin of replication in what is termed a “lifestyle island” (Kleerebezem *et al*., 2003). Thus, *L. plantarum* is capable of life in a great variety of environments which differ from their nutrient, especially sugar, content. Furthermore, the ability to utilise a great diversity of sugars is an advantage especially if the easy utilisable sugars become limited and only those sugars which are harder to degrade, i.e. pentoses or di- or trisachharides, are available. In addition, the utilisation of a greater variety of sugars can prolong the fermentative ability and thus may lead to as faster and greater pH lowering effect compared to other LAB. For this reason, the *L. plantarum* strains were especially favoured in this study as possible starter cultures.

The strains VE 20, VE 36, VE 65b, VE 77 and VE 82 were chosen for further study as starter cultures in chapter 4. These *L. plantarum* strains were chosen based on slight differences in their technological properties. Strain VE 20 showed proteolytic as well as aminopeptidase activity and also had good pH lowering activity (Fig. 3.6). The proteolytic/aminopeptidase activity might be important in supporting the growth of the starter and also in aroma production,

especially if cassava would be fortified with protein such as soy protein. Strain VE 36 was bile salt hydrolase positive, a functional or probiotic trait that has been linked with cholesterol lowering ability (Klaenhammer and Kullen, 1999; Naidu *et al.,* 1999). In addition, this strain also produced hydrogen peroxide, indicating that the production of this antimicrobial compound may bestow upon the strain a competitive advantage in the fermentation, allowing it to predominate. Strain VE 77 also produced hydrogen peroxide and was chosen as a potential starter based on this. In contrast to strain VE 36, it did not have BSH activity. Bile salt hydrolysis is also controversially discussed as primary bile salts produced by LAB can possibly be converted to secondary bile salts by gastrointestinal bacteria. These secondary (hydroxylated) bile salts are cytotoxic and procarcinogenic and thus may involve a health risk (Marteau *et al*., 1995). *L. plantarum* strain VE 65b was chosen, as it is able to degrade the alpha galactoside sugars raffinose and stachyose which both occur in cassava. These sugars are not sensitive to digestion by pancreatic and brush border enzymes (Quigley *et al*., 1999) of humans. Thus 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) *L. plantarum* strain VE 82 was chosen as it also breaks down raffinose and has a high capacity for organic acid production, lowering the pH of the broth to below 3.5 after 48 h.

Thus, in this study the majority of the isolates from Benin could be characterised as belonging to the *L. plantarum*-group and most of the isolates could be identified as *L. plantarum* strains on the basis of a polyphasic taxonomical approach. The strains were further investigated for technological properties which would be useful for their application as starters in the cassava fermentation, for example the ability for fast acid production, degradation of indigestible α-galactoside sugars, the production of antimicrobial (hydrogen peroxide and bacteriocin) or demonstration of functional activity (i.e. bile salt hydrolase activity). Five strains isolated from cassava from Benin (strains VE 20, VE 36, VE 65b, VE 77 and VE 82) were finally chosen on the basis of predominance and possession of suitable

technological properties. The investigation of this study was complemented by further, studies of a similar nature on *Gari* isolates by our German project partner. That study was done independently, but both served to select potential starter cultures for cassava fermentation for the production of *Gari*, as this was the common goal of the project. Thus, a wider final selection of potential starter cultures was decided on at the project level and this selection was further tested in fermentation experiments (Chapter 4).

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Chapter 4
Biomass production and small scale testing of freeze-dried LAB in cassava mash fermentations

This chapter has contributed to the following publications:

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Publication under submission in Biotechnology, Agronomy, Society and Environment: Development of a *Lactobacillus* sp. starter culture for the fermentation of cassava fortified with soybean and palm oil or coconut milk for the production of Gari. Carine Dortu, Amenan A. Yao, Cristina Pinto, **Vinodh A. Edward**, Melanie Kostinek, Charles M.A.P. Franz, Willhelm Holzapfel, Moutairou Egounlety, Samuel Mbugua, Moses Mengu, Philippe Thonart.

4.1 Introduction

Several microbial strains demonstrate properties that are useful in the food fermentation industry. Lactic acid bacteria (LAB) occupy a central role in many of these fermentation processes (Leroy and 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 *et al*., 2001; De Vuyst *et al*., 2002). A reason for this trend 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 a predominant isolate is well adapted for growth in the fermentation substrate (Klijn *et al*., 1995).

When LAB are isolated from fermentation processes, 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 feasible for industrial applications (Carvalho *et al*., 2002). Industrial applications also require the use of cheap raw materials in order for a process to be economically feasible. Freeze-drying is currently the most suitable and widely used technique for LAB preservation (Carvalho *et al*., 2002; Zayed and Roos, 2003). The freeze drying process imposes environmental stresses 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 *et al*., 1999; Carvalho *et al*., 2002; Morgan *et al*., 2006).

Fermentation processes in Africa, such as *Gari* production, usually take place at a household level. There is little starter culture use, if any, and most of these fermentations rely on back-slopping. Back-slopping involves the use of a residue (‘starter dough’) from a previous fermentation batch of acceptable

quality for inoculation (Holzapfel, 1997) of a fresh batch. However, retention of product characteristics over time may prove difficult due to changes in microbial types (Ray, 2001). 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 chapter focuses on the evaluation of the ability of the selected strains to be produced in fermenters, withstand the freeze drying process and their successful starter activity in small scale cassava mash fermentations. An ideal starter culture should be able to provide rapid acidification, remove linamarin and maintain the *Gari* texture and flavour.

4. 2 Materials and methods

4.2.1 Strains and their maintenance

The five selected starter strains from the previous chapter (strains VE 20, VE 36, VE 65b, VE 77 and VE 82), as well as 12 strains selected by the BFE in Germany (Table 4.1), were preserved in glycerol 20 % (v/v) at -80°C. As described previously, the BFE also conducted studies to select potential starter culture strains as an independent partner in the framework of the EU study. Stored vials for the selected strains were thawed as needed, and used as seed inoculum.

4.2.2 Biomass production in 2 L fermenters

Biomass production for all 17 strains was carried out in 2 L Biostat B (B. Braun Biotech International, 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. $1x10^7$ CFU/ml), in a 250 ml Erlenmeyer flask. Each flask was incubated aerobically at 30°C for 16 h without agitation and then used to inoculate the 1.3 L growth medium.

The raw material composition of the various media used in the fermentations is outlined in Table 4.2. MRS medium (Merck) is referred to as medium 1. Medium 2 contained an additional 20 g/L of glucose. Medium 3, which was specific for the biomass production of *L. plantarum* and *L. pentosus,* was provided by the Belgian partners, the Walloon Center for Industrial Biology, University of Liège. Media 4 and 5 had minor variations to medium 3. Medium 4 had no meat extract, but a higher yeast extract (1 g/l) content, whereas medium 5 contained CSL (Afprod) instead of CSL (Roquette), and also had no meat extract. The medium also contained a higher yeast extract content (1 g/l).

Prior to the 2 L fermentations, the 17 selected strains were grown in static flask cultures (100 ml in 250 ml Erlenmeyer flasks). This was conducted as a pilot experiment to test starter culture growth conditions. All *L. plantarum* and *L. pentosus* strains were grown using media 3, 4 and 5 (Table 4.2), while all of the *L. fermentum*, *W. paramesenteroides* and *L. mesenteroides* ssp. *mesenteroides* were grown using all 5 recipes (results not shown).

The rationale for this was that the media for *L. plantarum* and *L. pentosus* were already optimised from previous work by the Belgian partners. Parameters for these were only adjusted to use a locally produced CSL (Afprod). For the *L. fermentum, W. paramesenteroides* and *L. mesenteroides* ssp. *mesenteroides*, optimisation of media was required as this had not been investigated by the Belgian partner before.

Individual fermentation medium recipes were chosen based on the cell counts, OD and biomass produced in these small–scale pilot experiments for each strain (results not shown). A summary of the final fermentation medium selected for each strain for the production of biomass in 2 L fermenters can be seen in Table 4.3.

Table 4.1 Morphological and biochemical properties of selected strains used for starter culture development

Isolate Internal Collection No.	*Isolate BFE No.*	*Country of sample origin*	*Morphology*	*α-amylase activity*	*β-glucosidase activity*	*Presumptive Identification/group*	*Genotypic Identification*
G 2/10	BFE 6620	Benin	rods	-	-	obligatory heterofermentative rods	*L. fermentum*
G 3/5	BFE 6625	Benin	rods	-	-	obligatory heterofermentative rods	*L. fermentum*
Lb 61	BFE 7589	Benin	rods	-	+	*L. plantarum*-group	*L. pentosus*
Lb 68	BFE 7596	Benin	rods	-	+	*L. plantarum*-group	*L. pentosus*
G 5/28	BFE 6748	Benin	rods	-	+	*L. plantarum*-group	*L. pentosus*
Lb 50	BFE 6793	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
G 2/25	BFE 6710	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
G 3/29	BFE 6739	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
G 4/18	BFE 6688	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
VE 20	BFE 6711	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
VE 36	BFE 6713	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
VE 65b	BFE 7685	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
VE 77	BFE 7687	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
VE 82	BFE 7688	Benin	rods	-	+	*L. plantarum*-group	*L. plantarum*
Lc 11	BFE 7601	Benin	cocci	-	+	*Leuconostoc/Weissella*	*W. paramesenteroides*
Lc 18	BFE 7608	Benin	cocci	-	-	*Leuconostoc/Weissella*	*W. paramesenteroides*
Kenya M Rog 2	BFE 7668	Kenya	cocci	-	+	*Leuconostoc/Weissella*	*L. mesenteroides* ssp. *mesenteroides*

Table 4.2 Composition of different fermentation media used for biomass production in 2 L fermenters

Medium number	1	2	3	4	5
Fermentation Raw materials	**g/l**				
Yeast extract	5	5	20	21	21
Peptone	10	10	-	-	-
Beef extract	5	5	-	-	-
Potassium phosphate	2	2	-	-	-
Tween 80	1	1	-	-	-
Tri-Ammonium citrate	2	2	-	-	-
Manganese sulphate	0.05	0.05	0.026	0.026	0.026
Magnesium sulphate	0.1	0.1	0.026	0.026	0.026
Sodium acetate	5	5	-	-	-
Glucose	20	40	50	50	50
CSL[a] (Afprod)	-	-	-	-	50
CSL[a] (Roquette)	-	-	10	10	-
Meat extract	-	-	1	-	-
Iron sulphate	-	-	0.013	0.013	0.013

[a]Corn Steep Liquor

The medium was sterilised insitu at 121°C for 15 minutes. Glucose monohydrate was sterilized separately in an autoclave and added aseptically to the fermenter. All chemicals (anhydrous) were supplied by Merck (Darmstadt, Germany). 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 controlled with 1N HCl and 1N NaOH. The fermentation was run until glucose was depleted or for ~24 hours. Samples (2 ml) were taken every 2 to 4 hours for pH, glucose, OD_{660} (optical density at 660 nm) and viable cell counts (CFU/ml) determinations. Biomass was estimated using the dry weight method. However, due to interference of the production medium, which was slightly granulated after hydration, viable cell counts were instead used to estimate biomass formation and the dry cell weight method was only used for correlation. Glucose concentration was determined using the Accutrend® *alpha* glucose meter (Boehringer Mannheim), pH was measured using a Jenway 3310 pH Meter and the optical density was determined using the Spectronic® 20 Genesys™ at 660 nm.

Table 4.3 Summary of the raw materials used for the biomass production of each selected lactic acid bacterium starter strain for *Gari* fermentation

Strain number		G2/10	G3/5	Lb 61	Lb 68	G5/28	Lb 50	G2/25	G3/29	G4/18	VE20	VE36	VE65b	VE77	VE82	Lc11	Lc18	KM Rog 2
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	**Unit**	**Unit/l**																
Yeast extract	g	5	5	20	21	20	21	20	20	20	20	20	20	20	21	5	5	5
Peptone	g	10	10													10	10	10
Beef extract	g	5	5													5	5	5
Potassium phosphate	g	2	2													2	2	2
Tween 80	g	1	1													1	1	1
Tri-Ammonium citrate	g	2	2													2	2	2
Manganese sulphate	g	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	g	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	g	5	5													5	5	5
Glucose	g	20	20	50	50	50	50	50	50	50	50	50	50	50	50	20	40	20
CSL (Afprod)	g				50										50			
CSL (Roquette)	g			10		10	10	10	10	10	10	10	10	10				
Meat extract	g			1		1		1	1	1	1	1	1	1				
Iron sulphate	g			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*

4.2.3 Centrifugation and freeze drying process

The biomass produced from each organism was collected and centrifuged at 10 000 rpm in a Beckman ® Model J2 – 21, Rotor JA 14 for 10 minutes. 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 maltodexrin 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 subsequently making serial dilutions. At each stage, the pellets were weighed to keep track of mass balances. The cell material with cryoprotectants was then freeze-dried.

The freeze-drying process (Fig 4.1) was carried out in a Genesis 25L freeze-dryer (VirTis, United Scientific) as depicted in Fig. 4.2. The freeze-dried material obtained varied from strain to strain but were in the range of ca. 1-5g.

To test for cell viability of freeze-dried biomass, 0.1 g of this biomass was added to 0.9 g of sterile water. Serial dilutions were then made and 2µl plated onto MRS agar for plate counts and incubated at 30°C overnight. Plates counts were done in triplicate on MRS agar and mean values were calculated.

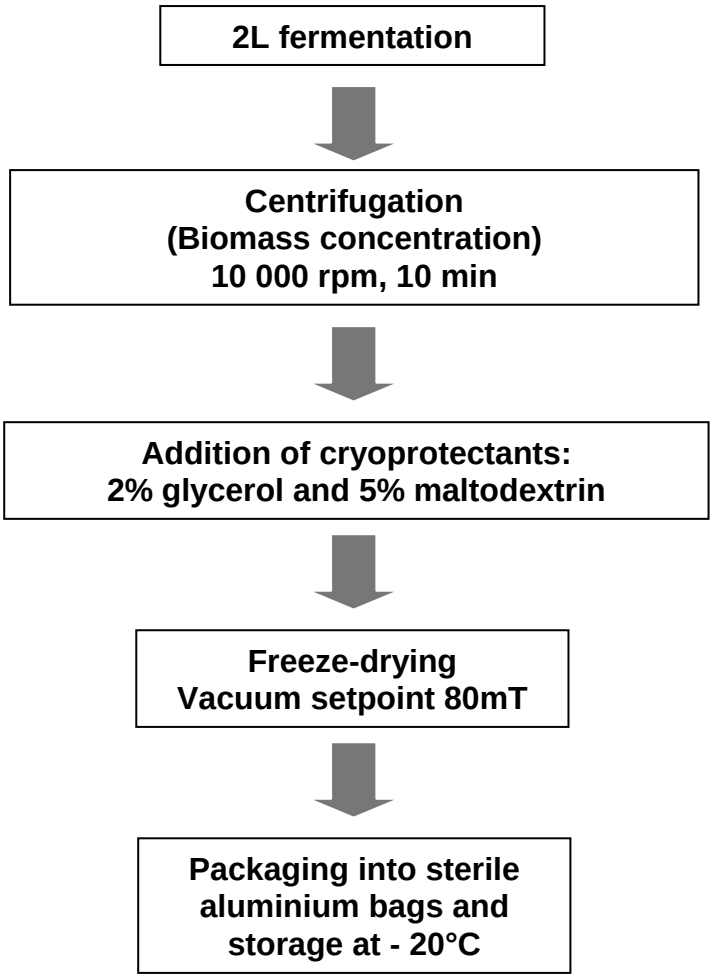

Fig. 4.1 Schematic of the freeze drying process used in this study for LAB

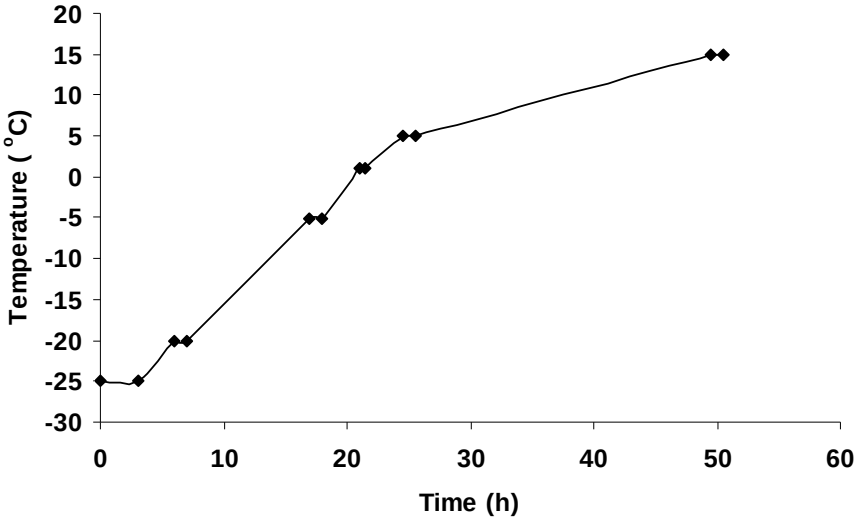

Fig. 4.2 Freeze drying protocol for LAB, showing the gradual increase of temperature in the freeze dryer over time

4.2.4 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 were provided by the Department of Nutrition and Food Science, University of Abomey-Calavi, Benin. Soybean and palm oil were added 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 8h), ground using a mechanical grinder and stored at ambient temperature before use. Palm oil was also stored at ambient temperature before use.

Ground cassava (500g) was mixed with 30 g of soybean and 5 ml of palm oil in 1 L buckets as indicated in Fig. 4.3. Each starter culture (17) was inoculated in separate experiments at an initial concentration of approximately $1x10^8$ CFU/g of cassava after rehydration of 1g of each freeze-dried starter culture in 10 ml of saline-peptone water (NaCl 5g/l, casein peptone 1g/l, Tween 80 1g/l) for 10 minutes, and diluting in saline-peptone water using a ten-fold dilution series to obtain the appropriate concentration ($1x10^8$ CFU/g) in the cassava mash. The uninoculated cassava mash served as a negative control. All the buckets were sealed tight with lids and incubated at 30°C for 48 hours. Two replicates were made for each culture.

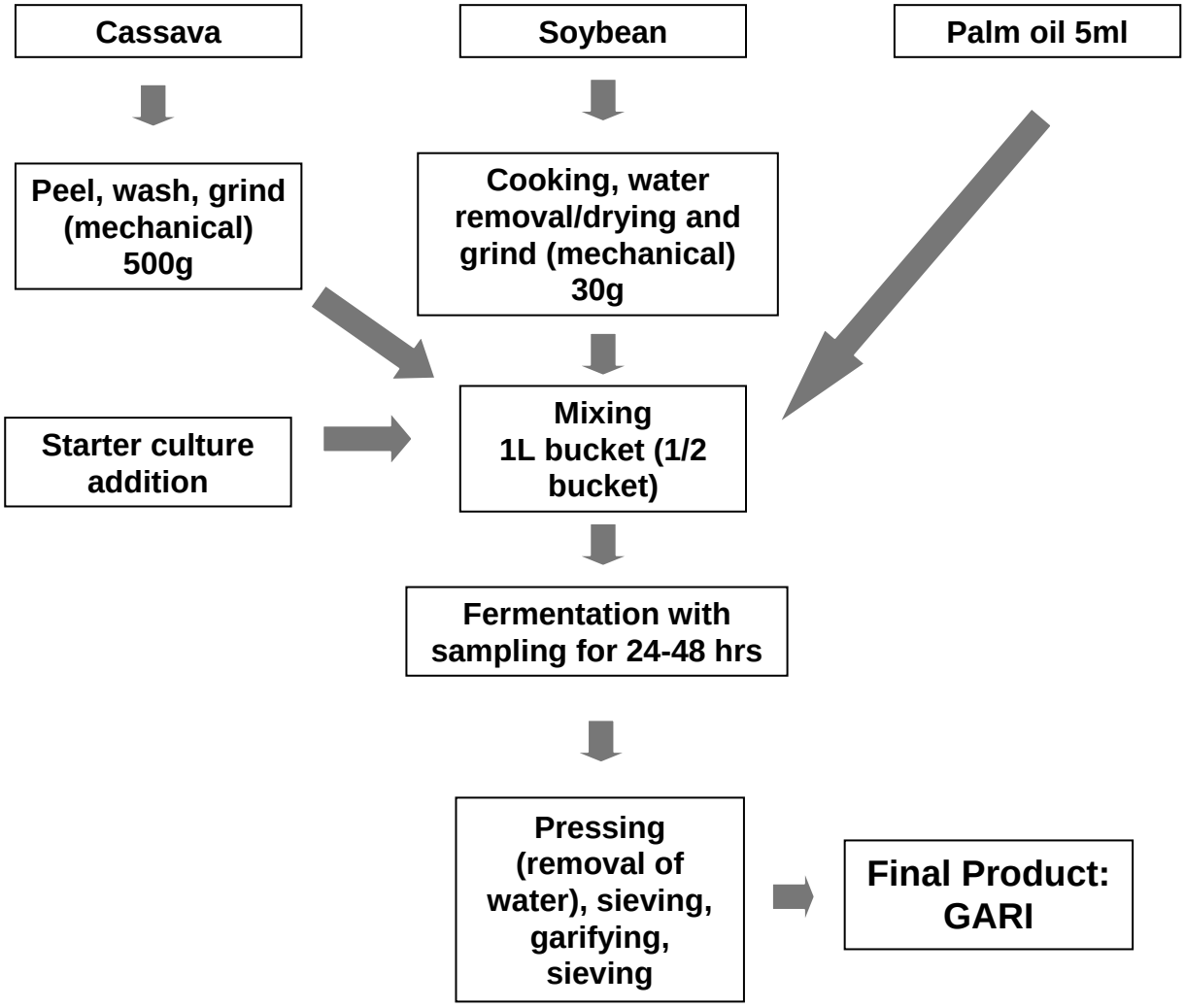

Fig. 4.3 Schematic of *Gari* fermentation conducted at small scale in the laboratory

Samples were removed at 0 h, 8 h, 24 h and 48 h during each 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.

For microbial analysis, samples were taken at 0, 24 and 48 hours 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 lactic acid bacteria counts and on Malt Extract Agar (malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l, pH 5.4) containing 0.5 µg/ml of chloramphenicol for yeast counts.

4.2.5 Cyanide determination

Total cyanide present in the cassava fermentation was determined using a picrate paper kit as described by Bradbury *et al*. (1999). The 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 10 level colour chart (indicating 0-800 ppm) to determine the presence of total cyanide.

4.3 Results

4.3.1 Biomass production in 2 L fermenters

A total of 17 fermentations were carried out in 2 L fermenters. Figures 4.4 to 4.9 show the pH, OD, biomass, cell counts and glucose utilisation for each of the fermentations, the results of which are also summarised in Table 4.4. Fermentation was stopped once the glucose was depleted as determined by glucose testing. The majority of the fermentations were terminated after 10 to 14 hours. The exceptions were *L. fermentum* strain G 3/5, *L. pentosus* strain Lb 68 and *L. plantarum* strain VE 82, which took 18, 25 and 25 hours respectively. *L. mesenteroides* ssp. *mesenteroides* strain KM Rog 2 and *W. paramesenteroides* strain Lc 18 completed the fermentation in 8 and 9.5 hours, 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 4.4. Variations were evident in terms of cell concentration after fermentation and after freeze-drying for all strains. *L. fermentum* strain G 2/10 produced 2.8 g/l biomass compared to 6.7 g/l produced by *L. fermentum* strain G 3/5 (Fig. 4.4). Strain G 3/5 was also 1 Log higher in terms of cell counts in the freeze dried powder compared to

strain G 2/10. However, strain G 2/10 had a high yield of 43 % after freeze drying compared to only 14 % for strain G 3/5. The biomass produced by the *L. pentosus* strains G 5/28, Lb 61 and Lb 68 (Fig. 4.5) 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 their yields after freeze drying ranged from 20 to 27 %. The majority of strains (9/17 = 53 %) studied were *L. plantarum*. The biomass produced by the *L. plantarum* strains ranged from 5.1 to 18.2 g/l (Figs. 4.6 and 4.7). Cell counts in freeze dried powder for these strains ranged from 11.1 to 13.0 Log CFU/g. Their yields after freeze drying ranged from 18 to 51 %. Biomass produced by *W. paramesenteroides* strains Lc 11 and Lc 18 were 1.4 and 3.7 g/l respectively (Fig. 4.8). Their cell counts in freeze dried powder were between 11.3 and 12.2, and their yield after freeze drying was between 20 and 34 %. *L. mesenteroides* ssp. *mesenteroides* strain KM Rog 2 produced 2.6 g/l biomass (Fig. 4.9), with a cell count of 11.2 Log CFU/g in the freeze dried form, and a yield of 24 % after freeze drying.

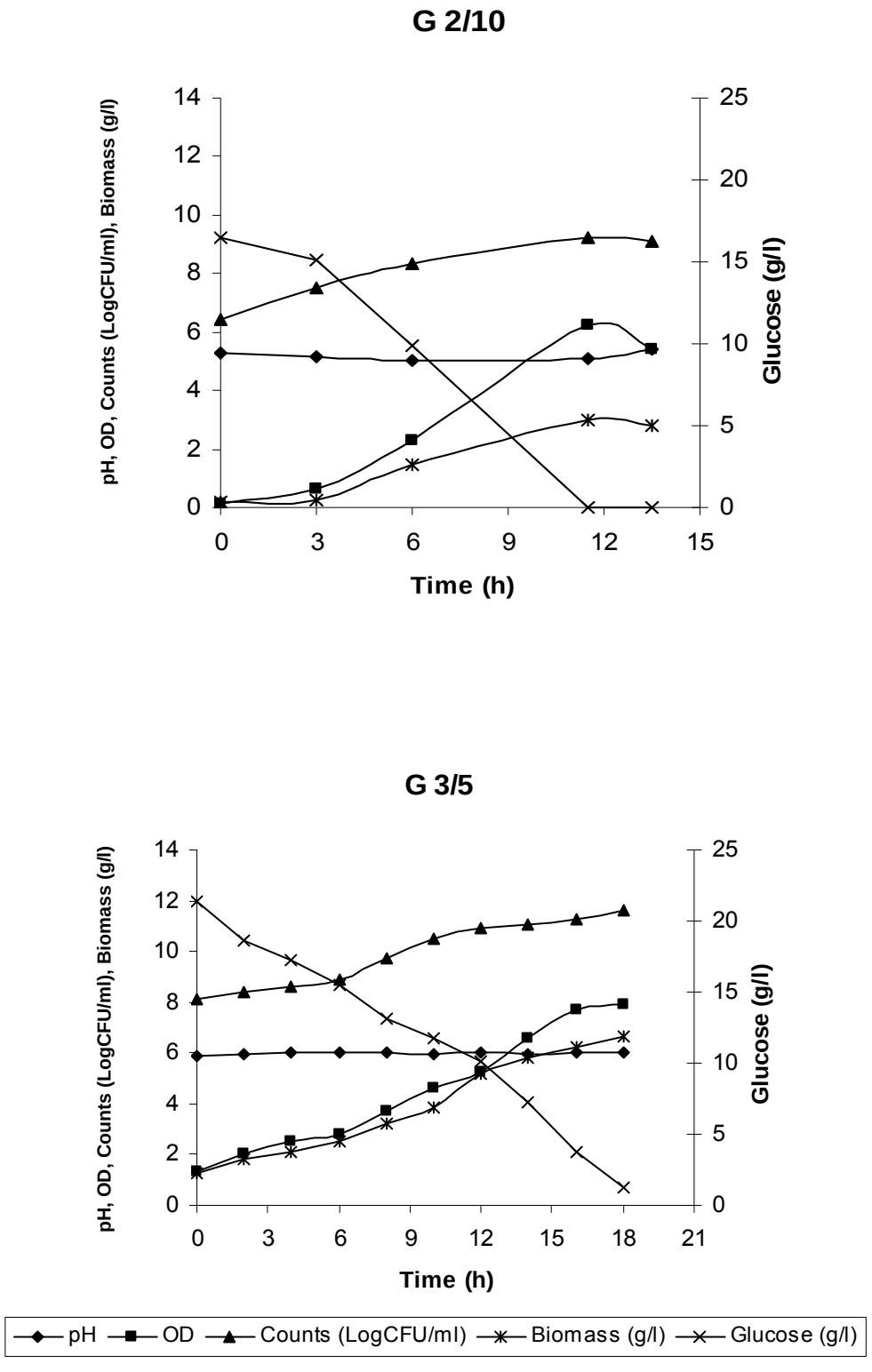

Fig. 4.4 Growth characteristics of *L. fermentum* strains G 2/10 and G 3/5, showing mean values for pH, OD, cell counts, biomass and glucose utilisation

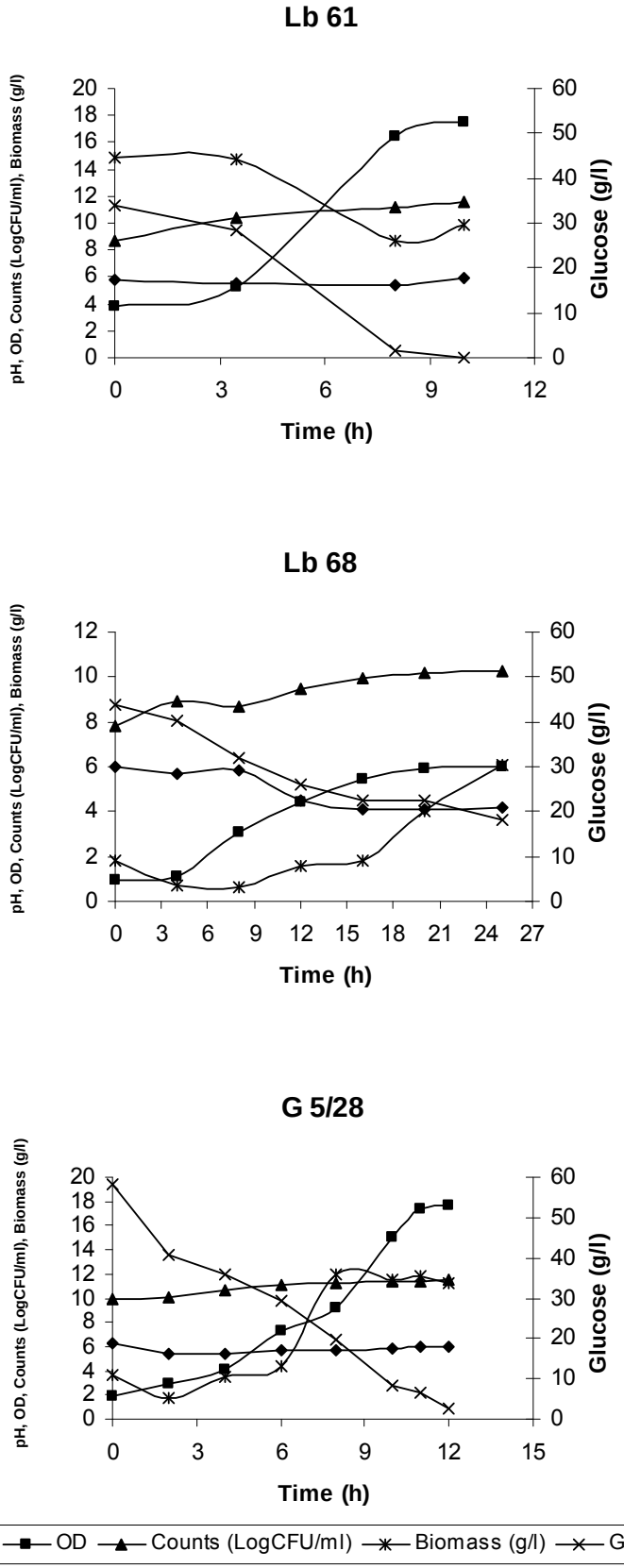

Fig. 4.5 Growth characteristics of *L. pentosus* strains Lb 61, Lb 68 and G 5/28, showing mean values for pH, OD, cell counts, biomass and glucose utilisation

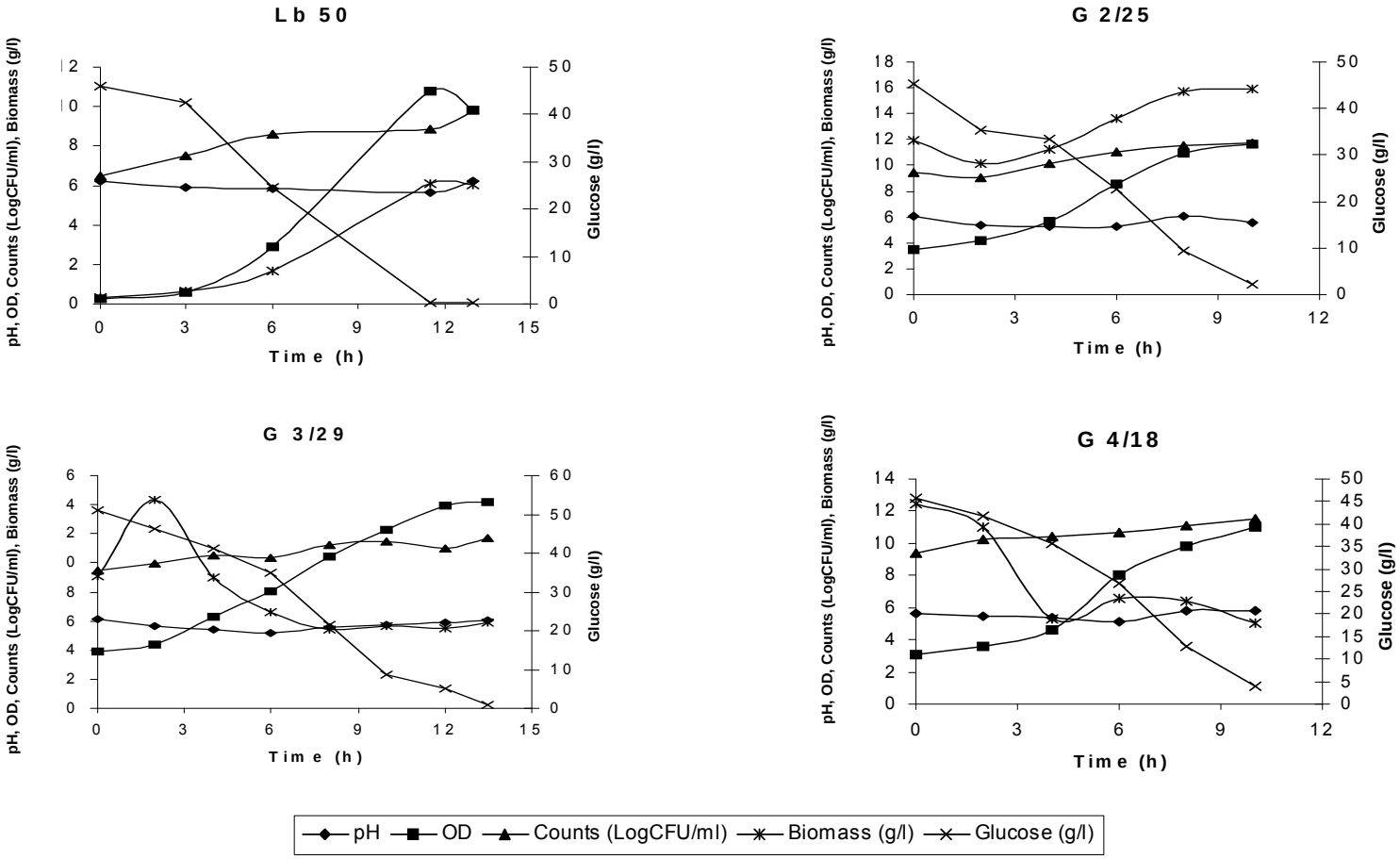

Fig. 4.6 Growth characteristics of *L. plantarum* strains Lb 50, G 2/25, G 3/29 and G4/18, showing mean values for pH, OD, cell counts, biomass and glucose utilisation

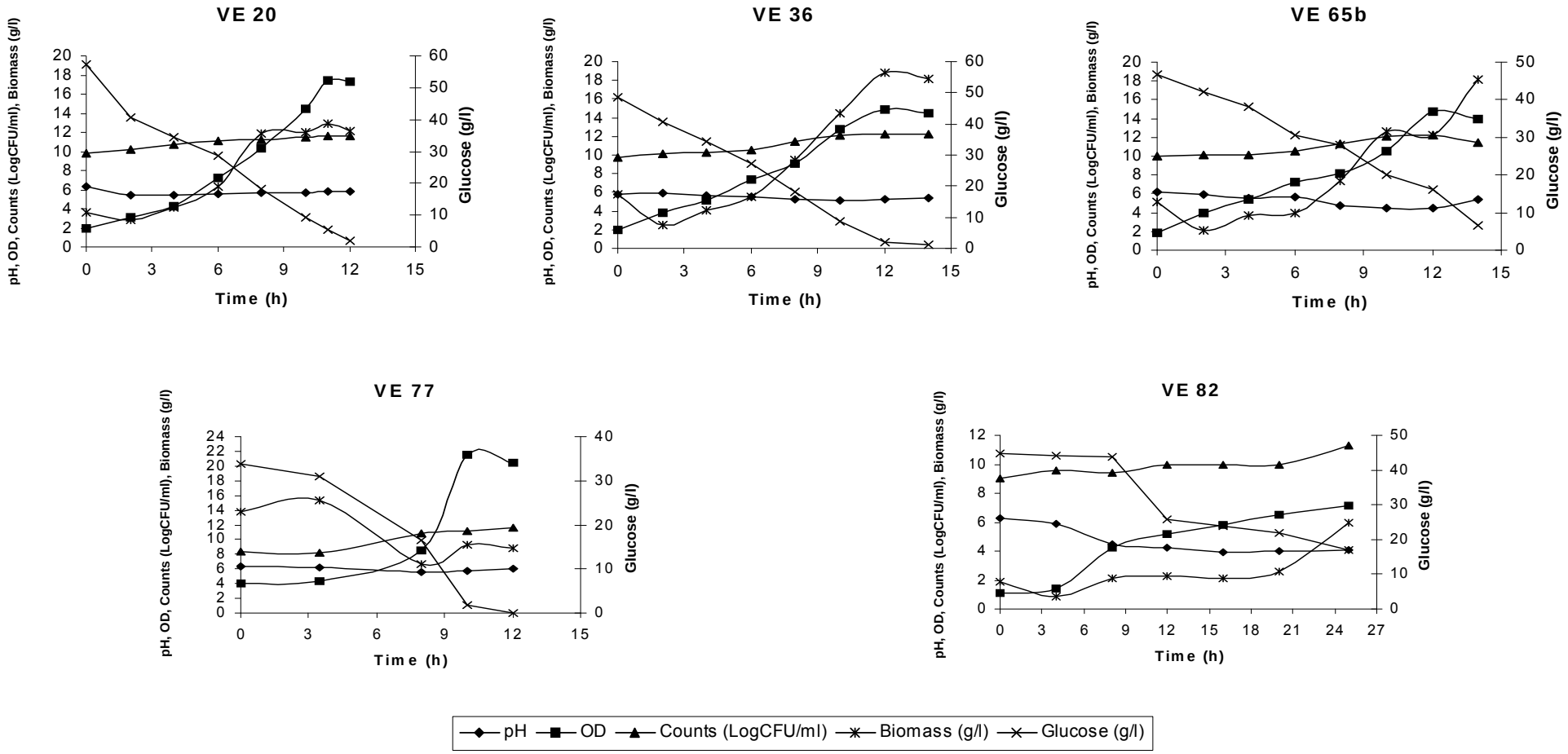

Fig. 4.7 Growth characteristics of *L. plantarum* strains VE 20, VE 36, VE 65b, VE 77 and VE 82, showing mean values for pH, OD, cell counts, biomass and glucose utilisation

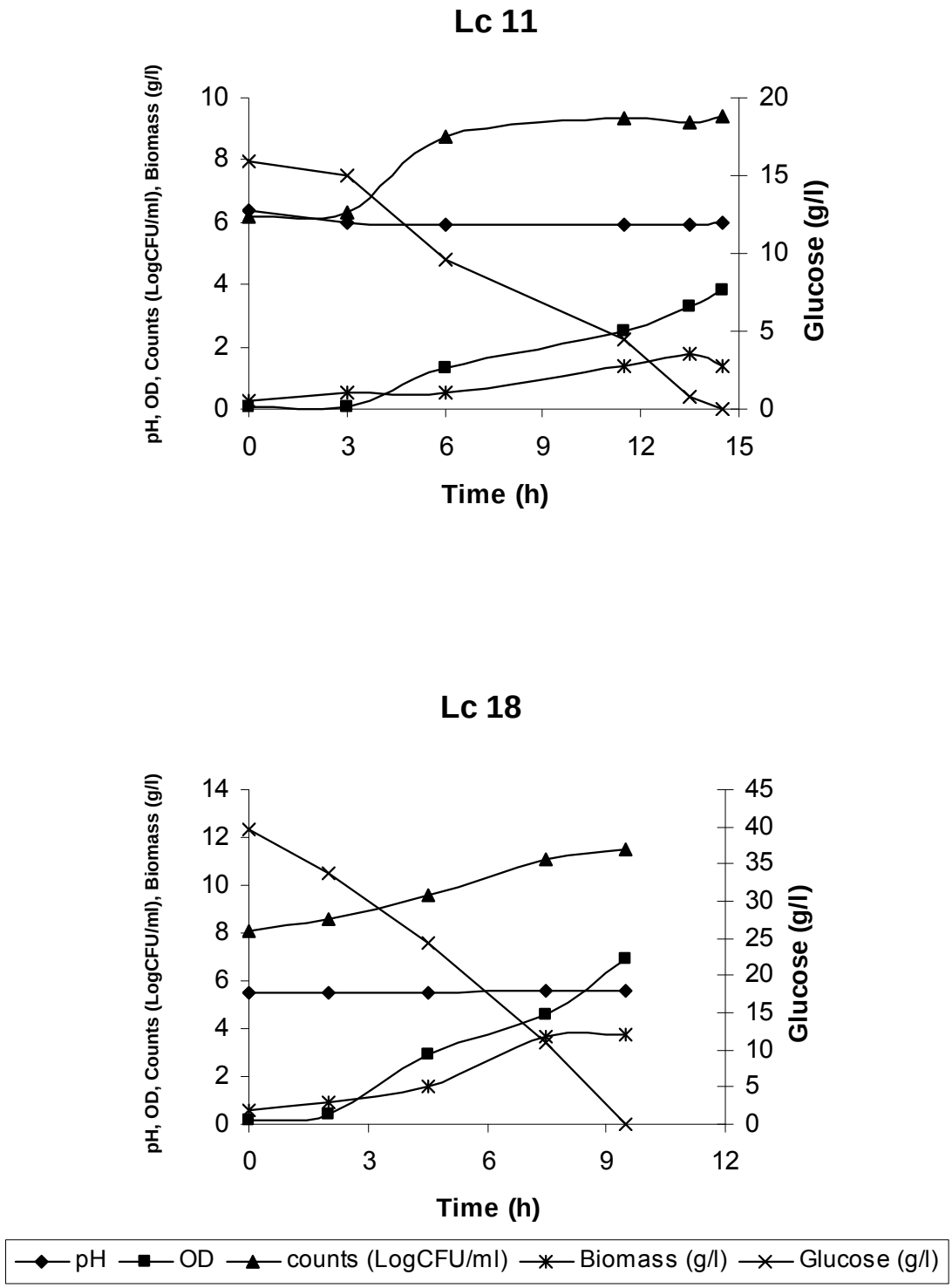

Fig. 4.8 Growth characteristics of *W. paramesenteroides* strains Lc 11 and Lc 18, showing mean values for pH, OD, cell counts, biomass and glucose utilisation

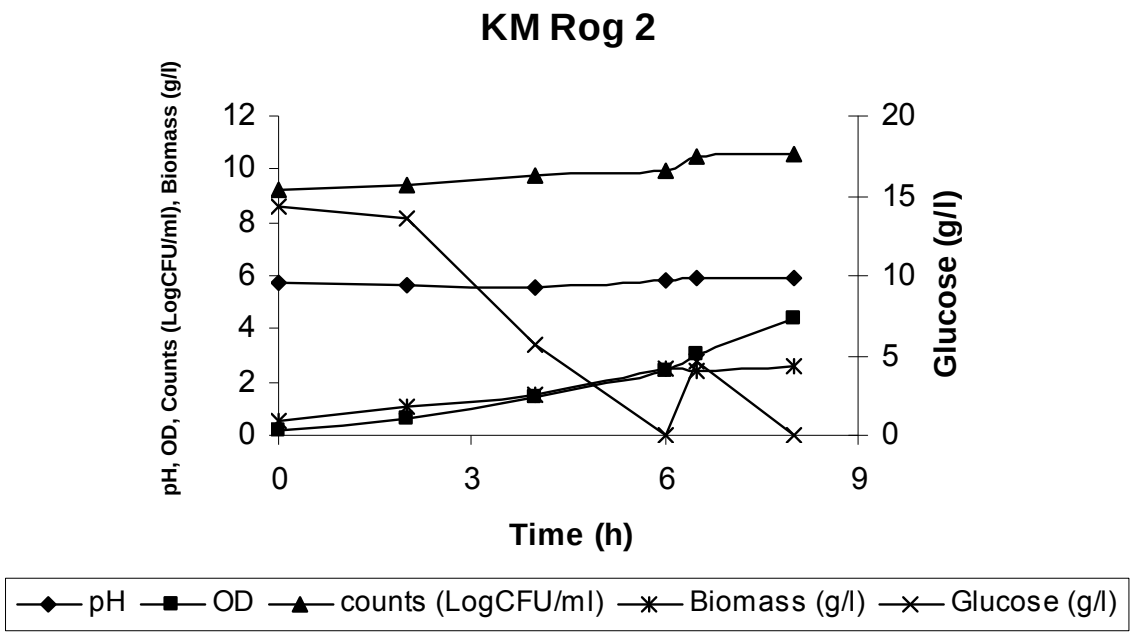

Fig. 4.9 Growth characteristics of *L. mesenteroides* ssp. *mesenteroides* strain KM Rog 2 showing mean values for pH, OD, cell counts, biomass and glucose utilisation

Table 4.4 Summary of the fermentations with the selected starter cultures in 2 L fermenters and yield after freeze drying

Strain	*Medium**	*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 (%)*
G 2/10	1	6.4	9.1	2.8	13.5	10.5	43
G 3/5	1	8.1	11.6	6.7	18.0	11.5	14
Lb 61	3	8.7	11.6	9.9	10.0	12.5	20
Lb 68	5	7.9	10.2	6.1	25.0	11.7	27
G 5/28	3	10.0	11.6	11.2	12.0	12.4	26
Lb 50	4	6.5	9.8	6.0	13.0	11.1	33
G 2/25	3	9.4	11.7	15.9	10.0	12.9	31
G 3/29	3	9.5	11.7	5.9	13.5	12.6	51
G 4/18	3	9.4	11.5	5.1	10.0	12.2	20
VE 20	3	9.8	11.7	12.2	12.0	12.4	20
VE 36	3	9.7	12.2	18.2	14.0	13.0	24
VE 65b	3	10.0	11.5	18.2	14.0	12.3	18
VE 77	3	8.4	11.6	8.8	12.0	12.3	31
VE 82	5	9.0	11.3	6.0	25.0	12.4	47
Lc 11	1	6.2	9.4	1.4	14.5	11.3	34
Lc 18	2	8.1	11.5	3.7	9.5	12.2	20
Kenya M Rog2	1	9.3	10.6	2.6	8.0	11.2	24

* Refer to media in materials and methods section (Table 4.2)

Table 4.5 Cost in South African Rands (ZAR) of raw materials and cost/g LAB starter strain biomass produced

Strain number			G2/10	G3/5	Lb 61	Lb 68	G5/28	Lb 50	G2/25	G3/29	G4/18	VE 20	VE 36	VE 65b	VE 77	VE 82	Lc 11	Lc 18	KM Rog 2
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-03	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-02	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-03	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*

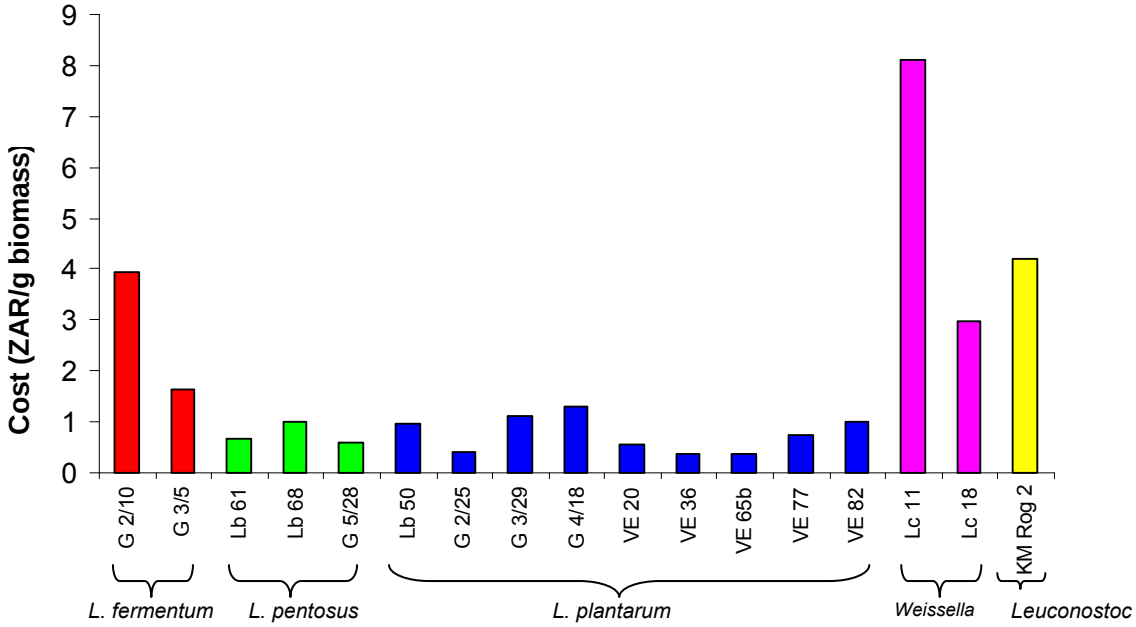

Fig. 4.10 Summary of the cost in South African Rands (ZAR) per gram of LAB starter strain biomass produced in 2 L fermenters

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. Thus energy and labour costs would need to be added. This, however, could not be calculated because of regional variances in labour and energy prices and the different energy efficiencies of different freeze drying equipment. The raw materials costs associated with biomass production of the *L. plantarum* and *L. pentosus* strains as calculated in this study clearly were much lower than those for the *L. fermentum* and *Leuconostoc/Weissella* strains (Table 4.5 and Fig. 4.10). The cost/g biomass produced for *L. fermentum* was between ZAR 1.64 and ZAR 3.95, for *L. pentosus* it was between ZAR 0.59 and ZAR 0.99, for *L. plantarum* it was between ZAR 0.36 and ZAR 1.30, for *W. paramesenteroides* it was between ZAR 2.98 and ZAR 8.13, and it was ZAR 4.22 for *L. mesenteroides* ssp. *mesenteroides*. However, biomass production for *Leuconostoc/Weissella* strains was much lower due to the smaller cell size. This affected the cost/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.

4.3.2 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 palm oil, to determine the starter potential for growth and acidification of the raw material.

The pH of the cassava before inoculation was 5.5 ± 1.0 and the titratable acidity was 0.5 ± 0.3%. The changes in cell concentration, pH, titratable acidity and cyanide can be seen in Table 4.6.

The pH was 5.2 for both control fermentations (control 1 and control 2) respectively after 24 hours of fermentation. At the same time point, the pH for the fermentations with the selected starter strains was between 4.3 and 4.8. The titratable acidity was 0.8 and 0.9 % for control 1 and 2, respectively, while it ranged from 1.1 to 1.3 % for the fermentations with the selected starter strains. Differences regarding pH and titratable acidity at 48 hours 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 G 4/18 had the highest titratable acidity at 1.6 %. The pH for the two controls was between 4.8 and 4.9 at 48 hours, while it ranged from 3.9 to 4.6 for the fermentations with the selected starter strains. *L. plantarum* strains Lb 50 and G 4/18 showed the greatest pH reduction at 48 hours, i.e. down to pH 3.9.

There was a considerable reduction in cyanide concentrations between the controls and the fermentations with the selected starter strains. After 48 hours of fermentation there was 60 ppm/kg cyanide detected in the control buckets. On the other hand, cyanide was detected at concentrations between 10-50 ppm/kg for the fermentations with the selected starter strains.

LAB counts varied between 9.1 and 10.6 Log CFU/g for the fermentations with the selected starter strains at 48 h. At the same time, counts for the control 1 and 2 fermentations left uninoculated were both at a level of 8.9 Log CFU/g. Thus there were noticeable differences between the LAB concentration in the control fermentation and the cassava fermentation inoculated with the selected starter strains. A difference could also be observed with regard to the pH, titratable acidity and cyanide reduction. Furthermore, the results showed clearly that most of the selected strains reached their peak cell concentration after 24 hours, and stabilised at this level until 48 hours of fermentation time. This was different from the situation in the uninoculated controls, in which the total count continued to grow up to 48 hours fermentation time.

Table 4.6 Summary of cassava mash fermentations (values are a mean of two independent trials) using various LAB strains

Starter strain	*Counts (Log CFU/g)*			*Titratable acidity (%)*		*pH*			*Cyanide content (ppm/kg)*	
	0h	24h	48h	24h	48h	0h	24h	48h	0h	48h
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
G 2/10	7.9	9.4	9.2	1.2	1.4	6.4	4.4	4.0	100	10
G 3/5	8.6	10.2	10.9	1.1	1.4	5.6	4.6	4.3	100	20
Lb 61	8.0	9.4	9.3	1.1	1.5	5.9	4.4	4.0	100	30
Lb 68	8.1	9.1	9.2	1.1	1.5	6.0	4.4	4.0	100	30
G 5/28	8.5	9.2	9.4	1.1	1.4	5.7	4.4	4.1	100	20
Lb 50	8.6	10.2	10.5	1.1	1.4	6.1	4.3	3.9	100	10
G 2/25	8.4	9.3	9.1	1.1	1.5	5.6	4.4	4.1	100	20
G 3/29	8.5	9.4	9.1	1.2	1.5	5.6	4.4	4.2	100	30
G 4/18	9.1	9.7	10.3	1.3	1.6	6.4	4.5	3.9	100	10
VE 20	8.3	10.2	9.9	1.2	1.4	5.4	4.4	4.1	100	10
VE 36	8.2	10.3	10.1	1.2	1.5	5.8	4.3	4.0	100	10
VE 65b	8.4	10.0	10.6	1.1	1.4	5.9	4.4	4.0	100	10
VE 77	7.9	9.6	9.8	1.2	1.5	6.0	4.5	4.0	100	50
VE 82	7.8	9.6	9.4	1.1	1.4	5.9	4.4	4.0	100	10
Lc 11	8.3	10.2	10.1	1.1	1.4	6.4	4.7	4.4	100	20
Lc 18	8.1	10.0	9.8	1.1	1.3	6.2	4.8	4.5	100	20
Kenya M Rog 2	8.3	9.7	9.9	1.1	1.3	6.0	4.8	4.6	100	40

4.4 Discussion

LAB were selected on the basis of their biochemical and technological properties as described in chapter 3, as well as by Kostinek *et al*. (2005, 2007) in Germany also on the basis of technological properties. The studies were done by different partners within the framework of the same EU studies on isolates from different African *Gari* producing countries. Further technological properties related to their growth in a fermenter, resistance to freeze drying in the presence of cryoprotectants, as well as the ability to recover their fermentation activity in fortified cassava, were investigated in this work.

It is well documented that the use of starter cultures 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 *et al*., 1998; Carvalho *et al*., 2004; Morgan *et al*., 2006). Generally, all strains performed well in the medium that was selected for the individual biomass production on the basis of a pilot experiment with different growth media. The survival of strains after freeze drying, however, appeared to be strain dependant. 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 *et al*., 2003; Schoug *et al*., 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 *et al*., 2005; Schoug *et al*., 2006). The strains maintained good cell counts after freeze drying (Table 4.4). *W. paramesenteroides* strain Lc 18 showed very good survival among the *Leuconostoc/Weissella* strains at 12.2 Log CFU/g. *L. fermentum* strain G3/5

showed the highest survival amongst the *L. fermentum* strains. For the *L. pentosus* strains, strain G5/28 and strain Lb61 showed the best survival, whereas *L. plantarum* strains VE 36, VE 82 and G2/25 yielded the best counts among the *L. plantarum* strains after freeze-drying. However, it must be noted that *L. plantarum* strain VE 82 took twice as long to complete the biomass production stage, which may be a disadvantage in terms of the overall production costs as a consequence of higher energy requirements for the freeze-drying process. From an economical point of view clearly the *L. plantarum* group strains (i.e., *L. pentosus* and *L. plantarum*) were the ideal candidates for utilisation as starter cultures as they could generally be produced at the lowest cost (below 1 ZAR/g biomass).

The major test for all the selected strains was to test them in cassava mash to evaluate their ability to ferment the cassava, reduce pH and remove cyanide. This was the first time, as far as we were aware, that starter cultures were used for the fermentation of fortified, bitter cassava after being freeze dried. The *Gari* processing was replicated as far as possible to match the processes documented in Benin (see chapter 2).

The strains performed well in the small scale bucket fermentations. There was a rapid acidification evidenced by the increase in titratable acidity, ranging from 1.1 to 1.3% at 24 hours, and 1.3 to 1.6% at 48 hours. This correlated well with a rapid decease in pH that was observed to range from 4.3 to 4.8 at 24 hours, and 3.9 to 4.6 at 48 hours. Quick acidification is important to prevent undesirable bacteria from growing during fermentation especially if the pH of the fermentation is reduced to approximately 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 and Ejiofor, 1985).

Starter culture addition provides important advantages when compared to spontaneous fermentation 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 products 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 microbes compete with the desirable microorganisms (Holzapfel, 2002). Adding a starter culture reduces fermentation times notably, as could be clearly seen in this work, by a fast decrease in pH, and noticeable increase in acidity when compared to the controls. This helps reduce the risk of contaminating microbes and also contributes to more control over aroma, texture and flavour of the final product (Holzapfel, 2002; Leroy and De Vuyst, 2004).

The kit for determining residual cyanide proved quite useful as it provided an indication of the strains ability to assist with the degradation of linamarin. It was evident that there was more cyanide reduction when the selected strains were used in the fermentations when compared to the two controls which did not have starter cultures added. Some argue that the role of starter cultures in the reduction of cyanogenic glucosides is negligible, and processing of the cassava actually plays a more important role (Agbor-Egbe and Lape Mbome, 2006). For bitter cassava, a 100 ppm/kg cyanide concentration is fairly low. It is possible that the initial reduction by the plants own linamarase indeed was due to processing, i.e. when this enzyme was released from the plants cells upon grating. Nevertheless, as seen from the data of this study, it appeared that both the processing and starter culture addition played a role in the removal of cyanide during processing of the cassava into *Gari*. This is evident from the lower cyanide values obtained for fermentations that included starter cultures (Table 4.6). A reason for this is the possibility that the starter cultures removed linamarin by utilising the sugar moiety by means of their enzyme *β*-glucosidase, which removes the glucose from the aglycone. This glucose is then available for bacterial metabolism, especially when other sugars are limited (Brimer, 1994). Indeed, 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 in the investigations of the strains technological properties (Table 4.1).

Although this work was based on pilot scale, laboratory fermentations, it provided great insight into the possibility 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 had the potential to lower the pH of the fermentation quickly and reliably, while at the same time they showed a positive technological effect in removal of cyanide from the raw materials during fermentation. Furthermore, the study determined quite well that the survival of strains upon freeze-drying was strain-dependent and that therefore, a strain's survival during freeze drying and storage must be studied before further development of a particular strain. 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. As this project formed part of a larger EU project, further work on large scale development was undertaken by the partners responsible for that deliverable, mainly the Belgian partners.

Overall the results of this study were crucial for the project in showing that a starter culture which is easy and economical to produce and which has the desired attributes is a feasible possibility for application in the field.

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Chapter 5
General discussion and conclusions

Commercial starter cultures generally originate from food substrates or from the processes in which they are applied (Holzapfel, 2002). There has also been a trend recently to isolate wild-type strains from traditional products for use as starter cultures in food fermentation (Beukes *et al*., 2001; De Vuyst *et al*., 2002; Leroy and De Vuyst, 2004). Currently researchers always search for multifunctional starters with a combination of functional as well as probiotic properties (Holzapfel, 2002; Leroy and De Vuyst, 2004). A similar approach, i.e. to isolate wild type strains from successful fermentations for development as starter cultures was used in this study. Thus it was aimed to achieve control over the fermentation using starter culture technology, in order to standardise the fermentation process and to obtain a fermented product with consistent quality. In our study, the use of probiotic cultures was not considered, as the fermented product is heated and viable probiotic bacteria, which could theoretically exert health beneficial activity, would not survive the heating process.

A total of 74 dominant lactic acid bacterial strains and 21 dominant yeast strains were isolated during a research visit to Benin. Twenty-six dominant LAB strains were also isolated at the CSIR, from cassava samples sent from Benin. These strains were all tentatively characterised phenotypically, and some technological properties were assessed. A selection of 24 strains was made on the basis of good acid production, as well as their ability to produce α-amylase or *β*-glucosidase. These traits were considered to be important as fast acid production to low levels serves to inhibit competitive and spoilage strains. Furthermore, the bacterial *β*-glucosidase activity was considered important for aiding in the breakdown of linamarin during the cassava processing. These tentatively identified strains were taken to the Federal Research Centre for Nutrition in Karlsruhe, Germany (BFE), where further taxonomic work was conducted to accurately identify the organisms to species level and to further characterise their phenotypic properties.

Usually strains isolated from products which predominated in the fermentation are the best adapted for fermentation of that particular product. Although

certain LAB species such as *L. plantarum* can be found in a variety of niches, such as e.g., the gastrointestinal tract, fermenting plant materials and meat (Kleerebezem *et al*., 2003; Siezen *et al*., 2009), this does not mean that individual strains are equally adapted to grow in all these different environments. Genetic studies on LAB have shown that the genomes of the different species have reduced, concomitant with adaptation to life in specific nutritionally rich environmental niches (Pfeiler and Klaenhammer, 2007). Thus, these bacteria have successfully adapted themselves to specific environments and as a result of this specialisation lost the genes required for certain nutrients (like amino acids) or certain transporters for sugars which do not occur in the environment. On the other hand, they gained certain genes for e.g. specific sugar transporters or proteinases, which are important for life in the specific niche as a result of horizontal gene transfer or gene duplication (Claesson *et al*., 2007). Different strains belonging to a single species thus may be very different. In a study on 185 different *Lactobacillus plantarum* strains from different sources, Siezen *et al*. (2009) showed that strains varied in gene content between 9 to 20% of that of the chosen reference strain in that study, i.e. the probiotic strain *L. plantarum* WCFS1. Despite the presence of a 'core' genome, the various strains appear to differ especially in 'genomic islands' that include genes for sugar metabolism, plantaricin production and exopolysaccharides biosynthesis, suggesting the presence of 'lifestyle' adaptation regions (Molenaar *et al*., 2005; Siezen *et al*., 2009). Therefore it is vital that strains well adapted to the substrate and stemming from successful fermentations are selected as potential starter cultures. Furthermore, there is a need to screen all strains isolated for their technological properties and select those with the most suitable technological and functional properties.

L. plantarum was the most dominant species isolated from fermenting cassava in this study. *L. fermentum*, *L. acidophilus*, *L. buchneri*, *L. pentosus*, *Lactococcus lactis* and *Leuconostoc* were also identified but to a lesser extent. *L. plantarum* has been shown previously to be the predominant LAB species in sour cassava starch (Lacerda *et al*., 2005). *L plantarum*, together with *L. paraplantarum* and *L. pentosus* make up the so-called *'L- plantarum group'* of highly related lactobacillus species. These are very difficult to

differentiate on the basis of phenotypic properties alone, or even by single genotyping techniques (Kostinek *et al*., 2005). All these species are Gram positive, catalase-negative, rod shaped, facultatively heterofermentative, produce DL-lactate and contain *meso*-diaminopimelic acid in their cell wall. Nevertheless, there are some differences in sugar fermentation patterns which together with genotyping techniques in a polyphasic taxonomical approach do allow an accurate identification of the different species. Indeed, this strategy was also adopted in this study and phenotypic identification tests in combination with RAPD-PCR and rep-PCR fingerprinting was successful in identifying strains belonging to the *L. plantarum*-group to the species level.

In this study, the production of α-amylase was a very rare trait (<5%), whereas more than 50% of strains were positive for production of *β*-glucosidase. In the case of the α-amylase activity this was quite surprising as cassava contains about 84% of the carbohydrates as starch (Ketiku and Oyenuga, 1972), representing an important energy source for the LAB. Nevertheless, only a few amylolytic LAB have ever been isolated from starchy fermented foods in Africa (Sanni *et al*., 2002). The relatively higher incidence of *β*-glucosidase production may be a reflection of the relative abundance of linamarin and other *β*-glucoside sugars in cassava. This may indicate that the strains utilise simple sugars (e.g. monosaccharides) in the fermenting cassava and when these get limited that they may liberate the glucose moiety from linamarin by virtue of their *β*-glucosidase activity rather than being able to break down starch in order to obtain more simple sugars for growth. *L. plantarum* strains are well-known to hydrolyse various *β*-glucosides of vegetable origin (Ciarfardini *et al*., 1994) and the incidence of *β*-glucosidase activity in *L. plantarum* strains was also found to be very high in another investigation (Kostinek *et al*., 2007). This may be one of the factors that may contribute to the predominance of *L. plantarum* strains in the fermentation of *Gari*. Another factor may be that these bacteria were determined to be fast acid producers which lower the pH to lower levels when compared with other LAB (Kostinek *et al*., 2007). *L. plantarum* strains are more acid resistant than *Leuconostoc* spp. or many other *Lactobacillus* spp., and thus the acid

production and corresponding resistance to low acid levels also explains why strains of *L. plantarum* often predominate in the late stages of vegetable fermentations (Stamer, 1975; Figueroa *et al*., 1995). Last but not least, the sequencing of a *L. plantarum* genome has shown that this species has the biggest genome of all the LAB species sequenced of about 3.3 Mbp. A large proportion of the genes encode sugar transport and utilisation and thus allows *L. plantarum* to exploit a wide diversity of sugars (Kleerebezem *et al*., 2003) which may be important when simple hexoses such as glucose get limited in the cassava fermentation and other, less common sugars are the only source of energy left for utilisation. All taken together, these factors can thus explain why *L. plantarum* was the most dominant isolated organism from the cassava fermentations.

Strains selected as potential starter cultures for the fermentation of cassava in the production of *Gari* were selected on the basis of their technological properties but before large scale production they were also used for testing their suitability as starters in lab fermentations. A total of 17 strains were selected from this study. These comprised five *L. plantarum* strains (VE 20, VE 36, VE 65b, VE 77 and VE 82), as well as 12 strains selected by the BFE who conducted a similar independent study. The 17 selected strains in this study were all grown in optimal media in 2 L fermenters, freeze-dried, and then tested as freeze-dried cultures in pilot scale, laboratory fermentations of cassava mash to select cultures, which showed a good capacity for fermentation as well as for economical starter production. The results indeed showed that the starter strains grew well in the laboratory fermentations, increasing titratable acidity and decreasing pH in a shorter time than when compared to the control. Furthermore, the starters also contributed towards the degradation of linamarin in the fermentation, confirming their role as processing aids for linamarin detoxification. An economic cost analysis showed that strains, especially *L. plantarum* and *L. pentosus*, could be produced at low costs, using cheap raw materials like CSL, yeast extract and glucose, and thus should be further developed as starter cultures for real fermentations in Africa in the field.

This starter culture technology is currently missing for many African food fermentations, as starters are seldom used. Knowledge in this field is extremely limited. Relatively few LAB have been isolated from starchy fermented foods in Africa and used as starter cultures. Giraud *et al*. (1993; 1998) and Kimaryo *et al*. (2000) mentioned the use of the amylolytic *L*. *plantarum* as a starter culture during cassava fermentation for *Gari* and *kivunde*, respectively. However, there are no reports that these were actually successfully developed and applied in the field. Okafor *et al*. (1998) inoculated *L*. *coryneformis* and a *Saccharomyces* sp. as starter cultures in cassava mash for *Gari* production. The survival of these microorganisms was studied when placed alone or mixed in different carriers (Okafor *et al*., 1999). In the EU study, to which this work contributed, we could thus select suitable starter cultures, based on predominance and technological properties, and showed that their production as starters were associated with relatively low costs.

The aim of the study was to contribute to improving the quality and nutritional status of *Gari*, by using a starter culture and fortification with soybean and palm oil. The objectives were to isolate lactic acid bacteria (LAB) (mainly *Lactobacillus* spp.) and yeasts from fermenting cassava; select LAB on the basis of technological traits such as lactic acid production, detoxification of cyanogenic glucosides and the production of α-amylase; identify LAB by the application of classical biochemical and molecular biological methods; grow the microorganisms in small scale fermenters for use as inoculum and test the selected starter cultures in small scale, fortified *Gari* fermentations. Thus, all the objectives of the study were met.

The *L. plantarum* VE 36 and G2/25, *L. fermentum* G2/10, and *W. paramesenteroides* Lc 11 strains were finally selected on the basis of this work and work done by other partners. These strains have been put into large scale production and were evaluated either alone or in combination, in soy-palm oil cassava mash fermentation for *Gari* production in Benin (Yao *et al*., 2009). Further work is also being conducted in Benin where small scale pilot plants are being built to assist women in local villages with *Gari* production.

Thus, a HACCP rural pilot plant was constructed in Ouèdo village in Benin to produce *Gari* using the selected freeze dried starter cultures. The plant is equipped with high standard hygienic locally-fabricated equipment and is run by a women's group of thirteen members. They were trained, also within the framework of the EU study, in *Gari* production, agricultural entrepreneurship and equipment management. The use of the selected LAB starter cultures mentioned above singly or in combination resulted in a rapid acidification of the mash ensuring a reduction of the fermentation period from 24 h to even 12 h with the *Gari* even having improved sensory properties. The commercial yield was also improved upon the application of the starter cultures (Egounlety *et al*., 2007). Thus this study laid the scientific basis for the successful implementation of starter cultures in real *Gari* fermentations in West Africa.

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