London Metropolitan University

Developing starter cultures for the optimisation of cassava (*Manihot esculenta* Crantz) fermentation

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

School of Human Sciences

Ms Brigitte Awamaria

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Declaration

I confirm that this is my own work and the use of all material from other sources has been clearly acknowledged.

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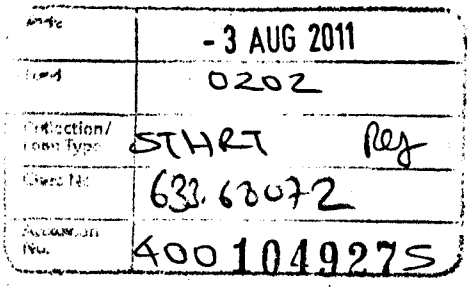

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Contents

Abstract

The overall objective of the study was to optimise natural fermentation of cassava through development of starter cultures of lactic acid cassava bacteria (LAB) and softening bacteria to address quality variations of the end product, in terms of acidification and softening inconsistencies. Several natural fermentations of cassava under various conditions were carried out to study the effects of time, temperature, substrate composition (cassava skin and leaves) on pH, cassava softening and microbial growth. Fermented cassava pieces were examined to detect reported bacteria (*Bacillus*, *Clostridium* spp.) associated with softening by conventional cultural and non-cultural methodology; to detect pectinolytic activity by viscometry and dinitrosalicylic acid reagent colorimetric methods; to evaluate α-glucuronase and β-glucosidase activities by API Zym tests. For selection of starter cultures study: LAB (33), non- LAB (55), and odour producers (three) were isolated at different stages of natural fermentations. The isolates were identified by phenotyping and genotyping using PCR amplification of the 16S-23S rDNA intergenic transcribed spacer (ITS-PCR), repetitive sequence-based PCR (rep-PCR) and DNA sequencing. Out of 33 LAB isolates, 13 representative strains were selected and further characterised for the desirable properties of starch fermentation, initiation of growth at different pH values, rapid acidification, antimicrobial & linamarase activities, degradation of oligosaccharides and probiotic activities. Non lactic acid isolates (55) were individually tested for the desirable property of causing reproducible softening by discriminatory tests. Representative softening isolates (13) and odour producer (1) were selected and further characterised for sugar fermentation by API CHB tests, pectinolytic activity by viscometry and colorimetric methods & detection of metabolitic activities by High Performance Liquid Chromatography & Gas Chromatography. Fermentations using starter cultures of various combinations using 13 LAB, 12 softening and one odour producer bacteria were developed and assessed by studying their interactive effects on pH, softening, colour and odour of cassava. Thirteen LAB of nine species, 12 softening bacteria of three species and one odour producer were selected for starter culture development. The study highlighted technological issues and the significance of culture inoculation concentration when using mixed cultures. The study demonstrated possibilities to develop potential starter cultures of LAB, softening bacteria and odour producer strains, using various combinations to produce cassava products of variable desirable attributes.

Introduction

1.1 Fermentation of foods

In general terms, fermentation is defined as the degradation of organic compounds in the absence of oxygen for the purpose of energy production by certain organisms such as lactic acid bacteria and yeasts (McCahill, 1991). Fermentation is called "natural" or "spontaneous" when it is initiated by endogenous microorganisms present in raw materials, while a "controlled" fermentation is manipulated with use of selected starter cultures (Holzapfel, 2002; Kostinek *et al.*, 2008). Fermentation is a convenient multifunctional process used for food preservation to prolong the shelf life through for example: production of compounds such as organic acids and alcohols: this particular important feature can significantly contribute to food safety. The low pH provides protection against enteropathogens through growth control and inhibition. However, some food pathogens implicated in human gastroenteritis, such as enterohaemorrhagic *Escherichia coli* and foodborne viruses such as rotavirus to acid have been demonstrated to be acid resistant, and may survive the fermentation process (Timmerman *et al.*, 2004), therefore, fermentation does not necessarily ensure complete elimination of microbial hazards, including mycotoxin contamination. Another major safety benefit is that it can be a potential tool to remove chemical hazards, such as cyanide naturally present in cassava and anti-nutritive factors such as enzyme inhibitors, phytates, lectins and tannins, which are known to interfere with digestion and absorption of nutrients (Adams and Nikolaides, 1997; Motarjemi, 2002; Holzapfel, 2002). At the same time, fermentation plays a significant part in human nutrition and health, as it improves nutrient availability of the product, digestibility through removal of non-digestible oligosaccharides, such as raffinose and melibiose, responsible for gastrointestinal disturbances. Moreover, fermented products can exert several health benefits through acting as carriers for probiotic microorganisms as in the example of many current commercial yoghurts (Fuller, 1994; Huis in't Veld and Havenaar and Havenaar, 1997). On the culinary side, fermentation enhances taste and flavour, which improves the palatability of foods (Oyewole, 1997).

1.2 African traditional fermentation

Fermented foods have been a long history in Africa, dating back to prehistoric times and constitute a significant component of African diets (Odunfa and Oyewole, 1998). Fermentation of food is one of the oldest known and most economical method of preserving food (Reddy *et al.*, 1986) and from time immemorial in many African countries, fermentation was a method to make the food storable and in this way attempt to have sufficient food in the lean seasons (Dirar, 1993).

Many fermented foods are known, some serve as main course meals, others as beverages, while others are highly-prized food condiments. Those which serve as main meals and beverages are usually products of carbohydrate-rich raw materials. Some of the most important in this group include *gari* from cassava, *ogi* and *mahewu* from maize and *kaffir* from sorghum. Those which serve as food condiments are usually made from fermentation of protein rich seeds. These include *iru* (*soumbala*) from African locust bean, *ugba* from African oil bean and *ogiri* from melon seeds, among others. All are known to be good sources of proteins and vitamins (Odunfa and Oyewole, 1998).

Today, foods are fermented for many other reasons also (Reddy *et al.*, 1986; Steinkraus, 1986; Odunfa and Oyewole, 1998):

- Improvement of sensory characteristics by development of diverse flavour and aroma compounds and texture alteration;
- Enhancement of nutritive value through breakdown of more complex substances such as proteins, carbohydrates and lipids to essential amino acids, easily digested sugars and essential fatty acids. Furthermore fermentation brings about synthesis of some vitamins;
- Improvement of safety (absence of toxins and partial and/ or complete elimination of antinutritional factors);
- Decrease in cooking time and thereby less fuel requirements;

Within the past few years, there has been increased scientific interest in traditional fermented foods, but the age-old techniques used for their production have not improved as a result. The importance of fermented foods in the nutrition of Africans is now better appreciated; as a result of which efforts are now being made to industrialize some of the processes in some cases (Steinkraus, 1986).

The term fermentation as it is commonly used can be defined as "a process in which chemical changes are brought about in an organic substrate through the action of enzymes

produced by microorganisms" (Jay, 1996). The word "ferment" comes from Latin, where it can be translated into "yeast" or "to seethe" (Andersen and Risum, 1994). The general principle behind fermentation is to create an environment that favours growth of one or more specific desirable microorganisms that have a beneficial effect on the food, rather than spoiling it (Andersen and Risum, 1994).

1.3 Cassava tuber

Cassava (*Manihot esculenta*, Crantz) is a tropical starchy crop cultivated in Sub-Saharan Africa, South East Asia and South America. It is a member of *Euphorbiaceae* family that originated from Latin America and was introduced during the 16th century into Africa, where it became the most popular root crop due to its culinary, commercial and industrial features (Asiedu, 1992). It possesses convenient agronomic advantages as it is able to grow on poor soils with very limited water requirements. Furthermore, cassava is known to be a perennial crop and is not subject to seasonal conditions, therefore adapts to a wide range of ecological conditions and requires very basic land preparation (Osunbitan *et al.*, 2000). Another characteristic of cassava is that upon maturity, it does not require immediate harvesting, instead the tuber can be left in the soil for a long time until needed. Cassava is highly productive, it has abilities to yield more carbohydrates per unit of land than other tropical crops, and it is, therefore, a convenient major source of dietary energy consumed by many people the in tropics (Oyewole and Sobowale, 2002).
For the general measurements, the cassava plant could be about 30-120 cm long and the tuber is 1-15 cm in diameter and weighs 1-8 kg or more, depending on cultivar. The peel is mainly composed of a dark coloured outer layer of cork cells and phellogen, and an inner part. The body of the tuber, which is the central portion, is composed mainly of starchy carbohydrates. In many tropical countries, particularly in Sub Saharan Africa, cassava is still used as subsistence food and is consumed in various forms of fermented products (Nout, 1999) depending on traditions and preference (Table 2, appendix 1). Although it plays a major part in the human diet, it has a nutritional constraint of protein and micronutrients limitations, and contains cyanogenic glucosides as well as high perishability after removal from the ground (Rosling *et al.*, 1993; de Bruijin and Fresco, 1989; Oyewole, 2002; Ogbo, 2003). These nutritional limitations can be overcome and the product improved by processing steps, which increase significantly the shelf life and sensory characteristics (Ogbo, 2003). The steps involved in the processing of cassava include peeling, grating, grinding, soaking, fermentation, cooking, steaming, pounding, roasting and drying. At present, there are numerous preparations of cassava products

(Appendix 1) characterised by cultural and geographical variations at household and industrial level.

1.3.1 Intrinsic limitations of cassava

1.3.1.1 Postharvest deterioration

The major concerns associated with cassava utilization and consumption are summarized as perishability, nutrition and toxicity limitations. The post-harvest deterioration of cassava is characterised by soft rot and by physiological spoilage (Agabor-Egber *et al.*, 1995). It is important to rapidly process cassava to prevent deterioration and minimise post harvesting losses.

Soft rot spoilage is characterised by a fungal or bacterial attack, such as *Erwinia carotovora,* following an external cut or bruise on cassava skin, and the high water content of cassava (60-70%) facilitates the microbial attack. The initial rot is seen as a localised softness of the skin and some parts of cassava tissue. As time advances, the rot invades the whole tuber and transforms it into unappealing wasted mash; for this reason farmers wax the skin of cassava to delay the onset of this type of spoilage.

In contrast, the physiological deterioration of cassava tissue happens soon after harvest and is triggered by stress-response reactions following wounding of a plant. Therefore, it is not caused by a microbial invasion but appears to result from a cascade of complex biochemical changes in phenolic compounds of the tuber, which are externally seen as blue-black, greenish vascular tissue streakings (Beeching *et al.*, 1998). The changes observed in cassava roots during postharvest deterioration, are similar to the natural wound responses in plant defensive mechanisms, which involves the phytohormone ethylene (Bennett and Wallsgrove, 1994). According to Beeching *et al.* (1998), once a plant is wounded, the wounding repair response mechanism is induced by combined actions of glucanases, chitinases and pytoalexins, together with secondary metabolites associated with anti-microbial defensive role; the synthesis of callose, lignin, suberin; and the insolubilization of hydroxyproline by hydrogen peroxide. The physiological deterioration is a result of lack or inadequacy of wounding repair response mechanisms in cassava plant (Beeching *et al.*, 1998).

1.3.1.2 Nutritional constraints of cassava: low protein and micronutrient content

Cassava root is known to be mainly starchy, and has the nutritional drawback of low protein and micronutrient content (Appendix 5). The contents of protein and micronutrients can be aggravated by processing impact; for example it has been reported that the processes involved in the removal of cyanogenic glucosides may further lower the protein and vitamin content, and the loss could be estimated to be 50-87% (Teles *et al.*, 2002). Raw and unprocessed cassava is reported to contain 1-5% of crude protein in dry matter; however, this can vary depending on cultivar (Tales *et al.*, 2002, 1993). The mono-diet is well established in many parts of Africa, and the diet consists of cereals, roots and tubers, particularly cassava, which is regarded a staple food and also as the base for the preparation of fermented complementary foods. As result, the nutritional limitation is one of the causes of infantile malnutrition, characterised by protein /energy deficiency, and micronutrient deficiency associated with vitamin A, iron, zinc and iodine (Beard, 2001; WHO, 2002).

1.3.1.3 Cyanogenic glucosides limitation

Linamarin (96%) and lotaustralin (4%) are present in cassava, and both compounds are initially synthesized from the amino acids valine and isoleucine in cassava leaves and are initially accumulated throughout the plant tissues and eventually stored in vacuoles (Mkpong *et al.*, 1990). The distribution of cyanogenic glucosides in cassava plant tissue varies, as the leaves including the petioles contain the highest level of cyanogenic potential, while the root parenchyma has a medium content, and the root cortex, the lowest. Some environmental factors such as water stress have adverse effects on cyanide concentration in cassava, for examples when cassava plant is exposed to long periods of draught, its cyanide concentration increases both in the glucosidic and in the free form, therefore, a higher level of toxicity is expected when cassava is grown in such ecological conditions (Lancaster *et al.*, 1982; Nwosu and Onofeghara, 1991).

1.3.1.4 Cyanogens hydrolysis

The hydrolysis of cyanogenic glucosides and the release of hydrogen cyanide are mediated by linamarase when the cells of cassava tuber are ruptured. There are four enzymes involved in the biosynthesis and the degradation pathways of cyanogenic glucosides. The *cytochrome P450* regulates the rate of conversion of the amino acids valine and isoleucine to the corresponding oximes, and *linamarase β-glucosidase* hydrolyses linamarin to

acetone cyanohydrins. The hydrolysis occurs at pH 4.0 and above, while below this pH range, the acidic environment prevents cassava detoxification from taking place. The *hydroxynitrile lyase* acts on the breakdown of acetone cyanohydrin to hydrogen cyanide (HCN), as consequence, exposing consumers in the long term to the free cyanide. Cyanide is toxic to consumers even though the human body possesses detoxifying mechanisms that can remove substantial amounts of cyanide by converting it into much less toxic thiocynate (Mkpong *et al.*, 1990; Vasconcelos *et al.*, 1990; Oyewole and Odunfa, 1992; Rosling *et al.*, 1993). *Glucosyltransferase* converts linamarin to its transport form linustatin.

1.3.1.5 Removal of cyanogens from cassava

After the cassava harvest, it is imperative that it is processed to make it safe and palatable for human consumption. Current methods for cassava post-harvest processing are known to reduce the cyanide level, but are not equal for effective removal. The best reduction of cyanide may involve many combinations of techniques such as slicing, grating, pounding, chipping, milling, soaking, boiling, drying. However, before the detoxification takes place, the cassava tissue must be ruptured to cause contact between the substrate cyanogenic glucosides and endogenous linamarase enzymes for the hydrolysis to happen. The disintegration of cassava cells can be achieved singly or in combinations of slicing, grating, crushing and microbial pectinolytic action during fermentation. Other combined processing methods can be applied to bring down the cyanogenic toxins to a very safe low level, for example: hydrogen cyanide produced following the hydrolysis of linamarin, is very volatile (boiling point: 25.7°C) and is easily removed during processing by volatilization into the air or by solubilization.

Drying cassava after soaking, pressing and heating are mechanical steps that remove water and lead to the reduction of the degraded soluble products from linamarin. Incomplete tissue disintegration will result in residual cyanogens, particularly linamarin, and incomplete drying or heating may result in residual cyanohydrins. Higher retention of glucosides tends to result from low impact slicing of roots that causes minimal tissue damage, followed by a rapid drying. It had been reported that the way the drying process is applied affects the cyanogenic loss, for example, high temperature could adversely contribute to a high retention of residual cyanogens in cassava due to rapid evaporation of water, while the effect of slow drying beneficially extends the linamarase activity, but at the same time, encourages mould growth that results in mycotoxin contamination (Muzanila *et al.*, 2000). Direct sun-drying of whole fresh roots leads to incomplete

removal of glucosides, and among all drying processes, drying at 45°C achieves the best results for elimination of cyanogens.

Although every step undertaken for the processing of cassava into products reduces or nearly eliminates the cyanogenic potential, substantial nutrients losses occur during grating and fermentation (Lancaster *et al.*, 1982; Mahungu *et al.*, 1987). The mechanism behind the removal of cyanide from cassava during submerged fermentation is linked first, to the leaching out of water soluble cyanogenic glucosides and to their breakdown compounds such as free cyanide; second, to increased activities of endogenous and microbial β-glucosidase enzymes (Bokanga, 1989); and third, to cyanide degradation properties exhibited by fermenting microorganisms such as *Lactobacillus plantarum* (Obilie and Amoa-Awua, 2004).

1.3.2 Traditional African fermentation of cassava

Fermentation is one of the oldest technologies that evolved through refining and diversification, for example the natural fermentation, was developed through experiences gained by trial and error by food producers, and despite progress made in this field, the technology is still often applied without understanding of the underlying principles of the process and requirements for ensuring quality and safety.

The main characteristics of natural fermentation are that it is spontaneous and follows its natural course without the application of starter cultures. The process is known to be unpredictable and likely to be exposed to quality inconsistencies such as variations observed in acidification rate, in cassava softening and in composition of the microflora. It is a slow process that takes time to bring out the desirable changes of the product, therefore the fermentation process is prone to failure. To alleviate these cassava fermentation faults, traditional processors use back-slopping systems, consisting of inoculating raw materials with a residue or aliquots from previous successful batches in order to accelerate the initial phase of fermentation, and eventually initiate the desired biotransformations.

Natural fermentation of cassava is generally associated with a complex microflora, where microorganisms such as lactic acid bacteria (LAB), *Bacillus, Clostridium, Enterobacteriaceae* and yeasts are commonly found. Dominant microorganisms are able to multiply and survive because they have acid tolerance, strong antimicrobial properties, and synergistic interaction effects between fermenting organisms, particularly yeasts such as *Candida krusei,* and also *Lactobacillus* strains such as *Lactobacillus plantarum,*

Lactobacillus fermentum, Lactobacillus brevis (Gobbetti and Rossi, 1994; Hounhouigan *et al.*, 1994; Holzapfel, 2002). The early stage of natural submerged fermentation of cassava for the production of various food products such as *foo-foo*, was reported to be commonly dominated by LAB and other organisms such as *Enterobacteriaceae* and *Bacillus*. Similar representative bacteria consisting of *Lactobacillus*, *Pediococcus*, *Clostridium*, *Propionibacterium* and *Bacillus* species were isolated from fermented cassava dough and 99% of the surviving total microflora at the end of fermentation were reported to be *Lactobacillus* species and yeasts (Oyewole, 2001; Miambi and Ampe, 2003).

There are two types of traditional African fermentation of cassava: a submerged fermentation also called wet fermentation or retting where the substrates must be soaked or retted in large volumes of water for the fermentation to take place, and a solid state or heap fermentation that uses moistened raw materials which are left to ferment without adding water. Both fermentations are prone to quality variations mentioned above, and differ in some fermentation characteristics. For example, higher numbers of LAB are routinely isolated at the start and end of submerged fermentation compared to solid state fermentation, where *Bacillus* spp., *Enterobacteriaceae* and yeast survive in higher numbers, due to the less acidic conditions occurring in submerged fermentation. With reference to cyanogenic detoxification level, submerged fermentation is reported to produce cassava with low residual cyanide levels and lower reducing sugars contents than solid state fermentation (Birk and Shoseyov, 1996). For this project, preliminary tests showed that submerged fermentation was most suitable for the type of experiments to be undertaken.

1.3.2.1 Cassava submerged fermentation

Submerged fermentation is an important process that improves cassava safety through antimicrobial activities of fermenting microflora and through substantial removal of cyanogenic toxins (Mensah, 1997). It is characterised by two major factors: a complex microflora and lack of reproducibility. The microflora complexity has been reported to be a succession of microorganisms present at the beginning but as the fermentation progresses, some species gradually become outnumbered and diminished by the surviving organisms such as LAB and yeasts. According to Amoa-Awua (1995) and Oyewole (2001), high numbers of *Bacillus*, *Klebsiella*, *Leuconostoc*, *Corynebacterium*, *Lactobacillus*, yeasts and moulds are found at the beginning of fermentation. *Lactobacillus*, *Corynebacterium*, *Leuconostoc* and yeasts appear in high numbers on the

second day and persist until the end of fermentation, while *Bacillus* and *Klebsiella* species tend to disappear on the third day. *Candida* species develop on the third day and survive in high numbers. Brauman *et al.* (1996) stated that spontaneous fermentation of cassava was a complex microbial succession in which a dynamic small number of homofermenting *Lactococcus lactis* and heterofermenting *Leuconostoc mesenteroides, Lactobacillus brevis* and *Lactobacillus plantarum* replace and outnumber the epiphytic microflora and lead to the fermentation of cassava. The end of fermentation is marked mostly by *Lactobacillus plantarum* dominance. Similar patterns of population succession and dominance of heterofermenting over homofermenting lactobacilli, observed during cassava natural fermentation was reported to occur in other vegetable fermentations (Daeschel *et al.*, 1987). The dominance of these wild LAB is attributed to several advantages: first, they are facultative anaerobes able to develop, grow rapidly and overcome other organisms in the presence of dissolved oxygen and available fermentable sugars such as sucrose, glucose, maltose, and fructose, produced by amylolytic organisms (*Bacillus*, *Clostridium* and yeast species) during starch degradation; second, they possess high cyanide resistance and strong linamarase activities; third, they produce high amounts of organic acids and volatile compounds responsible for acidity and inhibition activities. Lactic and other organic acids lower the pH to around 3.5 - 4.5, and cause the environment to become acidic, toxic and selective against less acid-tolerant microorganisms, but LAB seemed to adapt this environment because of their acid resistance property. This is reinforced by antimicrobial compounds produced by LAB especially *Lactobacillus plantarum* and *Lactococcus lactis* that produce bacteriocins such as lactocin 27, lactocin B, helveticin J and plantacin B, which are microbial inhibitors that limit the development of other fermenting organisms (Fricourt and Testin, 1994; Amoa-Awua and Jakobsen, 1995; Brauman *et al.*, 1996; Kimaryo *et al.*, 2000). The rapid drop in oxygen concentration is another feature of cassava submerged fermentation which causes the disappearance of the aerobic epiphytic microflora and enables a strict anaerobic microflora to develop, therefore *Clostridium* species has been isolated from fermented cassava and these isolates are able to produce typical fermentation products such as butyrate and to a lesser extent propionate, responsible for the production of cassava odour (Keleke *et al.*, 1996). It is important to note that the production of odour during cassava fermentation was reported to be also associated with other organisms such as yeasts and moulds (Amoa-Awua and Jakobsen, 1995; Jakobsen and Olsen, 1995; Brauman *et al.*, 1996; Kimaryo *et al.*, 2000; Oyewole, 2001). During submerged fermentation, cassava is expected to undergo a retting process, which is the softening of plant materials immersed in water, and is caused by the

breakdown of the pectin that give the plant cells their integrity. During fermentation, pectinases such as pectinesterase, lyase and polygalacturonase lyse the cell membranes of cassava tissue and causes the textural modification or softening that results in tissue disintegration (Okafor 1987). The softening reaction causes the soluble nutrients and cyanogens of cassava to leach out. For this reason, submerged fermentation is likely to reduce the nutritional value of cassava, and at the same time, it reduces the cyanogenic glucoside to low levels, which is reported to be directly proportional to the high degree of softening (Mathew and Moorthy, 1991; Giraud and Raimbault, 1994). The textural modification mechanisms of cassava tissue are current major intriguing issues, as a consequence continuous studies are undertaken to find the causative factors. In the current state, these studies show lack of uniformity and contradict each other, for example, some studies implied that endogenous pectinase contained in the cassava tissue and skin were responsible for cassava softening, and other studies implicated pectinolytic enzymes produced by the fermenting organisms such as *Bacillus* species, yeasts, moulds, *Corynebacterium* and some *Clostridium* strains because of their abilities to produce a wide range of enzymes that include cellulase, polygalactouronase, pectinesterase and pectin lyase. Other studies link the softening of cassava particularly with cellulase and not pectinase, while others implicate microbial pectinase. Other research also stated the inability of yeasts to cause softening, and additionally, a small number of studies that currently associate *Clostridium* species with softening are still ambiguous, irreproducible and inconclusive (Jakobsen and Olsen, 1995; Amoa-Awua and Jakobsen, 1995; Brauman *et al.,* 1996; Kimaryo *et al.*, 2000). Quality variation due to lack of reproducibility is another undesirable trait manifested in cassava submerged fermentation. Intrinsic, environmental and postharvest conditions, processing technique factors such as cultivar, age and quality of unprocessed cassava, regional variations and variability of individual processing methods primarily influence the quality of the fermented end products in relation to organoleptic characteristics, physicochemical parameters such as starch swelling capacity, pH, water activity; absence of chemical residues, residual cyanide level, microbiological quality and the presence of mycotoxins from moulds (Brennan and Muzanilla, 2000). Parameters such as the size of cassava pieces, temperature, and duration of fermentation affect the softening of cassava, the acidification rate, aroma, colour and the degree of cyanogenic glucoside detoxification. The smaller the size of cassava pieces, the higher the rate of acidification ~~is~~ achieved during the initial 12 – 48 h period of fermentation. The pH of smaller cassava pieces fermented over 24 h decreases from 6.3- 5.6 after 24 h to 3.8 – 4.2 over 60 h. However, the highly acidic conditions are

reported to affect the detoxification and retard the conversion of cyanohydrin to acetone and hydrogen cyanide during cassava fermentation. The initial rapid acidification rate encourages the dominance of LAB, yeasts, and disadvantages other fermenting organisms. This has an overall implication on growth and survival of fermenting organisms, which determine the biotransformations during fermentation. The rate of softening during 72 h increases with smaller cassava pieces. The temperature and duration of fermentation have an effect on the acidification rate and degree of cassava softening: a slow and low acidification rate occurs in cassava fermented under 30°C and high rate at 37 - 40°C; cassava fermentation at 30 - 40°C produced high degree of softening after 60 -72 h, and complete cassava tissue disintegration and sedimentation happens mainly in fermentation at 40°C. For sensory attributes, the typical odour of fermented cassava is produced with cassava fermented at 30 - 40°C over 48 h and cassava with poor visual appearances is produced in fermentation under 48 h. The best results in relation to quality accepted by consumers are achieved over 3 – 5 days at 30° - 37°C temperature, conditions similar to those in tropical climates where cassava is produced and consumed (Vasconcelos *et al.*, 1990; Holzapfel, 2002).

1.3.2.2 Lactic acid bacteria

Lactic acid bacteria belong to diverse genera and the principal ones are (Appendix 6: table 5): *Lactococcus, Leuconostoc, Lactobacillus, Enterococcus, Pediococcus and Streptococcus* (Okafor *et al.*, 1984; Amoa-Awua and Jakobsen, 1995; Holzapfel *et al.*, 1998; Kostinek *et al.*, 2008). They share a number of common features: they are Gram positive, catalase-negative non sporeforming rods or cocci, most are aerotolerant anaerobes, which lack cytochrome and are therefore oxidase negative. Some do take up oxygen through the mediation of flavoprotein oxidase and this is used to produce hydrogen peroxide. Cellular energy is derived from the fermentation of carbohydrate to produce principally lactic acid (Appendix 7). One important difference between subgroups of the LAB lies in the nature of the products formed from fermentation of sugars. One group is called homofermentative, produces a single glucose fermentation product, lactic acid, whereas the other group, called heterofermentative, produces lactic and acetic acids, ethanol, carbon dioxide from glucose. Currently the lactobacilli are subdivided into three groups (Appendix 5): obligate homofermenters (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii*), facultative heterofermenters (*Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus curvatus*) and obligate heterofermenters (*Lactobacillus brevis, Lactobacillus fermentum*, *Lactobacillus buchneri*) (Adams and Moss, 1995; Holzapfel,

2002). Lactobacilli are usually more resistant to acidic conditions than are other LAB as they are able to grow well at pH values as low as 4.0. The acid resistance allows them to continue growing during natural lactic fermentation when the pH value has dropped too low for other microorganisms to grow. Lactic acid bacteria are often inhibitory to other microorganisms and this is the basis of their ability to improve the keeping quality and safety of many food products. The principal factors which contribute to this inhibition are: low pH and production of organic acids, bacteriocins, hydrogen peroxide, ethanol and nutrient depletion. By far the most important are production of lactic and acetic acids and the consequent decrease in pH (Adams and Moss, 1995). Furthermore, they produce lactic acid and other metabolites compounds such as alcohols, short-chain fatty acids, exopolysaccharides that contribute to the organoleptic and textural characteristics of fermented foods products (Welman and Archer, 2009). Lactic acid bacteria are generally recognised as safe (GRAS) status due to their long use in traditional fermented foods and their association with human gastrointestinal (GIT) as they confer beneficial (probiotic) effects. For these reasons, LAB are intensively used in industries to ferment milk, vegetables, grains and meats to produce yogurts, cheese, sauerkraut, millet porridge, cassava, sourdough breads and in most African fermented products such as *foo-foo, gari, kivunde, atieke* and *ogi* (Appendix 3).

1.3.2.3 *Bacillus* species

The genus includes such diverse species such as *Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus sphaericus, Bacillus cereus, Bacillus thuringiensis* and many others. They are Gram positive or only positive in early stages of growth, catalase positive for most species, oxidase- positive or negative, aerobic or facultatively anaerobic, psychrophilic to thermophilic, acidophilic to alkaliphilic (Claus and Berkeley, 1986). They are able to produce spores under stressful environmental conditions that can be dormant for a long period of time. Some *Bacillus* species are amylolytic, proteolytic, and many produce a wide range of extracellular hydrolytic enzymes such as polygalactouronase that break down complex polymers such as polysaccharides, nucleic acids, and lipids, permitting the organisms to use these products as carbon sources and electron donors. *Bacillus* genus includes both free living and pathogenic species, and a small number of species such as *Bacillus cereus* have been associated with foodborne illness, albeit the incidence is much lower compared to *Salmonella* cases (Adams and Moss, 1995; Madigan *et al.*, 2005). The metabolic diversity

of *Bacillus* spp. has led to representatives of this group being used in a wide range of industrial processes (Berkeley *et al.*, 1984). *Bacillus* spp. mainly *Bacillus subtilis* are commonly associated with alkaline fermentations for production of foods such as the Asian *natto, kinema* and African *ugba, ogiri, kawal, soumbala* (also called *dawadawa* or *iru*) where they play a dominating role (Campbell-Platt, 1980, 1987; Steinkraus, 1986; Ouoba *et al.*, 2004). They have been also involved in lactic fermentation of cassava for production of African foods such *foo-foo, gari, atieke* and *agbelima* (Okafor and Ejiofor, 1990; Oyewole, 1995; Coulin *et al.*, 2006; Amoa-Awua and Jakobsen, 1995).

1.3.2.4 *Clostridium* species

Clostridium species are Gram positive, catalase negative, generally strict anaerobic spore-forming rods mainly found in the soil. They possess pectinolytic enzymes such as pectinesterases, which contribute to the destruction of plant cell walls; and they are also known to be cellulose-decomposing organisms (Madigan *et al.*, 2000). From fermentation of sugars and cellulose, they produce butyric acid, acetone and butanol and alcohols (Appendix 15), and their fermentation metabolites are associated with the production of flavour of many fermented products (Adams and Moss, 1995). They have been reported to be involved in the softening of cassava and the production of the characteristic odour during fermentation, and also in the retting of linen flax and hemp and olives (Oyewole, 1992; Brauman *et al.*, 1996; Keleke *et al.*, 1996; Ogbo, 2003).

1.4 Selection of starter cultures for cassava fermentation

A starter culture may be defined as a preparation or material containing a high concentration of one or more specific microorganisms, which may be added to accelerate a fermentation process and obtain products with desirable characteristics (Holzapfel, 1997). Because of the unpredictability and irreproducibility of cassava fermentation, particularly the softening process, there have been attempts to use biotechnological approaches to ensure that the softening of cassava occurs on constant basis whenever is desirable. Use of an appropriate selected mixed cultures of *Lactobacillus cellobisus, Streptococcus* spp., *Lactococcus lactis, Corynebacterium* spp. and *Pichia membranaefaciens* have been already developed for *in vitro* cassava fermentation, and it was found that not only did the cultures induce softening of cassava, but also accelerated the softening in 48 h of fermentation, and produced a fermented, softened cassava without the usual characteristic odour. The report emphasizes the combined role of both cellulolytic and pectinolytic actions of the mixed cultures in the softening of cassava (Mathew and Moorthy, 1991).

The study of Okafor (1986) demonstrated that inoculating sterile cassava pieces with a culture of *Bacillus* and *Corynebacterium* species produced softening, while a mixture of *Bacillus*, *Leuconsostoc* and *Lactobacillus* isolates produced similar results, as well as the characteristic odour. Among a wide range of *Lactobacillus* species that have been isolated from fermented cassava, *Lactobacillus plantarum* is dominant under most conditions and seems to have superior performance and a functional role in the cassava fermentation because of its ability to lower pH, degrade cyanogenic glucosides, metabolise oligosaccharides such as raffinose, stachyose, have antimicrobial activities, and possess potential probiotic properties (Steinkraus, 1996, 1997; Oyewole, 1997; Holzapfel, 1997, 2000; Kimaryo *et al.*, 2000).

Therefore, the incorporation of these organisms into starter cultures for cassava fermentation optimisation will significantly stabilise the fermentation and improve the quality of the end products. To select suitable starter cultures, the following desirable properties of strains should be initially assessed: interaction capabilities of the bacteria in mixed cultures, their sensitivity behaviours and substrate adaptability under changing fermentation conditions such as temperature and substrate composition. It is also vital to study their viability and competition behaviours, their antagonism against pathogens and spoilage organisms, their ability to degrade cyanogenic glucosides, to induce organoleptic changes such as cassava texture and odour, and to possess probiotic features (Holzapfel, 1997).

1.4.1 Antimicrobial properties

The bacterial antagonism refers as a combination of effect of different biological factors resulting from metabolic activities of microorganisms and their competitive interactions, which contribute to the safety of lactic fermented foods. Antimicrobial compounds produced by LAB are classified into the following three structural groups based on the physicochemical and level of antimicrobial properties: organic acid, bacteriocins, hydrogen peroxide, low molecular metabolites (Adams and Moss, 1995; Holzapfel, 2002). An effective microbial inhibition can be caused by individual inhibitory compounds or by a combination of all antagonist compounds (Holzapfel, 2002).

1.4.1.1 Low pH and organic acids

At lower pH values, undissociated organic acids such as lactic and acetic acids have strong antagonistic effects that provide effective inhibition particularly to pathogenic Gram-negative bacteria.

The acidity of an environment has a profound effect on the activity and stability of macromolecules such as enzymes, so it is not surprising that the growth and metabolism of microorganisms are influenced by pH. In general, bacteria grow fastest in the pH range 6.0-8.0, yeasts 4.5-6.0 and filamentous fungi 3.5-4.0. The acidity of a product can have important implications for its microbial ecology and the rate and type of spoilage. Low pH that prevents bacterial growth and spoilage in fermented foods is generally a consequence of growth and ultimate domination by LAB. Lactic acid inhibits putrefactive and Gram negative bacteria and some fungi. Acetic acid also has inhibitory effects against putrefactive bacteria, clostridia, some yeasts and fungi (Adams and Moss, 1995; Holzapfel, 2002).

1.4.1.2 Bacteriocins

Bacteriocins are antimicrobial substances of proteinaceous nature that are active against closely related bacteria. They generally show a narrow range of antimicrobial activities, which are not effective for the inhibition of Gram-negative bacteria. Bacteriocins produced by LAB are more effective in inhibiting the growth of pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus, Bacillus cereus* and *Clostridium difficile* (Holzapfel and Schillinger, 1995, Holzaphel, 2002; Mante and Amoa-Awua, 2003). Some bacteriocins of lactobacilli are effective against closely related species of mesophilic *Lactobacillus* and therefore are considered potential natural food preservatives (Daeschel *et al.*, 1991). *Lactobacillus brevis* and *Lactobacillus plantarum* have heat stable bacteriocins that stay stable after storage for 60 days at -20°C (Ogunbariwo and Onilude, 2003). Several types of bacteriocins from food associated with LAB have been identified and characterised, and the most important are diplococcin, acidophilin, bulgaricin, helveticins, lactocin and plantacin (Barefoot, 1983; Klaenhammer and Kullen, 1993; Maldonado *et al.*, 2002), pentocin and fungistatic bacteriocins like substance produced by *L. pentosus* (Okkers *et al.*, 1999). Nisin produced by *Lactococcus lactis* is currently the only commercial available bacteriocins reported to be more active against Gram positive bacteria particularly the sporeformers (Delves-Broughton, 1990).

1.4.1.3 Hydrogen peroxide

Although LAB lack the enzyme catalase which degrades hydrogen peroxide (H_2O_2) produced during metabolism, they still can produce low quantity of H_2O_2 in the presence of dissolved oxygen which is usually in low levels. Hydrogen peroxide has been found to be detrimental to most bacteria, but less harmful to LAB. Therefore, H_2O_2 accumulation could provide an additional selective advantage in relation to microbial competition, but its effect is likely to be generally minor (Adams and Moss, 1995). Hydrogen peroxide targets pathogens and spoilage organisms. When hydrogen peroxide is contact with microorganisms, it reacts very fast and releases oxygen radicals, and causes death of microorganisms through oxidation that allows oxygen to combine with other molecules.

1.4.1.4 Ethanol and low molecular weight metabolites

Ethanol is a well established antimicrobial, although its concentration in lactic fermented products is generally low compared to the production of organic acids by LAB. Ethanol provides some contribution to the inhibition of competitors, and gives LAB a selective advantage. On the other hand, low-molecular weight metabolites such as reuterin (3-OH-propionaldehyde) inhibit a wide spectrum of bacteria, moulds and yeasts, and diacetyl targets Gram negative bacteria.

1.4.2 Degradation or inactivation of natural toxins, antinutritive factors and oligosaccharides

Cassava naturally contains cyanogen glucosides, and if not processed, the cyanogenic toxins may lead to severe cyanide intoxication. Fermentation is reported to be the most effective tool that achieves the greatest degree of cassava detoxification, primarily through microbial activity as a consequence of linamarase produced extracellularly by *Lactobacillus plantarum* (Lei *et al.*, 1999), yeasts and moulds and through leaching out as a result of cassava softening. In addition, lactic fermentation can lower the levels of antinutritive factors such as tannins, phytates, polyphenols, disulphide cross linkages, amylase and protease inhibitors such as trypsin found in cereal and other plant materials known to reduce starch, protein and mineral bioavailability (Svanberg and Sandberg, 1993, 1997). During fermentation, the metabolism of antinutritive compounds by strains of *Lactobacillus plantarum, Leuconostoc mesenteroides* and some yeasts significantly decreases the presence of these compounds in the product (Holzapfel, 1997; Kimaryo *et al.*, 2000; Holzapfel, 2002). Oligosaccharides, which possess α-D-galactosidic bonds resistant to heating (cooking), are hydrolysed by α-galactosidase produced by a number of

moulds and bacteria associated with the digestive tract or with microorganisms such as LAB present in consumed fermented foods (Gobbetti *et al.*, 2000). Therefore, a mixed culture of LAB and yeasts are likely to improve the quality of fermented products (Westby, 1994; Amoa-Awua and Jakobsen, 1995; Essers, 1995; Holzapfel, 1997; Kimaryo *et al.*, 2000; Holzapfel, 2002).

1.4.3 Probiotic properties

A probiotic can be defined as a live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance (Gibson and Ziemer, 1998; Narvhus *et al.*, 2004). Some current requirements for a successful probiotic are survival of acid and bile stress, antimicrobial activity against intestinal pathogens, ability to adhere and colonise the intestinal tract, cholesterol reduction or assimilation and regulation of cytokine expression (Vijendra and Prasad, 2005; Bamias *et al.* 2005; Butler *et al.*, 2007). Some dairy LAB used in yoghurts, such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus pentosus*, *Bifidobaterium* spp. are reported to have probiotic properties (Gibson and Ziemer, 1998), and their growth and metabolic activity could have valuable implications on the human physiological, biochemical and nutritional well-being. The consumption of live probiotic strains is believed to assist in the prevention of diarrhoeal diseases, stimulate the immune system, and protect the host from invading bacteria and viruses (Fuller, 1994; Fuller and Gibson, 1997). Therefore developing starter cultures that incorporate key selected organisms that possess desirable properties is a step forward to the fermentation optimisation.

1.4.4 Possession of pectinolytic activities

One of the goals of submerged fermentation of cassava is to achieve softening through the breakdown of pectin, to facilitate the removal of cyanogenic toxins, and improve the organoleptic quality. Therefore, the decomposition of pectin is an important reaction that results in the textural modification of cassava during fermentation. Pectin is a complex of polysaccharides based on chains of α-1,4-linked galacturonic acid units in which the carboxylic acid groups are variably esterified with methanol and the uronic acid residues variably substituted at carbon -2 with acetyl group. Pectin is one of polysaccharide constituents of primary plant cell wall and the middle lamella of plant materials such as vegetable and fruits (Downie *et al.*, 1998; Bekri *et al.*, 1999; Mustapha and Belarbi, 2001). The plant cell wall is composed of cellulose, hemicellulose, pectin and lectin, thus, the role

of pectin is to maintain cell to cell cohesion, and when it decomposes, the cell wall collapses and loses its rigidity and the breakdown results in the maceration or softening of plant tissue (Alebeek *et al.*, 2003). Some studies have characterised cassava softening as a means of dissociation of cellulose fibres from their pectin cement through the action of pectinolytic enzymes on the pectin glycosidic linkages by depolymerisation, transelimination and esterification reactions which cleave the ester bonds between the carboxyl and methyl groups of pectin (Mustapha and Belarbi, 2001; Ceci, 1998). Pectinase enzymes are classified as methylesterases such as polygalacturonase, which catalyses removal of methanol, and lyases which polymerise pectin by cleaving the α-1,4- linkages of polygalacturonic acid or methylesterified pectin through a trans-eliminative mechanism that results in the production of 4,5- unsaturated oligogalacturonic acid (Dartora *et al.*, 2002). Pectinolytic enzymes have been studied extensively because of their commercial interest in food technology, and are used in extraction and clarification of fruit juices, wine, maceration of vegetables (Bruhlmann *et al.*, 1994; Kapoor, 2002), and in the extraction of vegetable oils (Whitaker, 1994; Demir *et al.*, 2001). Apple and citrus pectin are used as textural food ingredients for gelling marmalades and jams, and as thickening agents for other fruit based applications (Ceci, 1998; Downie *et al.*, 1998; Bekri *et al.*, 1999). Microbial pectinase enzymes produced during cassava submerged fermentation have been reported to be responsible for cassava softening, and among fermenting pectinolytic organisms, *Bacillus* and *Clostridium* spp have been implicated (Oyewole, 1990; Amoa-Awua *et al.*, 1996; Keleke *et al.*, 1996; Kimaryo *et al.*, 2000; Ogbo, 2003).

1.5 Aims of the research

The overall objective is to optimise cassava fermentation through the development of multifunctional starter cultures that address the variations associated with natural cassava fermentation. The selection of starter cultures will take into account the acidification and softening inconsistencies that affect the quality of the cassava fermentation end products. The specific objectives are:

- To investigate the cassava natural fermentation process by studying the effect of time, temperature and substrate composition on the overall inconsistency of changes in pH, cassava softening, microbial growth, and to detect presence of reported softening microorganisms, enzymes activities associated with softening and detoxification of cassava.
- To identify LAB isolated from cassava by phenotypic and genotypic characterisation and to select representative LAB and carry out further characterisation based on ability of the

selected isolates to ferment starch, initiate growth at different pH values, cause a rapid acidification, possess antimicrobial activity, possess linamarase activity, degrade oligosaccharides and possess probiotic activities.

- To individually assess non-LAB isolates for ability to cause cassava softening using discriminatory tests. To identify successful isolates by phenotypic and genotypic characterisations and to select representative bacteria and carry out a further biochemical characterisation to determine their sugar fermentation patterns, pectinolytic activity and metabolic compounds by chromatographic qualitative analysis.
- To develop starter cultures using the preselected LAB, softening bacteria shown to possess one or more desirable properties, and to a lesser extent, one odour-producing bacterium, and then to select suitable potential starter cultures from these for optimal fermentation of cassava, based on their assessed interaction effects on pH, softening, colour and odour of fermented cassava in mixed cultures.

CHAPTER I: NATURAL FERMENTATION OF CASSAVA

1.1 Introduction

Cassava is mostly processed under a natural or spontaneous fermentation, and is characterised by two major factors: initiation of development of a complex microflora, and its unpredicability. It suffers from irreproducibility in terms of acidification, microbial composition, and failure to produce desirable characteristics such as high acid production, softening of cassava, thus inflicting an overall negative impact on the quality of end product. Natural fermentation accompanied with acidification is considered an important factor in control of enteropathogens (Oyewole, 1995; Holzapfel, 1997). Therefore, in this research, several natural fermentation batches of cassava were set up to study and establish the patterns of acidification, occurrence of softening and development of microbial population represented by significant variation of pH values; frequency of inconsistency of cassava softening between fermentation batches and within batches; and variability of microbial composition and population. To a lesser extent, the aim was to detect reported softening bacteria such as *Bacillus* and *Clostridium* species (Keleke *et al.*, 1996; Amoa – Awua and Jakobsen, 1995; Oyewole, 2000) and key enzymatic activities involved in texture modification of cassava, and in cassava detoxification.

Preliminary cassava fermentation methods were assessed under different variables to identify suitable *in vitro* fermentation models to use in this study. Assessment of fermentation conditions was based on the optimum temperature, fermentation duration, use of peeled or unpeeled cassava pieces, grated and shredded cassava and solid state or submerged fermentation. As lactic acid bacteria (LAB) are largely involved, atmosphere requirement tests were carried out to confirm whether the fermentation occurred optimally under anaerobic or aerobic conditions. Preliminary experiments showed that anaerobic and aerobic conditions gave similar results at the end of fermentation. Consequential to the preliminary results the following conditions were selected: submerged fermentation at 37°C under aerobic conditions for five days and use of either peeled or unpeeled cassava cut in cylindrical pieces of approximately 5 mm diameter. The submerged fermentation consists of peeled or unpeeled cassava tuber, cutting and washing, then soaking the cassava pieces in substantial volume of water for five days in aerobic conditions. Based on preliminary fermentation results, extending and backslopping can occasionally lead to the softening of cassava fermentation that failed to produce it initially. Traditional

backslopping could be technically be considered use of a starter cultures where an aliquot of previous successful fermentation that produced desirable characteristics such as softening, was collected and kept for future use to initiate a new fermentation batch. In an attempt to study the possible causes driving these variations, models of natural fermentation batches of cassava were established under various parameters. The first approach was to study the time or duration of fermentation such as the early course (24, 48 h) and other varying intervals including an extension of 4 more days to 9 days. The second aspect was to carry out cassava fermentations using different incubation temperatures. The third approach was to modify the substrate composition by adding other components of cassava, such as skin using unpeeled cassava and inclusion of cassava leaves to evaluate the overall influence on microbial growth, pH and softening (biochemical) changes. It was expected that the results might indicate the most characteristic pH values, time and temperature ranges at which high acidification and microbial growth rates were optimally achieved and the softening was likely to materialise.

The main objective of this study was to investigate the cassava natural fermentation process by studying the effect of time, temperature, and substrate composition on the overall behaviours of pH, cassava softening, microbial growth, and to detect presence of reported softening microorganisms, enzymes activities associated with the softening and the detoxification of cassava.

1.2 Materials and Methods

1.2.1 Natural fermentation of cassava

Imported waxed skin raw cassava and frozen cassava leaves were purchased from local markets in London, and the former were used in most experiments throughout this study. The *in vitro* cassava fermentations were prepared by peeling or not peeling, cutting cassava into cylindrical pieces of approximately 5 mm diameter and 1 cm width and then washing. A total of 100 g of each type was placed in sterile lidded Kilner^{TM}- type jars containing 200 ml of sterile distilled water. The jars were closed and incubated aerobically at 37°C for 5 days. Samples were collected initially (0 h) and at intervals for a total of 5 days for measurement of pH value, degree of cassava softening, microbial isolation and growth assessment, enzymatic activity for detection of α-glucuronidase, β-glucosidase and pectinolytic enzymes.

1.2.2 Natural fermentation of cassava: effect of temperature

In this study, laboratory natural fermentations were set up as above with the modification and were fermented at 25°C, 30°C, 37°C and 40°C for 5 days. Samples were collected initially and at intervals during fermentation and at the end for measurement of pH value, cassava softening and microbial isolation and growth assessment.

1.2.3 Natural fermentation of cassava: effect of time

The first part of this experiment was to carry out fermentations at 37°C for 5 days and critically examine the first 24 h in order to study the early course of fermentation changes in terms of acidification rate, succession of microbial population and softening occurrence. Samples were collected and analysed at initial time, every 3 hours or 6 hours for 24 hours, and at day 5. The second part of this experiment was to study the effect of time under different temperatures of 25°C, 30°C, 37°C and 40°C for 5 days. The third part was to manipulate any of the above fermentations that failed to produce the softening of cassava after 5 days: first by extending the fermentation without backslopping for a further 4 more days, and second by extending with backslopping and leave the fermentation for 4 more days. The unsuccessful fermentations were divided into two samples: one sample was re-incubated and the fermentation extended by an additional 4 days (EXT), the second sample was backslopped and extended by 4 days (BCS) by adding an aliquot (10%: g/g) of previous fermented softened cassava that have been stored at -20°C, and kept at room temperature on the day of the experiment. After a total of 9 days, the pH values were remeasured and the softening, microbial status and growth assessment were reassessed.

1.2.4 Natural fermentation: effect of skin and leaves of cassava

Peeled and unpeeled cassava i.e. (skin on) pieces (100 g) were mixed with 20 g of leaves (unpeeled cassava pieces are hereafter referred to as "cassava skin"). All cassava pieces were fermented at 25°C, 30°C, 37°C for 5 days, while 50 g each of cassava skin and leaves as controls were fermented at 37°C for 5 days. The pH values, cassava softening and microbial counts were all determined initially and at the end of fermentation.

1.2.5 Measurement of pH during fermentation

Unfermented and fermented cassava samples were collected at initial time and at different stages of fermentation (0h, 24 h, 48 h, 72 h, 120 h, 9 days), and samples for analysis were prepared by weighing 10 g of each type of cassava into a sterile plastic bag, then homogenised with 90 ml of distilled water in a stomacher (Colworth stomacher 400, A.J

Seward & Company Ltd). The pH values of blended cassava homogenates and the fermenting liquid were determined electronically using a pH meter with a glass electrode (Jenway, Hanna Instruments) calibrated against buffers at pH 4.0, 7.0 and 10.0.

1.2.6 Evaluation of cassava softening

In the early stage of this experiment, the evaluation of cassava softening was determined quantitatively using an Instron Strength Telemometer. The machine is composed mainly of a compressor cell and a measurement cell, known as reading graph. The reliability and the accuracy of the quantitative measurement results were questionable, since the machine was particularly insensitive and not discriminative when obviously well-softened cassava pieces were measured. In contrast, measurements of raw unsoftened cassava pieces gave accurate and reproducible results. Therefore, this method was abandoned and the evaluation of cassava softening was instead achieved by manually pressing cassava pieces with a metal spatula or a glass rod.

1.2.7 Microbial isolation, enumeration and growth assessment by spread techniques

A 10 g sample of each type of cassava (unfermented and fermented) was collected initially, at different stages and at the end of fermentation. The collected samples were respectively homogenised with 90 ml of sterile maximum recovery diluent (MRD; Oxoid CM0733) or ringers solution (Oxoid BR0052), for 2 min for softened cassava and 5 min for unsoftened cassava. Serial ten- fold dilutions were prepared and 100 µl of appropriate dilutions were spread plated on various solid media all from Oxoid, Basingstoke, Hampshire, UK: nutrient broth, and agar (NB, CM0001B; NA, CM003B) for general aerobic count, violet red bile glucose agar (VRBGA, CM485B) and *Escherichia coli* chromogenic medium (ECC, CM0956) for the selection of *Escherichia coli*, and general isolation of *Enterobacteriaceae*, *Bacillus cereus* selective agar base (CM0617) with polymyxin/pyruvate (SR0099E)/egg yolk (SR0047C) supplement (PEMBA) for *Bacillus* spp., blood agar base (CM0055) supplemented with horse blood (SR0050C) (HBA), modified Wilkins – Chalgren Anaerobe agar (CCY) made of 25 g/l of nutrient broth no 2 (CM0067B); 1 g/l of glucose;1 g/l of L-arginine; 1 g/l of sodium pyruvate; 5 g/l of yeast extract (LP0021); 10 g/l of agar (LP0013B) and G-N anaerobe selective supplement (SR0108B) for Gram-negative anaerobes, and 25 ml of horse blood (SR0050C). Ferguson Perfringens (SFP, CM0587B), supplement (SR0093E) for *Clostridium* spp., De Man Rogosa, Sharpe broth, and agar (MRB, CM0359B; MRS, CM0361B) for LAB, Sabouraud dextrose agar (SAB, CM0041B), Rose-Bengal chloramphenicol agar (RBC,

CM0549B), chloramphenicol supplement (SR0078E) for yeast and moulds. NA, VRBGA and ECC plates were incubated aerobically at 37°C for 24 h, MRS, RCA, SFP and HBA were incubated anaerobically (Oxoid gas kit BR0038B) at 37°C for 48 h, and SAB and RBC plates were incubated aerobically at 25°C for 5 to 7 days. After incubation, the plates were observed for microbial growth and colonies counted. Selected colonies were picked off and purified. All isolates were maintained on cryobeads at -20°C for further tests. Purification was achieved by growing colonies in appropriate liquid and solid media until isolated single colonies were obtained. They were phenotypically characterised by Gram staining and microscopic examination and by catalase reaction (by selecting and placing a pure colony on clean glass slide, then covering with one to two drops of 30% hydrogen peroxide). Positive results were indicated by frothing of the colony due to evolution of oxygen.

1.2.8 Bacteria associated with cassava softening

1.2.8.1 Detection of Bacillus species in fermented cassava

The experiments were carried out primarily to isolate *Bacillus* species in parallel with the isolation of LAB using various cassava samples: previous stock of fermented softened cassava, heated (70°C, 1 h) fermented softened cassava, fermented unsoftened cassava, unsoftened cassava from backslopped and extended fermentation, pasteurised MRS broth culture of LAB isolated from previously fermented and softened cassava.

1.2.8.2 Detection of Clostridium species by classical cultural methodology in the fermented cassava

Use of the most probable number (MPN) technique

The detection by MPN was done using sterilised 4.5 ml of reinforced clostridial basal medium (RCM) dispensed in bijoux bottles and cooled down before adding 0.5 ml of equal volumes of filter-sterilised sodium sulphite (4% w/v) and ferric citrate (7% w/v) solutions. These solutions make the medium differential (DRCM) for sulphite-reducing clostridia. Heated cassava sample (70°C for 1h) was homogenized in maximum recovery diluent (10% w/v) to make the first dilution, the second was made by transferring 10 ml of the first dilution into 90 ml of diluent and 10 ml of the second dilution into 90 ml of diluent to obtain the third dilution. These dilutions were further each diluted by serial dilutions (10^{-1} – 10^{-4}) each time by transferring 0.5 ml into 4.5 ml of DRCM medium contained in bijou bottles. The bottles were filled with sterile liquid paraffin and incubated at 37°C for 7 days. After incubation, DRCM cultures with positive reactions (blackening) indicated evidence

of sulphite-reducing *Clostridium* species in the cassava sample. In the case of a positive reaction, confirmatory tests were carried out by heating (at 70°C for 1 h) the positive DRCM cultures and cooling. Then 100 µl of tenfold serial dilutions were spread on pre-reduced HBA plates and incubated anaerobically at 37°C for 7 days. The presumptive isolated *Clostridium* spp. were subjected to aerobic growth tests on NA at 37°C for 48 h, and visual observations based on colony and cell morphology of these isolates on HBA were carried out.

Use of Omeliansky /cellulose medium and double strength reinforced clostridial medium (RCM).

In this experiment, a new submerged fermentation of cassava was set up at 37°C for 5 days. The detection of *Clostridium* species was carried out by inoculating 1 ml of fermented and unfermented cassava homogenates each into 90 ml of pre-reduced sterilised (at 121°C for 20 min) Omeliansky medium ($(NH_4)_2SO_4$: 1g, K_2HPO_4: 1g, $MgSO_4.7H_2O$: 0.5g, CaO_3: 2 g, NaCL: trace, 1000 ml of distilled water that contained 2 g of sterile filter paper strips in screw-capped bottles. The bottles were topped up with fresh pre-reduced Omeliansky medium to increase anaerobiosis and incubated anaerobically at 37°C for 45 days. After the incubation, the paper strips were examined for the presence of patches to indicate cellulose-digesting activities, and followed by isolating and enumerating *Clostridium* spp. first on RCA, SFP, HBA and NA under anaerobic conditions at 37°C for 48 h. Second: 1 ml of each Omeliansky culture was inoculated into 9 ml pre-reduced double strength RCM (Oxoid CM149) and incubated anaerobically at 37°C for 6 days. After incubation, two types of isolation were made by streaking: firstly on RCA (Oxoid CM151) plates directly from the double strength RCM cultures, secondly on NA, HBA and RCA from double strength RCM cultures that had been heated at 70°C for 15 min and incubated anaerobically at 37°C for 48 h after the pasteurisation. All plates were incubated anaerobically at 37°C for 24 – 48 h. The isolated presumptive *Clostridium* spp. were purified, morphologically examined and tested for their ability to grow aerobically on NA at 37°C for 24 - 48 h.

1.2.8.3 Detection of Clostridium species by non-cultural methodology using fluorescent in-situ hybridization (FISH)

The FISH technique was described by (Rycroft *et al.* 2001). For fixation of the sample specimen, 1 ml each of fermented softened cassava and pasteurised fermented softened cassava homogenates were centrifuged 10000 rpm for 5 min, 375 µl of supernatant was removed and added to 1125 µl of filter-sterilised paraformaldehyde (4%: w/v) solution pH

7.2, and suspensions were mixed and stored overnight at 4°C to fix the cells. Fixed samples (1.5 ml) were centrifuged at 13000 rpm for 5 min, the supernatants were carefully removed and pellets resuspended in 1 ml of filter-sterilised 1x phosphate buffer saline (PBS). The harvested pellets were recentrifuged and resuspended two more times as above, and the pellets resuspended thoroughly in 150 µl of filtered 1 x PBS. After adding 150 µl of ethanol 99%, the suspensions were mixed and stored at - 20°C before hybridisation. For the hybridisation: the fixed cells and genus-specific 16S rRNA-targeted oligonucleotide probe (His 150, Molecular Probes Europe BV, Netherlands) labelled with cyanine dye (Cy3), a fluorescein derivative, were left at room temperature for 20 min before use. The fluorochromes Cy3 are superior dye which provide significant brighter staining and are very stable to photo bleaching. Fixed cells (16 µl) were added to micro-centrifuge tubes containing 264 µl of double strength filtered hybridisation buffer (30 mM Tris-HCl (Sigma); 1.36 M NaCL; 10% SDS), and then pre-warmed in a hybridisation oven at 50°C. A volume of 135 µl of prepared fixed cells was added to microcentrifuge tubes containing 15 µl of the probe (50 ng /µl). The tubes were vortexed and left overnight in the oven to hybridise at 50°C. For washing: 100 µl of hybridisation mix were transferred to sterilin tubes containing 5 ml of single strength filtered prewarmed wash buffer (0.9 m mol^{-1} NaCL, 20 mM l^{-1} Tris-HCL pH 7.2) and 20 µl of an aromatic diamidine blue fluorescent counterstaining 4',6 diamidino-2-phenylindole dihydrochloride (500 mg/ µl). DAPI prevents unnecessary binding of other materials to DNA. The tubes were incubated in the hybridisation oven at 50°C for 30 min. After incubation, the hybridisation mix was filtered through 0.2 µm pore size black filters GTBP filters (Millipore). After filtering the cells, the tubes and the filters were washed with 50 ml of prewarmed (50°C) wash buffer using a syringe to ensure that all of bacteria were washed onto the filters. For mounting and visualisation: filters were placed onto glass slides and a drop of Slow Fade (S-2828, Molecular Probes), an antifade kit, was deposited on top before covering with cover slips. The glass slides were examined using a microscope fitted with green light filter for DAPI stain was excited at 359 nm and emitted at 461 nm and the Cy3 dye was excited at 550 nm and emitted at 565 nm.

1.2.9 Detection of enzymes activity of the fermented cassava

1.2.9.1 Detection of endo-polygalacturonase activity by viscometry.

Following the method described by Blandino *et al.* (2000), 100 g of unfermented and fermented, peeled and unpeeled cassava; backslopped fermented peeled and unpeeled cassava samples were each homogenised in 1000 ml of distilled water. Each homogenate was mixed using a rotary shaker at 30°C, 200rpm for 30 min. For the extraction of crude enzymes, each homogenate was centrifuged at 5000 x g for 10 min. After centrifugation, 150 ml of supernatant were mixed with 450 ml of pectin solution (1%: w/v) in 0.1 M acetate buffer pH 5.0), and each mixture heated in a water bath at 45°C for 30 min. Each solution was transferred to a 600 ml beaker and the reduction of viscosity was monitored with a Brookfield DV-I+ viscometer. One unit of endo-polygalacturonase activity was defined as the amount of enzyme that reduced the viscosity of the pectin solution by 50%.

1.2.9.2 Detection of exo-polygalacturonase activity by modified dinitrosalicylic acid reagent (DNS).

Exo-polygalacturonase (Exo-PG) activity, based on the hydrolytic release of reducing groups from polygalacturonic acid, was detected using a modified dinitrosalicylic acid method by Wang *et al.* (1997). The results were expressed as galacturonic acid concentrations derived from a calibration curve, and one unit of exo-polygalacturonse activity was defined as the amount of enzyme that produced 1 µmol galacturonic acid/min. DNS reagent solution (without phenol) containing 44 Mm dinitrosalicylic acid, 4 mM sodium sulphite, 375 mM sodium hydroxide and polygalacturonic acid (PGA) solution containing 0.5 g of PGA (3889, Sigma) and 100 ml acetate buffer (100 mM, pH 5.0) were prepared. Standards were made by mixing DNS solutions (1:1, v/v) in test tubes. The tubes were heated for 10 min in a boiling water bath at 100°C. Volumes were adjusted to 4 ml using distilled water immediately after heating. The solutions were cooled to room temperature, and the absorbance read at 575nm using a spectrophotometer (Cecil CE 1021, 1000 Series). The samples of fermented peeled and unpeeled cassava, fermented backslopped peeled and unpeeled cassava were each homogenised (10%: w/v) in 0.5 mM acetate buffer pH 5.0. The homogenates were centrifuged at 12,000 RPM for 10 min. For the assay, two different volumes of polygalacturonic acid were used: for the first reaction mixture, 0.8 ml PGA (0.5%) were mixed with 0.2 ml of each cassava sample supernatant (extracted crude enzymes) in test tubes; and the second reaction mixture, 1.6 ml PGA (0.5%) were mixed with 0.4 ml of supernatant of each sample in test tubes. The tubes were

heated at 30°C for 4 h, then 1 ml DNS was added to each tube to terminate the reaction, and the tubes reheated in a water bath at 100°C for 10 min. After heating, each volume was adjusted to 4 ml by adding distilled water, cooled to room temperature, and the absorbance of the solutions after reaction with galacturonic acid was read at 575 nm.

1.2.9.3 Detection of pectinolytic enzyme activity by top agar method.

Following the method described by Amoa-Awua and Jakobsen, (1995), standard solutions of commercial pectinase, cellulase and polygalacturonase enzymes were prepared as controls by mixing 10 units of each with 10 ml of sterile distilled water (w/v). Peeled and unpeeled cassava pieces, each 10 g, were collected before and at the end of fermentation and homogenised with sterile distilled water (10%: w/v). For detection of pectinase activity of the cassava samples: 100 µl of each cassava homogenate and 100 µl pectinase solution were transferred into the wells of approximately 90 mm diameter cut in pectin agar plates (5 g pectin, 7.5 g agarose in 500 ml sodium phosphate buffer pH 8.0, 7.5 g, sterilised at 121°C for 20 min). The plates were incubated aerobically at 45°C for 4 h. For detection of cellulase activity of fermented cassava samples, 100 µl of each cassava homogenate and 100 µl cellulase solution were pipetted into the wells of approximately 90 mm diameter cut in cellulose agar plates (5 g of carboxymethyl cellulose, 6 g agarose in 500 ml sodium phosphate buffer pH 6.0, sterilised at 121°C for 20 min). The plates were incubated aerobically at 45°C for 6 h. For the detection of polygalacturonase, 100 µl of each cassava homogenate and 100 µl polygalacturonase solution were pipetted into wells of approximately 90 mm diameter cut in polygalacturonic agar (5 g polygalacturonic acid, 20 g nutrient agar powder in 500 ml sodium phosphate buffer pH 8.0, sterilised at 121°C for 20 min) plates, and the plates were incubated aerobically at 30°C for 4 days.
After incubation, the pectin agar plates were checked for appearance of precipitation surrounding the wells. Cellulose and polygalactouronic acid agar plates were examined for presence of clear zones after staining the surface with Congo red solution 0.1% (w/v) for 10 min, followed by twice decolorizing in 1 M sodium chloride solution for 10 min each time.

1.2.9.4 Detection of α-glucuronidase and β-glucosidase activities.

The assessment of change in enzyme activity over 5 days was determined by semi-quantitative assay of 19 key enzymes using API-Zym kits (BioMerieux, Basingstoke, UK) which are commercial ready-to-use media designed to detect enzymatic activity in complex samples which has not been purified. Strips are composed of cupules or wells

containing different enzymes. The cupules are inoculated with a dense suspension of freshly-prepared test organism. After the incubation, the metabolic end products formed can be detected by colour changes. The colour development is spontaneous or triggered by addition of reagents. The colour reactions are read according to the API Biomerieux Reading Table. For this test, set up according to the manufacturer's instructions, isolated on MRS plates were subcultured (10%: v/v) in MRS broth (Oxoid, Basingstoke, Hampshire, UK) and grown on MRS agar for 48 h to obtain a bacterial lawn, LAB colonies were used to prepare densely turbid microbial suspensions in 5 ml sterile distilled water. Aliquots of 80 µl of the suspension were inoculated into the wells of the strips and incubated for 48 h at 37°C.

1.3 Results and Discussion

In this study, the pH values, which reflect the acidification level during natural fermentation, were measured at intervals throughout the duration of fermentation, starting from initial to five days and from five to nine days when the fermentation has been extended and backslopped. The impact of temperatures of fermentation (25°C; 30°C; 37°C and 40°C) and substrate composition (i.e. use of cassava leaves and peeled or unpeeled cassava) of cassava were also examined. The values of pH were recorded at initial to nine days at various intervals.

All results demonstrated that the pH profiles during natural fermentation in varying conditions displayed a catalogue of continuous variation between and within fermentation batches. There was considerable variability in the initial pH (5.6-7.28) of cassava used in this study. A rapid pH decrease with inconsistent pH values for both peeled and unpeeled cassava under natural fermentation was displayed. On some occasions, lower pH values were observed in peeled cassava (Figure 1.2), and at other times were displayed in unpeeled cassava (Figure 1.1). Figure 1.3 shows a rapid pH drop over 6 h during unpeeled cassava fermentation, but after this there was no difference of pH fall between peeled and unpeeled cassava.

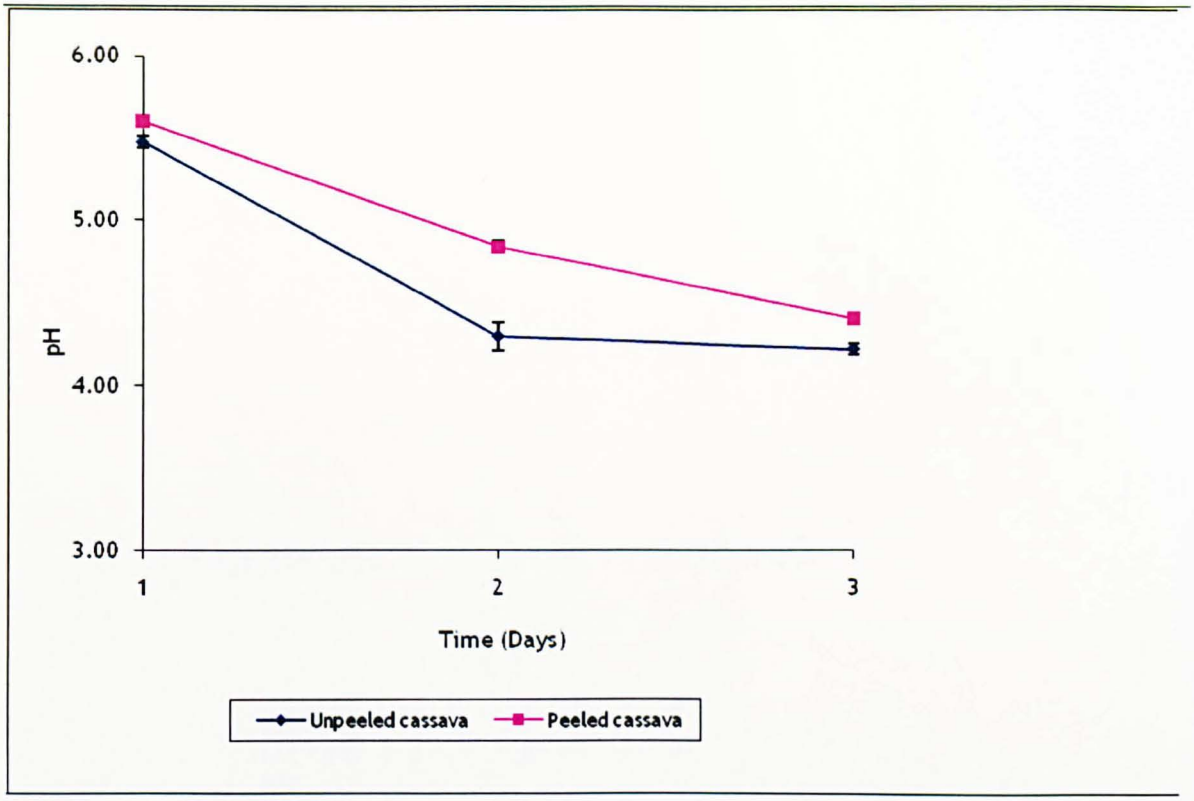

Figure 1.1: Changes in pH values recorded during natural fermentation of cassava initially and at day 3. Data represent the mean of 3 replicates. Errors bars indicate the standard deviation of the mean: 5.48, 4.30, 4.22 (Unpeeled cassava) and 5.60, 4.84, 4.41 (Peeled cassava).

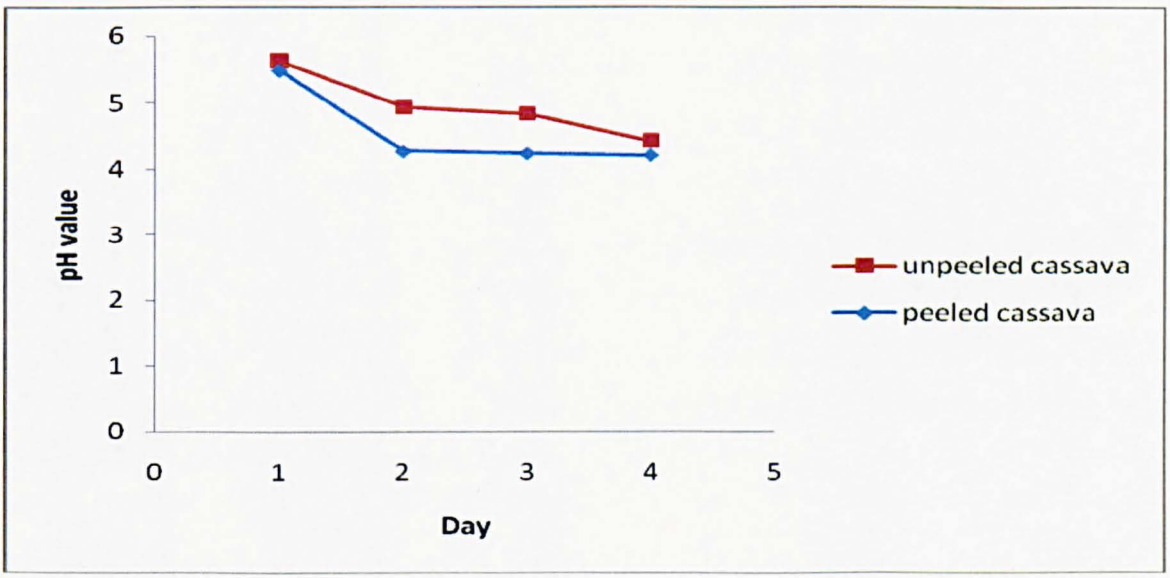

Figure 1.2: Changes in pH value of peeled and unpeeled cassava during natural fermentation

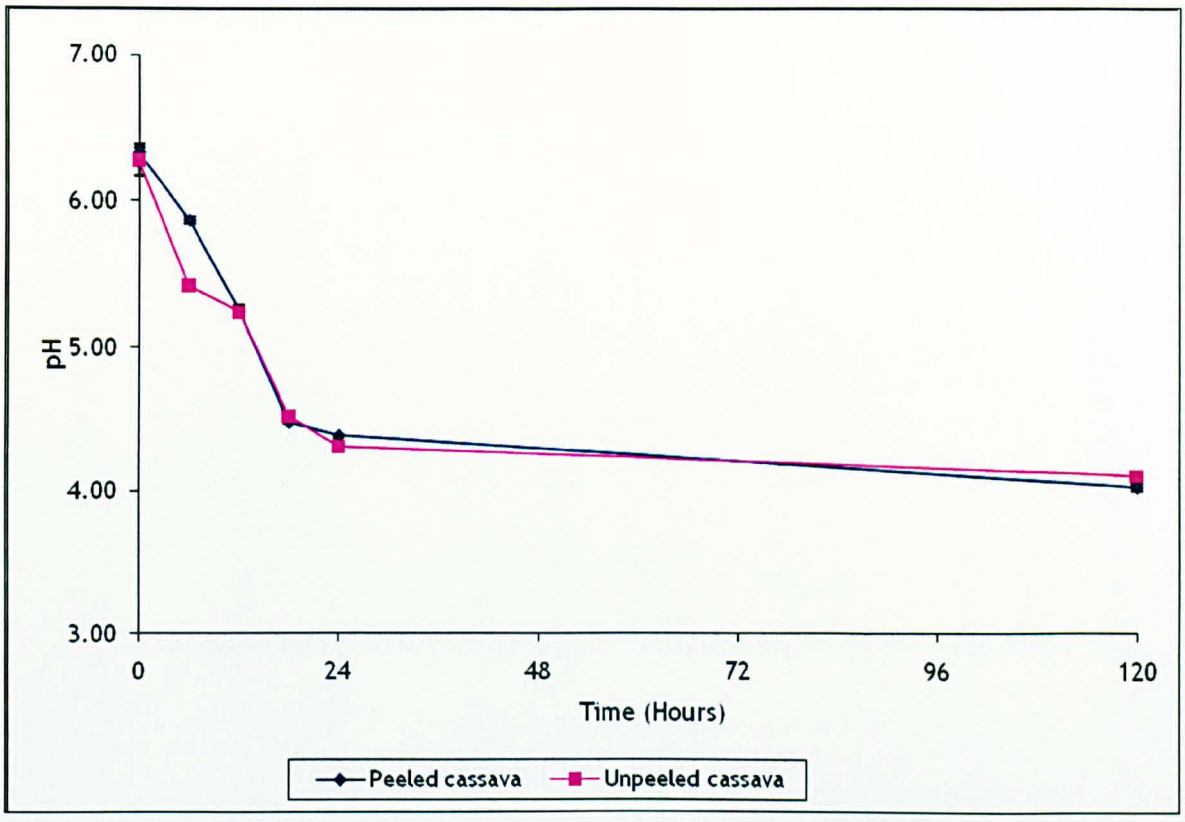

Figure 1.3: Changes in pH values during 24 h at 6 h intervals and at day 5. Data represent the mean of 3 replicates. Errors bars indicate the standard deviation of the mean: 6.33, 5.87, 5.24, 4.48, 4.39, 4.02 (Peeled cassava) and 6.28, 5.41, 5.23, 4.52 (Unpeeled cassava).

The first case study of the effect of time and temperature on pH (Figures 1.1, 1.2, 1.3, and Tables 1.1, 1.2) indicate a sharp and significant drop of pH values from 6.32 (+ 0.67) to 4.6 1 (+ 0.76) within the first 48 h, then continued to decrease slightly to the third day. A minor pH change was observed towards the end of fermentation, and there was no major pH difference between peeled and unpeeled cassava. These findings suggest that the early course of fermentation was the most crucial stage, where a major decrease of pH values occurred and similar results were recorded by Brauman *et al.* (1996), Amoa Awua *et al.*, (1996); Obilie and Amoa-Awua, (2004). According to Blanshard *et al.* (1994), low pH reduced the numbers of *Enterobacteriaceae* to below the limit of detection, and also affected the type of microflora.

Table 1.1: Effect of time on pH value

Time	Peeled cassava	Peeled shredded cassava	Unpeeled cassava	Unpeeled shredded cassava
Initial	6.32 - 7.2	6.32 - 7.0	6.19 - 7.28	6.19 - 7.28
3 h	6.1 - 6.7	6.68 - 6.7	6.05 - 6.58	6.55 - 6.58
6 h	5.88 - 6.51	6.66 – 6.7	5.44 - 6.29	7.12 - 7.36
9 h	5.5 - 6.28	6.6 - 6.96	5.42 - 6.04	6.92 - 6.94
12 h	5.24 - 6.08	6.5 - 6.63	5.24 - 5.98	6.31 - 6.38
15 h	5.3 - 5.64	6.6 - 6.64	5.41 - 5.74	6.19 - 6.22
18 h	4.49 - 4.78	5.88 - 5.92	4.52 - 4.79	6.04 - 6.16
21 h	4.1 - 4.25	5.74 - 5.81	4.2 - 4.30	5.72 - 5.83
24 h	4.0 - 4.40	5.36 - 5.40	4.09 - 4.43	5.42 - 5.50
48 h	3.4 - 3.85	5.11 - 5.31	3.81 - 4.09	5.05 - 5.30
Day 5	3.75 - 4.04	5.0 - 4.90	3.73 - 4.07	4.86 - 4.92

Range of pH values recorded during four cassava fermentations replicates

It was observed that shredded cassava fermentation at all conditions of temperature and time was characterised by higher pH values than cassava cut in cylindrical pieces, dropping from 6.58 (+0.12) to 4.90 (+0.02) (Table 1.1). This finding demonstrated that the size into which cassava was cut had no influence on the acidification rate. On the contrary, however, Vasconcelos *et al.*, (1990) confirmed that small cassava pieces led to high acid production during cassava fermentation.

Table 1.2: Effect of time and temperature on pH value

Natural fermentation	Time	25°C	30°C	37°C	40°C
Peeled cassava	Initial	5.55+1.2			
	5 days	3.99+0.33	3.76+0.6	3.56+0.56	3.63+0.63
peeled cassava (normal)	5 days			4.30	
Extended peeled cassava (4 more days)	9 days	4.22	3.40	3.52	3.39
Extended peeled cassava with backslopping	9days	4.10	3.46	3.50	3.40
Unpeeled cassava	Initial	5.55+1.2			
	5 days	4.01+0.41	3.96+0.19	3.44+0.75	3.45+0.72
Unpeeled cassava (normal)	5 days			4.22	
Extended unpeeled cassava (4 more days)	9 days	4.24	3.44	3.61	3.41
Extended unpeeled cassava with backslopping (4 more days)	9 days	4.15	3.43	3.54	3.40

Range & average of pH values recorded during four cassava fermentations experiments

Effects of time and temperature on pH value were assessed and it was observed that a further slight decline of pH value at all temperatures was caused by the action of extending the fermentation (Table 1.2). Moreover, a combination of backslopping and extending the fermentation caused further pH drop of almost 1 unit from 4.22 (+ 0.08) to 3.3 9 (+ 0.15). Thus a combination of extending and backslopping the cassava fermentation could positively influence the pH value or acidification level.

It was concluded that the time or duration of fermentation had a positive effect on the pH values; as a result, it was recommended that cassava should be fermented at least for 72 h to achieve adequate pH of > 4.2 for the safety of product.

With regard to the effect of temperature on pH, Table 1.4, Figures 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0 all display overt pH irregularities. Generally cassava fermentation at 25°C produced high pH values that decreased from between 5.55 & 6.75 to 4.27 + 0.15 compared with low final pH values in the range of 3.44 - 4.32 at 37°C. The results revealed an inverse correlation between pH and temperature suggesting that the higher the temperature, the lower the pH values were recorded. It was concluded that higher temperatures promoted a decrease in pH value (or increase in acidification rate) and 37°C was considered to be optimal for cassava fermentation models in this study.

The effects of cassava skin and leaves on pH were studied and Figure 1.6 shows that the skin had no effect on pH value, suggesting that it had no role in the acid production. The results in Figure 1.7, 1.8, 1.9, 2.0 demonstrate that adding leaves to cassava before fermentation increased slightly the initial pH value, however, at the end of fermentation lower pH values were recorded. This suggested that supplementing cassava with leaves might have an impact on decreasing the pH value and thus the acidification rate.

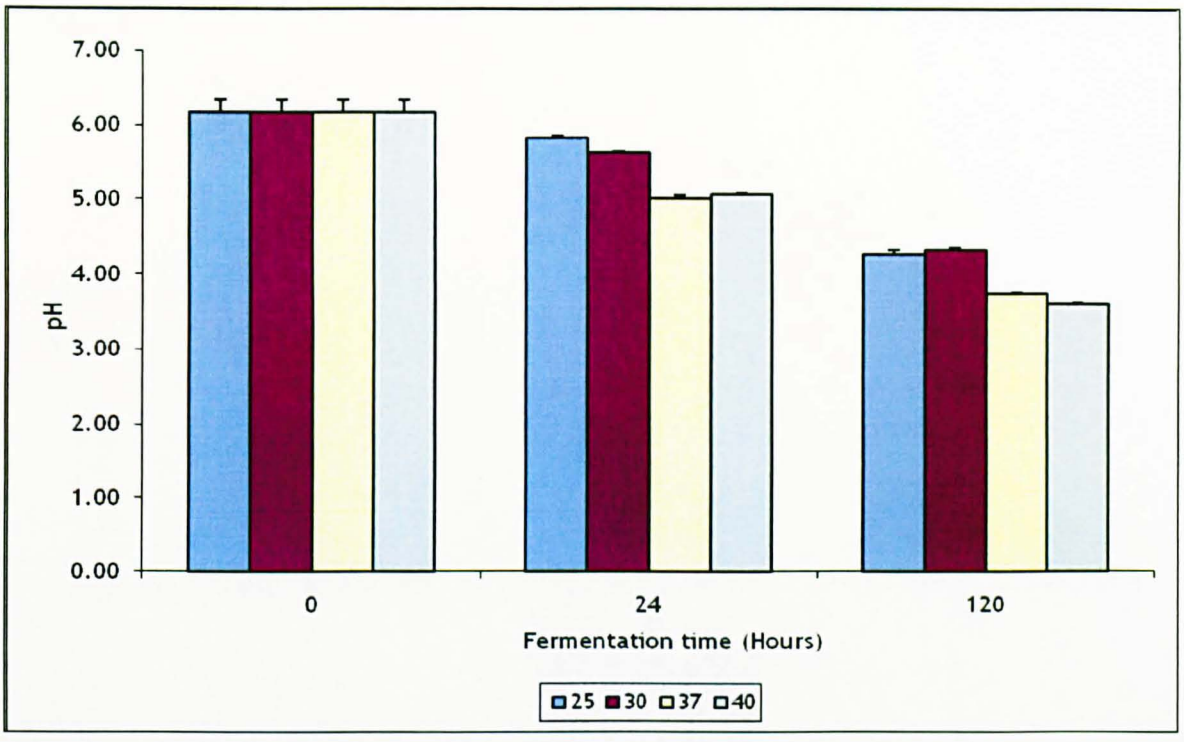

Figure 1.4: Peeled cassava

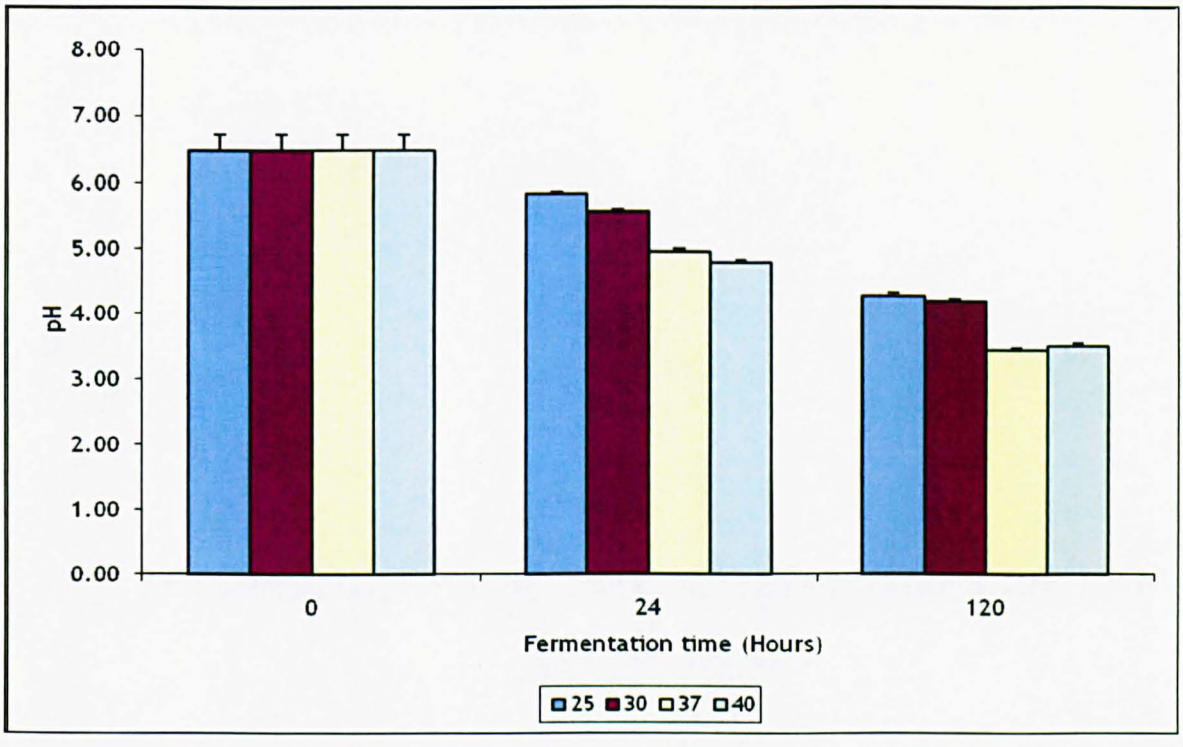

Figure 1.5: Unpeeled cassava

Figures 1.4, 15: Effect of temperature on pH of peeled and unpeeled cassava. Data represent the mean of 3 replicates. Errors bars indicate the standard deviation of the mean: Peeled cassava: 6.17, 5.83, 4.27 (25°C), 6.17, 5.63, 4.32 (30°C), 6.17, 5.01, 3.73 (37°C), 6.17, 5.06, 3.61(40°C). Unpeeled cassava: 6.48, 5.83, 4.27 (25°C), 6.48, 5.56, 4.19 (30°C), 6.48, 4.94, 3.43 (37°C), 6.48, 4.79, 3.50 (40°C).

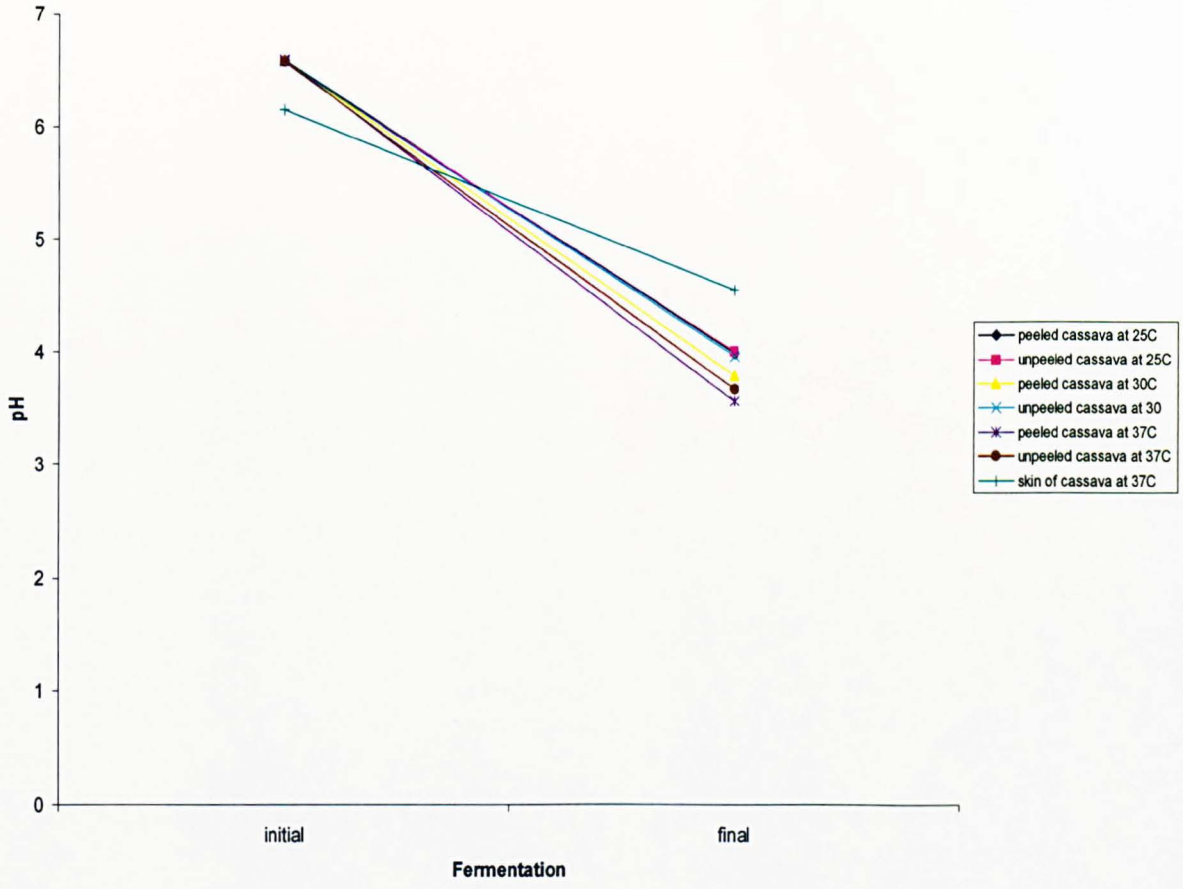

Figure 1.6: Effect of temperature and presence of cassava skin on changes in pH during fermentation

Peeled cassava

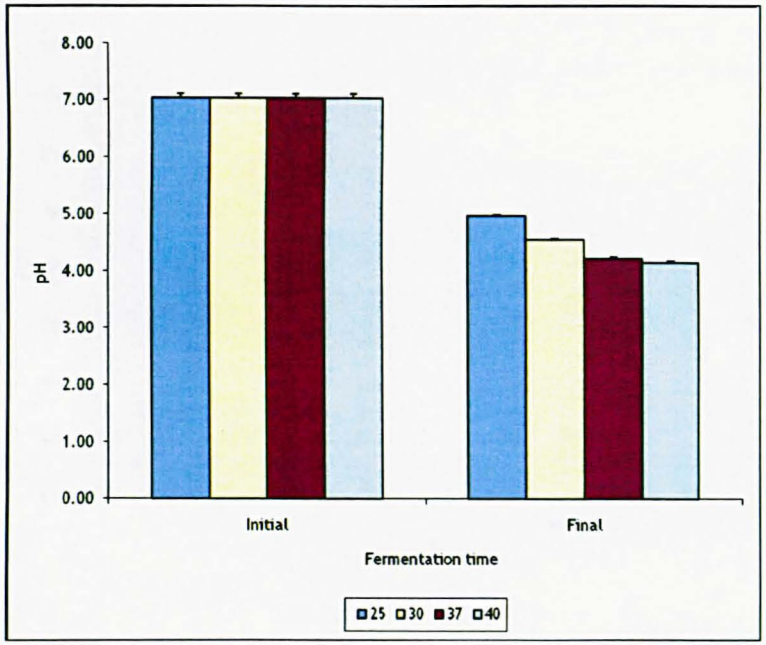

Fig. 1.7

Peeled cassava + leaves

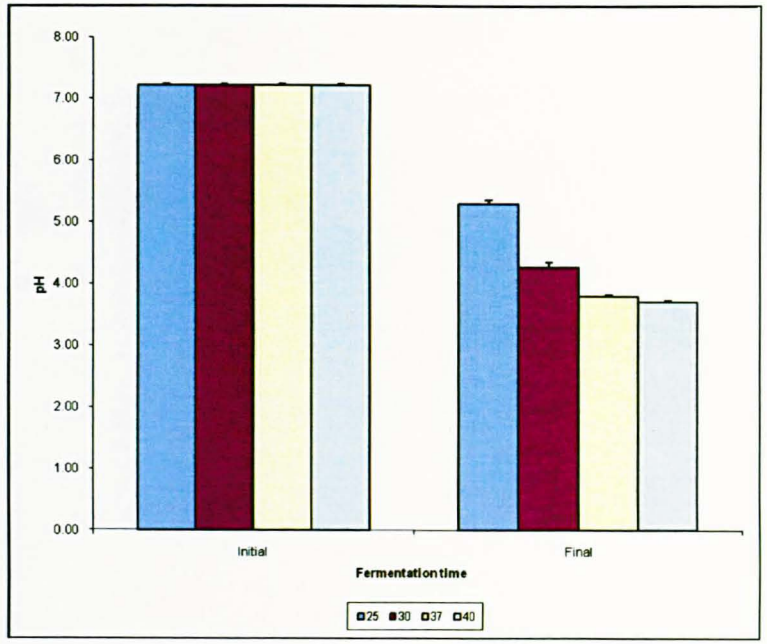

Fig. 1.8

Figures 1.7, 1.8: Effect of cassava leaves on changes in pH during fermentation. Data represent the mean of 3 replicates. Errors bars indicate the standard deviation of the mean: Peeled cassava: 7.05, 4.98 (25°C), 7.05, 4.56 (30°C), 7.05, 4.23 (37°C), 7.05, 4.16 (40°). Peeled cassava + leaves: 7.21, 5.29 (25°C), 7.21, 4.26 (30°C), 7.21, 3.80 (37°), 7.21, 3.72 (40°C).

Unpeeled cassava

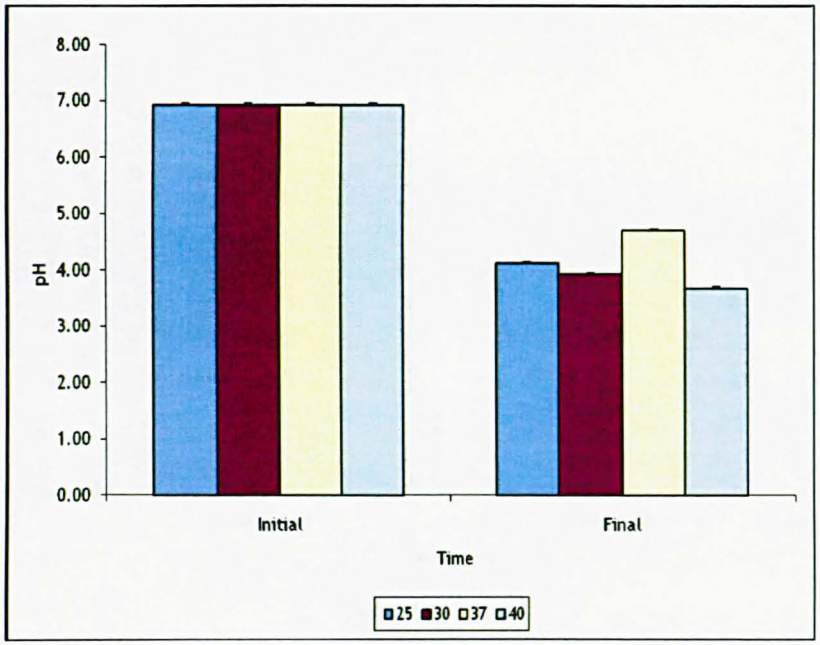

Fig. 1.9

Unpeeled cassava with leaves

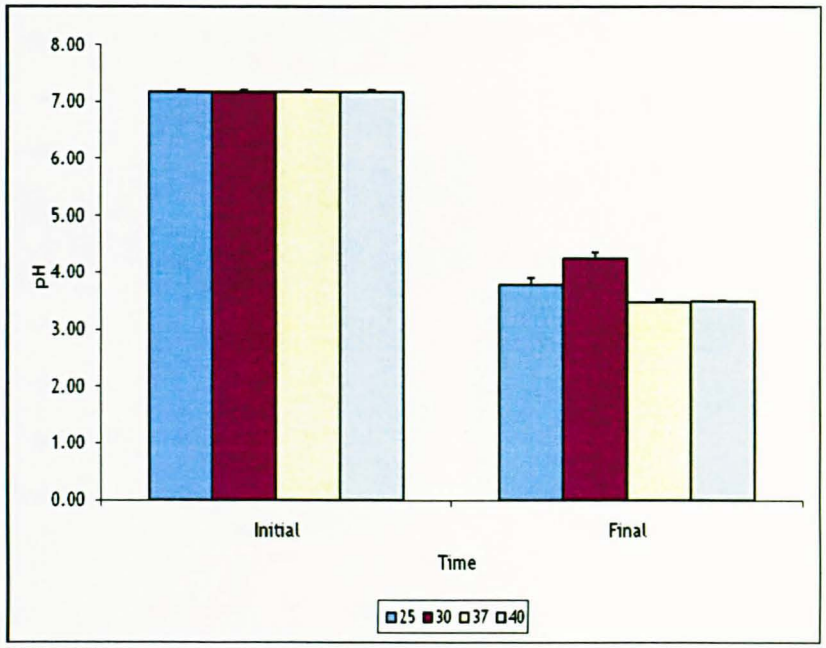

Fig. 2.0

Figures 1.9, 2.0: Effect of cassava leaves on changes in pH during fermentation. Data represent the mean of 3 replicates. Errors bars indicate the standard deviation of the mean: Unpeeled cassava: 6.94, 4.15 (25°C), 6.93, 3.92 (30°C), 6.93, 4.71 (37°C), 6.93, 3.68 (40°C). Unpeeled cassava + leaves: 7.18, 3.78 (25°C), 7.18, 4.36 (30°C), 7.18, 3.49 (37°C), 7.18, 3.51 (40°C)

The effect of time on cassava softening was evaluated at 24h, 48h, 72 h and 5 days of natural fermentation at 37°C. Table 1.3 reveals that both even and uneven softening of cassava occurred over 72 h for both peeled and unpeeled cylindrical cassava pieces and shredded cassava, and there was no softening at 24 and 48 h.

Table 1.3: Effect of time on cassava softening at different stages of natural fermentations at 37°C

Fermentation time	Peeled cut cassava	Peeled shredded cassava	Unpeeled cut cassava	Unpeeled shredded cassava
24 h	NS	NS	NS	NS
	NS	S	NS	NS
48 h	NS	NS	NS	NS
	NS	NS	NS	NS
72 h	NS	NS	*S	*S
	S	NS	NS	NS
5 days	NS	NS	*S	*S
	S	NS	NS	NS

Natural fermentations (two replicates) S: softening NS: no softening *: partial softening

Table 1.4: Effect of time and temperature on cassava softening evaluated during natural fermentation

Fermentation time	Peeled cassava 25°C	Peeled cassava 30°C	Peeled cassava 37°C	Peeled cassava 40°C
24 h	NS (3/6-)	NS (6/6-)	NS (6/6-)	NS (6/6-)
48 h	NS (6/6-)	NS (6/6-)	NS (6/6-)	NS (6/6-)
72 h	*S (3/6+)	*S (4/6+)	*S (1/6+)	*S (3/6+)
	NS (3/6-)	NS (2/6-)	NS (5/6-)	NS (3/6-)
5 days	*S (3/6+)	*S (4/6+)	*S (2/6+)	*S (3/6+)
	NS (3/6-)	NS (2/6-)	NS (4/6-)	NS (3/6-)

Fermentations: six replicates S: softening NS: no softening *: partial softening

Table 1.5: Effect of time and temperature on softening of peeled and unpeeled cassava evaluated at the end of fermentation

Temperature	Peeled cassava	Unpeeled cassava
25°C	NS (3/4-) S (1/4+)	NS (4/4-) S (0/4+)
30°C	NS (2/4-) S (2/4+)	NS (4/4-) S (0/4+)
37°C	NS (2/4-) S (2/4+)	NS (3/4-) S (1/4+)
40°C	NS (2/4-) S (2/4+)	NS (3/4-) S (1/4+)

Fermentations: four replicates **S: softening** **NS: no softening**

Table 1.6: Effect on softening of 4-day fermentation extension with and without backslopping

Temperature	Peeled cassava	Unpeeled cassava	Peeled cassava	Unpeeled cassava
	Extended		**Extended and backslopped**	
25°C	NS	NS	NS	NS
30°C	NS	NS	NS	NS
37°C	NS	NS	NS	NS
40°C	NS	NS	NS	NS

Fermentations: two replicates **NS: no softening** **S: softening**

Table 1.7: Effect of leaves and skin on cassava softening

Fermentation temperature	Peeled cassava	Peeled cassava + leaves	Unpeeled cassava (skin)	Unpeeled Cassava (skin) + leaves
25°C	S	NS	S	NS
30°C	S	NS	NS	NS
37°C	S	NS	S	NS
40°C	S	NS	S	NS

Fermentations: two replicated **S: softening** **NS: no softening**

Scenario one

Tables 1.10, 1.11, 1.12, 1.13 and Figure 1.7 generally show a microbiological succession with rapid microbial growth from 3 h onwards, and Table 2.0 demonstrated modest numbers of LAB ($10^2 - 10^3$ CFU/ml) and yeasts (10^5 CFU/ml), and low numbers of *Enterobacteriaceae* (10^1 CFU/ml) that were recovered at initial fermentation, and were steadily increasing up to 6 h. From 6 h to 48 h, LAB exponentially multiplied, becoming dominant, but their population decreased at the end of fermentation. Slightly different trends were observed with the yeasts and *Enterobacteriaceae*. *Bacillus* species were not detected during the first 6 h, but gradually increased and had reached significant numbers (10^6 CFU/ml) by 24 h of fermentation. The final stage of fermentation was characterised by decreased numbers (10^4 and 10^3 CFU/ml respectively) of LAB and yeasts and by no recovery of *Bacillus* species and *Enterobacteriaceae,* which might have been inhibited. This observation was supported by the research of Amoa-Awua and Jakobsen, 1995; Amoa-Awua *et al*., 1997); Mante and Amoa-Awua, (2003); Obelie and Amoa-Awua, (2003); *Bacillus* species were isolated during their studies and were confirmed to be one of the dominant species at the early stage (0 – 24 h) of cassava fermentation, but at the end of the fermentation process their numbers decreased to below detection. The 24 h fermentation exhibited a microbiological development where the primary microflora consisted of predominantly LAB, *Enterobacteriaceae* and yeasts, while *Bacillus* species formed the second a secondary microflora. Visual observations based on colony phenotype suggested diversity amongst LAB. A positive correlation between a rapid pH fall (Tables 1.10, 1.11, Figures 1.1, 1.2, 1.3) and rapid growth rate of LAB at the early course of fermentation was observed, and this proved that these organisms contributed to

the production of acids during the fermentation. This agreed with the findings of Amoa-Awua and Jakobsen, (1995); Amoa-Awua *et al.*, (1997). From the point of view of reproducibility, the results were generally found to be inconclusive, as Figure 1.7 indicates that the *Enterobacteriaceae* formed the primary dominant populations, while Gram positive anaerobes and yeasts were the secondary microflora over 24 h.

Scenario three

The results of the study on the effect of time following extension of fermentation (4 days) without and with backslopping are shown in Table 1.13. Substantial populations of *Bacillus* species ($10^5 - 10^8$ CFU/ml), *Clostridium* species (10^6 - 10^9 CFU/ml) and yeasts ($10^5 - 10^6$ CFU/ml) were recovered from extended and backslopped fermentation compared to unextended fermentation of 5 days. The latter was characterised by very low numbers of *Bacillus*, *Clostridium* species and yeasts ($10^1 - 10^2$ CFU/ml) in unpeeled cassava and no detection in peeled cassava. Although *Bacillus*, *Clostridium* species have been reported to be associated with cassava softening (Amoa-Awua *et al.*, 1995), their high numbers at the end of extended and backslopped fermentation did not improve, enhance or initiate desirable fermentation characteristics, including softening of cassava. This study demonstrated that the duration of natural fermentation was crucial and influenced by type, composition, development and survival of the fermenting microorganisms. For these reasons, cassava should be fermented for at least 3 to 5 days to guarantee microbiological safety of the end product.

Table 1.10: Effect of time on composition of microflora: total count analysis

Fermentation time	MRS LAB	PEMBA *Bacillus* spp.	ECC *E.coli*	SAB Yeasts
		PEELED CASSAVA		
Initial	5.0×10^2	ND	5.0×10^1	ND
3 h	5.0×10^3	ND	1.0×10^2	ND
6 h	1.5×10^6	ND	2.0×10^2	1.0×10^4
9 h	1.1×10^7	ND	1.6×10^4	1.0×10^4
12 h	2.0×10^7	4.0×10^4	1.5×10^6	1.1×10^6
15 h	8.0×10^7	2.4×10^6	2.5×10^6	5.0×10^6
18 h	1.2×10^8	6.5×10^6	2.0×10^6	2.1×10^6
21 h	1.0×10^9	3.8×10^6	2.0×10^5	1.0×10^6
24 h	6.0×10^9	2.4×10^6	1.1×10^6	1.0×10^5
Day 5	4.0×10^4	ND	ND	1.0×10^3
		UNPEELED CASSAVA		
Initial	1.0×10^3	ND	ND	1.0×10^5
3 h	5.0×10^3	ND	6.0×10^1	ND
6 h	5.0×10^3	ND	1.5×10^5	3.1×10^5
9 h	1.3×10^5	ND	3.0×10^4	1.7×10^6
12 h	5.0×10^6	1.2×10^2	1.5×10^5	6.5×10^6
15 h	2.0×10^7	8.0×10^3	3.3×10^6	2.7×10^6
18 h	2.5×10^8	7.0×10^4	2.3×10^6	8.0×10^6
21 h	5.0×10^8	4.4×10^5	4.3×10^6	5.0×10^5
24 h	9.0×10^8	3.4×10^4	3.0×10^6	4.0×10^5
Day 5	4.0×10^4	ND	ND	1.0×10^2

Microbial enumeration: average of three cassava fermentations repeats **ND: not detected**

Table 1.11: Effect of time on composition of microflora of fermenting peeled cassava

Time	3 h	6 h	9 h	12 h	15 h	18 h	21 h	24 h	Day 2	Day 5
PEMBA *Bacillus* spp.	ND	ND	ND	ND	6.5×10^6	4.2×10^6	2.4×10^6	2.5×10^6	2.6×10^4	ND
SAB Yeasts	ND	ND	1.0×10^4	1.0×10^4	1.1×10^6	1.2×10^6	2.1×10^6	1.0×10^6	2.6×10^6	1.0×10^5
ECC *E.coli*	5.0×10^1	1.0×10^2	2.0×10^2	1.6×10^4	1.5×10^5	2.5×10^6	2.0×10^6	1.1×10^6	4.0×10^6	ND
CCY Gram – anaerobes	ND	ND	ND	ND	ND	ND	ND	1.0×10^7	ND	ND
SFP *Clostridium* spp.	ND	ND	ND	ND	ND	ND	ND	2.5×10^4	ND	ND
HBA *Clostridium* spp.	ND	ND	ND	ND	ND	ND	ND	3.0×10^5	1.2×10^1	ND
VRBGA *Enterobacteriaceae*	ND	ND	ND	1.0×10^4	1.3×10^4	3.7×10^4	1.0×10^5	5.0×10^4	1.0×10^5	ND

Microbial enumeration: average of three cassava fermentations repeats **ND: not detected**

Table 1.12: Effect of time on composition of microflora of fermenting unpeeled cassava

Time	3 h	6 h	9 h	12 h	15 h	18 h	21 h	24 h	Day 2	Day 5
PEMBA *Bacillus* spp.	ND	ND	ND	ND	7.0×10^4	1.5×10^6	4.4×10^5	5.0×10^6	3.4×10^4	ND
SAB Yeasts	ND	ND	1.0×10^4	1.0×10^4	1.1×10^6	1.2×10^6	2.1×10^6	1.0×10^6	2.6×10^6	1.0×10^5
ECC *E.coli*	1.0×10^5	ND	1.0×10^4	1.0×10^4	3.1×10^5	1.0×10^6	1.7×10^6	6.5×10^6	2.7×10^6	ND
CCY Gram – anaerobes	ND	ND	ND	ND	ND	ND	ND	7.8×10^8	ND	ND
SFP *Clostridium* spp.	ND	ND	ND	ND	ND	ND	ND	5.0×10^6	ND	ND
HBA *Clostridium* spp.	ND	ND	ND	ND	ND	ND	ND	ND	8.0×10^5	2.0×10^1
VRBGA *Enterobacteriaceae*	ND	ND	ND	6.0×10^3	4.0×10^4	6.0×10^4	6.0×10^5	2.7×10^4	3.8×10^5	ND

Microbial enumeration: average of three cassava fermentations repeats **ND: not detected**

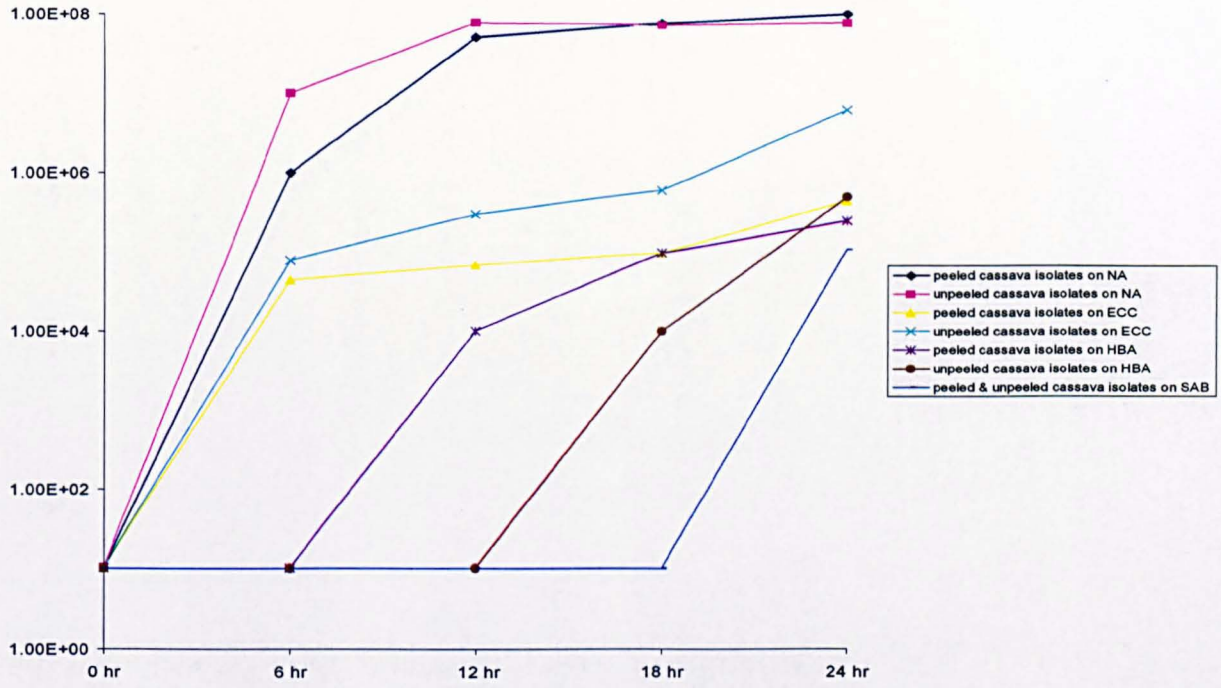

Figure 1.7: Changes in microbial population during the first 24 h of fermentation at 37°C *Enterobacteriaceae* on ECC, Gram positive anaerobes on HBA, yeasts and fungi on SAB, and general aerobic microflora on NA

Table 1.13: Effect on composition of microflora of extending fermentation to 9 days with and without backslopping

Fermentation Time	NA Total viable count	MEA Yeasts	RBC Yeasts	MRS LAB	RCA *Clostri* spp.	SFP *Clostri* spp.	PEMBA *Bacillus* spp.	HBA *Clostridium* spp.
				Peeled cassava fermentation				
Normal (5(days)	7.0×10^1	2.0×10^2	1.0×10^2	5.2×10^4	ND	1.0×10^1	ND	ND
Extended with backslopping (9 days)	7.0×10^3	4.0×10^4	1.0×10^4	1.8×10^6	5.0×10^2	1.0×10^3	1.0×10^3	4.0×10^3
				Peeled cassava fermentation				
Normal (5(days)	7.0×10^1	2.0×10^2	1.0×10^2	5.2×10^4	ND	ND	ND	ND
Extended without backslopping (9 days)	1.0×10^3	3.9×10^2	1.4×10^2	8.6×10^4	8.0×10^1	3.5×10^1	1.0×10^1	0.5×10^1

Microbial enumeration: average of three replicated experiments **ND: not detected**

Table 1.14: Effects of time on microbial growth during 5-day fermentations at a range of temperatures

Time	Media	Peeled cass:25°C	cass:30°C	cass:37°C	cass:40°C	Unpeeled cass:25°C	Unpeeled cass:30°C	Unpeeled cass:37°C	Unpeeled cass:40°C
	PEMBA								
	Bacillus spp.								
24 h		8.0×10^5	2.6×10^6	6.0×10^5	1.2×10^5	2.1×10^4	1.3×10^6	1.0×10^6	3.0×10^4
Day 2		$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$	ND	$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$
Day 5		1.0×10^5	1.1×10^5	ND	ND	1.4×10^5	1.7×10^5	ND	ND
	HBA								
	Clostridium spp.								
24 h		9.0×10^5	3.0×10^4	4.0×10^5	3.8×10^5	3.2×10^5	1.3×10^5	6.2×10^5	1.7×10^5
Day 2		$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$	$>1.0 \times 10^6$
Day 5		1.1×10^5	1.3×10^5	2.0×10^5	ND	2.5×10^5	4.0×10^5	8.0×10^5	ND
	VRBGA								
	Enterobacteriaceae								
24 h		1.2×10^6	1.1×10^6	1.9×10^5	1.0×10^5	1.8×10^6	2.0×10^6	7.0×10^5	1.8×10^5
Day 5		1.4×10^5	1.3×10^5	ND	ND	8.0×10^4	1.6×10^5	ND	ND
24 h	SAB	ND	ND	ND	ND	ND	ND	ND	ND
	Yeasts								
Day 2		3.0×10^6	1.0×10^6	1.7×10^6	ND	2.5×10^6	6.0×10^6	6.5×10^5	5.0×10^3
Day 5		5.0×10^5	ND	2.4×10^5	ND	5.8×10^5	1.7×10^5	3.7×10^4	1.0×10^5
24 h	ECC *E.coli*	1.4×10^6	4.0×10^6	4.0×10^6	ND	6.2×10^6	6.0×10^6	1.1×10^6	0.9×10^1
Day 2		4.0×10^5	5.1×10^5	6.0×10^5	ND	7.7×10^5	5.0×10^5	3.9×10^5	ND
Day 5		ND	5.0×10^4	ND	ND	ND	ND	ND	ND

Microbial enumeration: average of three cassava fermentations repeats **ND: detected**

Media	25°C Peeled cassava	25°C Unpeeled cassava	30°C Peeled cassava	30°C Unpeeled cassava	37°C Peeled cassava	37°C Unpeeled cassava	40°C Peeled cassava	40°C Unpeeled cassava
MRS	light	light	moderate	moderate	heavy	heavy	moderate	moderate
LAB	light	light	moderate	moderate	heavy	heavy	moderate	moderate
	light	moderate	moderate	moderate	heavy	heavy	light	light
PEMBA	ND	ND	light	light	ND	ND	ND	ND
Bacillus spp	moderate	moderate	light	light	ND	ND	ND	ND
	moderate	moderate	ND	ND	ND	ND	ND	ND
VRBGA	heavy	heavy	moderate	moderate	ND	ND	ND	ND
Enterobacteriaceae	moderate	moderate	moderate	moderate	ND	ND	ND	ND
	ND	ND	light	ND	ND	ND	ND	ND
HBA	ND	ND	light	light	ND	ND	ND	ND
Clostridium spp.	moderate	moderate	moderate	moderate	light	light	light	light
	heavy	ND	ND	light	ND	ND	ND	ND
RCA	ND	ND	ND	ND	light	light	light	light
Clostridium spp.	ND	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	light	light	ND	ND
NA	limited	moderate	moderate	moderate	moderate	heavy	moderate	moderate
Total viable count	light	moderate	moderate	moderate	moderate	heavy	moderate	moderate
	heavy	light	moderate	light	light	light	moderate	moderate
SAB	moderate	moderate	moderate	moderate	light	light	ND	ND
Yeasts	moderate	moderate	moderate	moderate	light	moderate	light	light
	moderate	moderate	moderate	*heavy	moderate	*heavy	light	light
ECC	light	ND	ND	light	ND	ND	ND	ND
E.coli	ND	light	light	light	limited	limited	ND	ND
	ND	ND	ND	ND	ND		ND	ND

Microbial growth assessment of three cassava fermentations replicates **ND: not detected**

The quantitative effects on the microbial population of inclusion of cassava leaves and skin were assessed by enumeration on a range of media. Table 1.16 demonstrates that initial numbers of LAB, yeasts, *Enterobacteriaceae*, *Bacillus* and *Clostridium* species ranged from 10^3 to 10^5 CFU/ml on peeled cassava (skinless) and unpeeled cassava (skin on), and from 10^1 to 10^5 CFU/ml on the skin on its own. At the end of fermentation at 37°C for 5 days, unpeeled cassava yielded numbers of LAB, *Bacillus* and *Clostridium* species of 10^2 CFU/ml, yeasts 10^3 CFU/ml, with a more numerous population of *Enterobacteriaceae* of 10^6 CFU/ml. Peeled cassava at the end of fermentation produced *Bacillus* species populations of 10^4 CFU/ml; yeasts numbers of 10^5 CFU/ml and 10^3 CFU/ml *Enterobacteriaceae* populations, while LAB and *Clostridium* species were not detected. The skin alone at the end of fermentation produced relatively high numbers of *Clostridium* species (10^6 CFU/ml), a yeast population of 10^4 CFU/ml and *Enterobacteriaceae* numbers of 10^4 CFU/ml and no detection of LAB or *Bacillus* species.

Table 1.16: Effect of skin on microbial numbers during fermentation at 37°C

Medium	Substrate	Initial time	Final time
MRS (LAB)	Unpeeled cassava	2.0×10^4	1.0×10^2
	Peeled cassava	2.5×10^3	ND
	Skin	7.5×10^1	ND
PEMBA (*Bacillus* spp.)	Unpeeled cassava	1.1×10^4	3.0×10^2
	Peeled cassava	1.0×10^3	1.0×10^4
	Skin	1.0×10^5	ND
HBA (*Clostridium* spp.)	Unpeeled cassava	1.0×10^5	3.0×10^2
	Peeled cassava	1.0×10^3	ND
	Skin	1.0×10^4	1.1×0^6
VRBGA (*Enterobacteriaceae*)	Unpeeled cassava	2.5×10^4	3.0×10^6
	Peeled cassava	1.5×10^3	2.3×10^3
	Skin	1.5×10^3	6.4×10^4
SAB (Yeasts)	Unpeeled cassava	8.0×10^4	1.0×10^3
	Peeled cassava	8.0×10^4	1.0×10^5
	Skin	2.2×10^3	6.0×10^4
NA (Total viable count)	Unpeeled cassava	1.2×10^5	ND
	Peeled cassava	2.0×10^6	ND
	Skin	1.7×10^5	ND

Microbial enumeration (average) of three cassava fermentations replicates

The effects of inclusion of cassava leaves on the microbiological profile during fermentation of unpeeled and peeled cassava are shown in Tables 1.17 and 1.18 respectively. Tables 1.17 and 1.18 indicate that *Bacillus* species were not detectable at initial fermentation in all samples.

Table 1.17 shows that the numbers of yeasts recovered were between 10^5 & 10^6 CFU/ml) from unpeeled cassava without leaves at 25°C and 30°C, and when leaves were included at 30°C. Unpeeled cassava without leaves was characterised after fermentation by high numbers of LAB and yeasts ($\geq 10^6$ CFU/ml) at 25°C, 30°C and 37°C. Inclusion of leaves after fermentation, was associated with numbers of 10^5 CFU/ml of LAB and yeasts at 25°C and 37°C and somewhat higher numbers in the range of 10^6 - 10^7 CFU/ml at 30°C. Recovery of yeasts (10^4 CFU/ml) and *Bacillus* species (10^6 CFU/ml) were recorded after fermentation from leaves alone in the unpeeled cassava experiment, while LAB were not detected in leaves alone before or after fermentation. There was no isolation of *Bacillus* species from unpeeled cassava without leaves at all temperatures, but were recovered (10^6 CFU/ml) when leaves were included, and in leaves alone at 30°Cand 37°C.

Table 1.18 indicates that recoveries followed the same patterns but were generally lower in peeled cassava, with and without leaves.

Table 1.17: Effect on microbial population of adding cassava leaves to unpeeled cassava fermentation at different temperatures

Media	Unpeeled cassava 25°C	Unpeeled cassava 30°C	Unpeeled cassava 37°C	Unpeeled cassava +leaves 25°C	Unpeeled cassava +leaves 30°C	Unpeeled cassava +leaves 37°C	Cassava leaves 37° C
MRS LAB							
Initial	ND	ND	ND	ND	ND	ND	ND
Final	1.0×10^7	6.0×10^6	1.1×10^6	1.0×10^5	3.7×10^7	8.0×10^5	ND
SAB Yeasts							
Initial	1.9×10^6	6.0×10^5	ND	ND	3.0×10^6	ND	ND
Final	1.0×10^6	1.0×10^6	1.5×10^6	ND	3.0×10^6	3.0×10^5	6.0×10^4
PEMBA *Bacillus* spp.							
Initial	ND	ND	ND	ND	ND	ND	ND
Final	ND	ND	ND	ND	2.0×10^6	2.0×10^6	3.0×10^6

Microbial enumeration (average): three cassava fermentations replicates **ND: not detected**

Table 1.18: Effect on microbial population of adding cassava leaves to peeled cassava fermentation at different temperatures

Media	Peeled cassava 25°C	Peeled cassava 30°C	Peeled cassava 37°C	Peeled cassava +leaves 25°C	Peeled cassava +leaves 30°C	Peeled cassava +leaves 37°C	Cassava leaves 37° C
MRS LAB							
Initial	ND	ND	ND	ND	ND	ND	ND
Final	ND	5.0×10^5	2.0×10^4	2.0×10^7	7.0×10^5	9.0×10^4	ND
SAB Yeasts							
Initial	ND	ND	ND	ND	ND	ND	ND
Final	8.0×10^4	ND	ND	2.4×10^6	ND	3.0×10^5	ND
PEMBA *Bacillus* spp.							
Initial	ND	ND	ND	ND	ND	ND	ND
Final	ND	ND	1.0×10^6	3.0×10^7	2.0×10^7	3.0×10^7	ND

Microbial enumeration (average): three cassava fermentations replicates ND: not detected

For the effect of substrate composition on microbial growth, the question was whether supplementing peeled cassava with leaves and/or skin, was a positive approach to improve or optimise the fermentation. The findings displayed inconsistent and inconclusive results for growth and survival of fermenting organisms. Table 1.16 indicates that significant numbers between 10^5 & 10^3 CFU/ml of non-lactic acid microorganisms were already present on the skin at the initial fermentation, and high populations (>10^6 CFU/ml) were recovered at the end of fermentation. These results revealed that the presence of the skin could be one of the significant factors that contributed to microbial load at initial time, and consequently at the end of fermentation of unpeeled cassava. Tables 1.17, 1.18 show comparable viable counts and pattern of fermentation, between the fermentations of cassava without, and with inclusion of leaves. The final fermentations of unpeeled and peeled cassava with added leaves resulted in higher microbial numbers, particularly presumptive *Bacillus* species (10^6 in unpeeled and 10^7 CFU/ml in peeled cassava) than cassava fermented without the inclusion of leaves. Therefore, inclusion of leaves could have been an undesirable influence that contributed to high numbers of *Bacillus* species at the end of fermentation compared to low numbers recovered from fermentation of cassava without leaves. Adding leaves to cassava might also have an adverse impact on the population of LAB. These results suggest that supplementing cassava with leaves generally enhanced growth of microorganisms during fermentation, but there was no

guarantee that this would lead to development of desirable attributes, such as softening of cassava.

Various samples of fermented cassava were microbiologically analysed to detect *Bacillus* and *Clostridium* species and Table 1.19 shows that presumptive *Bacillus* species were isolated from all fermented softened cassava samples with the exception of fermented unsoftened cassava. Therefore, these results demonstrated that presumptive *Bacillus* species could be involved in softening of cassava during fermentation. LAB were generally recovered from all type of cassava substrates regardless whether cassava was softened or unsoftened and this suggested a non- involvement in cassava softening.

Table 1.19: Detection and growth evaluation of *Bacillus* species and LAB

Sample	MRS LAB	PEMBA *Bacillus* spp.	NA Total viable count
Fermented soften cassava	Growth	growth	growth
Heated fermented softened cassava	Growth	growth	growth
Fermented unsoftened cassava	Growth	ND	growth
Softened cassava from backslopped extended fermentation	growth	growth	growth
MRS broth cultures of LAB isolated from fermented softened cassava (as control)	growth	ND	ND

Growth evaluation (average): three replicated experiments **ND: not detected**

Tables 1.20 & 1.21 demonstrate a detection of *Clostridium* species suspects in all cassava samples at initial fermentation. However, after five days they were recovered in moderate numbers between 10^4 & 10^2 CFU/ml) in peeled cassava, and 10^5 CFU/ml in unpeeled cassava, and numbers in the range of 10^4 to 10^5 CFU/ml in heated cultures. Use of standard anaerobic jars and unreduced media for the initial isolations and enumerations resulted in low detection of presumptive *Clostridium* species by classical cultural methods. The recovery of numbers between 10^1 & 10^5 CFU/ml subsequently improved when pre-reduced media and anaerobic workstation were used but these isolates were not expected to be sufficient to cause cassava softening at such population levels (Table 1.21). In spite of the results however, there was circumstantial evidence to support metabolic activity, particularly since a strong butyric odour associated with fermented softened cassava was

observed, as reported by Keleke *et al.*, (1996) and Ogbo (2003). It was observed that a greater degree of cassava softening and intensification of the characteristic odour were directly proportional to increasing numbers of isolated *Clostridium* spp.

Table 1.20: Detection and growth evaluation of *Clostridium* species in double strength RCM medium

Sample	RCA	HBA	SFP
Fermented peeled cassava	crowded	crowded	crowded
Fermented unpeeled cassava	crowded	crowded	crowded
Unfermented peeled and unpeeled cassava	no growth	no growth	no growth

Growth evaluation (average): three replicated experiments RCA, SFP, HBA: media for detection of presumptive Clostridium spp.

Table 1.21: Detection of *Clostridium* spp in double strength RCM medium

Substrate	Fermentation Time	RCA	SFP	HBA	NA
Peeled cassava:	initial	ND	ND	1.0×10^3	ND
	final	8.0×10^4	5.0×10^4	2.0×10^2	ND
Unpeeled cassava:	initial	ND	1.0×10^1	1.5×10^2	9.0×10^2
	final	1.6×10^5	1.4×10^5	1.6×10^5	ND
Heated Omeliansky cultures	initial	ND	0.8×10^1	7.6×10^3	7.8×10^3
	final	2.8×10^4	8.6×10^5	4.4×10^5	ND

Growth evaluation (average): three replicated experiments
RCA, SFP, HBA: media for detection of presumptive Clostridium spp.
NA: for total viable count (for comparison)

The results (not shown) showed only one positive blackening reaction of the DRCM cultures by MPN cultural method. Profuse growth in liquid medium and heavy growth on solid media respectively before and after DRCM cultures were heated were displayed, suggesting circumstantial evidence of *Clostridium* species present in the fermented cassava. Detection using Omeliansky medium showed no appearance of holes in or disintegration of the filter paper strips to indicate cellulose digestive activity, suggesting

absence of *Clostridium* species. Nevertheless, double strength RCM broth for recovery of *Clostridium* species exhibited profuse growth and growth occurred after streaking/plating on solid media, which strongly suggested presence of significant numbers of presumptive *Clostridium* species population between 10^2 & 10^5 CFU/ml were recovered from heated and non-heated DRCM cultures, filter paper strips and from double strength RCM (Tables 1.20, 1.21). The aerobic growth, colony and cell morphology examination revealed that the presumptive *Clostridium* isolates in fact fitted the typical colony and cell morphology of *Bacillus* species, with the exception of the double strength RCM cultures and unfermented unpeeled cassava isolates. These isolates displayed inability to grow aerobically, vigorous spreading ability and distinctive colony phenotype characteristics, compared to other isolates.

Although it is postulated that *Clostridium* species are implicated in cassava softening (Amoa-Awua and Jakobsen, 1995, Amoa-Awua *et al.*, 1997; Keleke *et al.*, 1996; Oyewole, 2000), they were not detected in this research by cultural methodology or using FISH techniques. This result generally agreed with most isolation findings using classical cultural methodology carried out in this study.

FISH has been successfully used in studies of human gut microflora, but has not previously been used with traditional fermentation processes.

Detection of enzyme activity of the fermented cassava

Cassava samples were tested to detect endo-polygalacturonase activity by a viscometry method and spectrophotometrically.

Table 1.22 shows detection of endo-polygalacturonase activity by viscometry in fermented cassava samples. The pectin solution incubated with crude enzymes extracted from initial samples of unfermented cassava had a high viscosity (25.6 – 26.8 cP), while after incubation with the crude enzyme extracts of fermented cassava showed a reduction of more than 50% of pectin solution, i.e. the reading decreased to a value of 10.0 – 12.4 cP, indicating a substantially lower viscosity compared to the initial value, consequential to the activity of endo-polygalacturonase. A similar result was recorded for 9-day fermentations with backslopped peeled and unpeeled cassava.

Table 1.22: Detection of endo-polygalacturonase by viscosity (cP)

Substrate	Speed	Spindle	Reading (cP) Maximum range
Unfermented unpeeled cassava at initial time	100	2	25.6 – 26.8
Unfermented peeled cassava at initial time	100	2	25.6 – 26.0
Fermented unpeeled cassava over 5 days	100	2	10.1 – 10.9
Fermented peeled cassava over 5 days	100	2	10.0 – 10.7
Backslopped fermented unpeeled cassava over 5 days	100	2	10.8 – 12.2
Backslopped fermented peeled cassava over 5 days	100	2	10.8 – 12.4

cP: centipoise (viscosity measurement unit): average readings of three replicated experiments

Table 1.23 demonstrates that exo-polygalacturonase activities were not detected in all samples at initial time, but were detected in the range of 4.5 – 19.1 µmol (Figure 1.8) in fermented cassava and backslopped cassava at the end of fermentation.

The DNS method is one of the methods used to detect polygalacturonase activity and is widely used for the assay of galacturonic acid residues liberated during hydrolysis of pectin. Endo-polygalacturonase is responsible for breakdown of the α-glycosidic bonds of pectin chains, while exo-polygalacturonse hydrolyses the terminal residues of the chains, causing release of oligogalacturonic acid, which was measured spectrophotometrically at 575 nm (Wang *et al.*, 1997). Based on OD results (Table 1.23), a standard curve was used to estimate the readings, Figure 1.8 shows that galacturonic acid of approximately between 4.5 - 19.1 µmol were detected among all cassava samples, and peeled and unpeeled fermented cassava displayed high exopolygalacturonase activities (Table 1.23). Absorbance readings of peeled, unpeeled, fermented and backslopped peeled and unpeeled backslopped cassava indicated that there was no detection of pectinase, cellulase and polygalacturonase enzymes in unfermented cassava at initial fermentation, and only pectinase were detected in the fermented soften cassava after 5 days (Table 1.14). The results suggested that pectinase enzymes could be responsible for the cassava softening in this study. However, other studies (Amoa-Awua and Jakobsen, 1995, Amoa-Awua *et al.*, 1997; Mathew and Moorthy, 1999) revealed that the cassava softening was caused by both pectinolytic and cellulolytic actions.

Table 1.23: Detection of exo-polygalacturonse (PGA) activity by DNS assay

Sample	OD Reading	OD Reading
PGA standard (0.8 ml)	2.13	2.10
PGA standard (1.6 ml)	2.54	2.51
Peeled cassava	0.106 (0 d)	0.098 (5 d)
Unpeeled cassava	0.104 (0 d)	0.091 (5 d)
Backslopped fermented peeled cassava	0.130 (5 d)	0.046 (9d)
Backslopped fermented unpeeled cassava	0.133 (5 d)	0.063 (9 d)
Blank	0.00	0.00

PGA activity (average) of three replicated experiments d: day

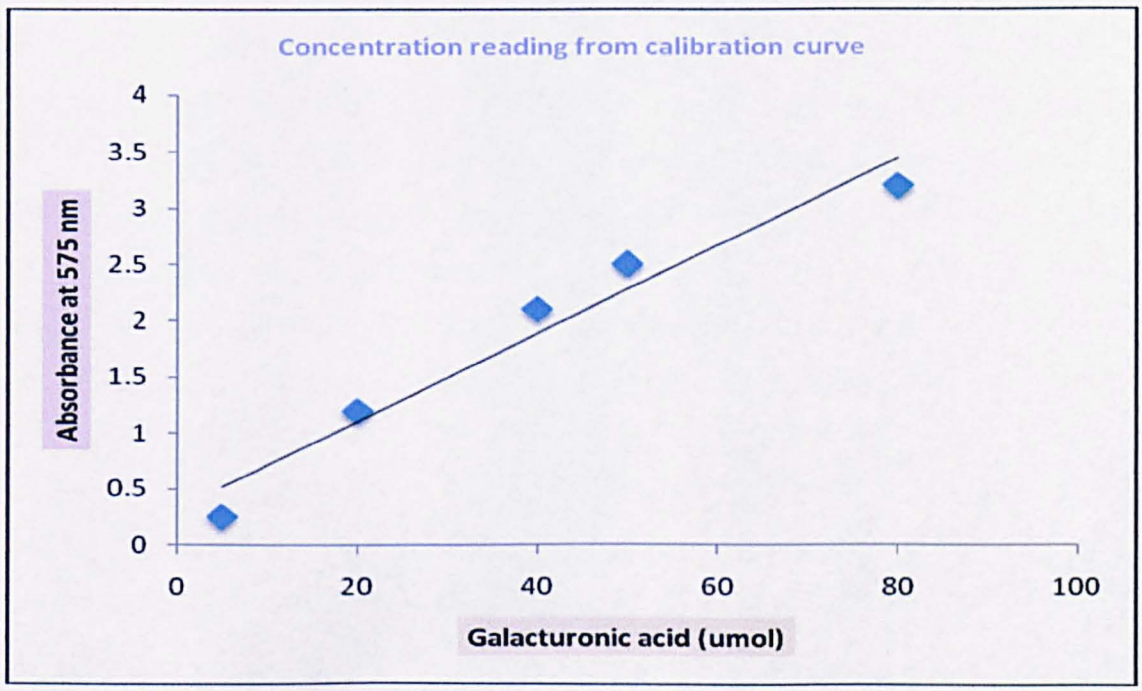

Figure 1.8: Concentration (umol) of released polygalacturonic acid by exo-polygalacturonase contained in fermented cassava.

Peeled and unpeeled cassava samples were tested to detect pectinase, cellulase and polygalacturonase activities by the top agar method. Table 1.24 demonstrates that there were no signs of enzymatic activity initially and only pectinase activity was detected in all fermented cassava samples after five days.

Table 1.24: Detection of pectinase, cellulase, and polygalacturonase activities by top agar method.

Sample	Pectinase		Cellulase		Polygalacturonase	
	initial	final time	initial	final time	initial	final time
Peeled cassava	ND	positive	ND	ND	ND	ND
Standard solution	ND	positive	ND	ND	ND	ND
Unpeeled cassava	ND	positive	ND	ND	ND	ND
Standard solution	ND	positive	ND	ND	ND	ND

Enzymatic activity (average): three replicated experiments ND: not detected

The principles of fermentation process involve the enzymatic activities of fermenting organisms such as LAB and tissue modification organisms, and their metabolic activities lead to the production of various enzymes such as β- glucosidase, and - α glucuronidase. The β-glucosidase enzymes play an important role in the hydrolysis of linamarin, a toxic cyanogenic glucosides, therefore their presence is crucial for the detoxification of cassava. While α -glucuronidase could be of the cassava softening transformation. Detection of changes in amounts of α-glucuronidase involved in cellulose degradation that in some cases results in tissue softening, and β-glucosidase responsible for the hydrolysis of cyanogenic glucosides were determined during fermentation by API Zym tests. Table 1.25 demonstrates that α-glucuronidase was present in all unfermented cassava samples at varying levels (2 – 4 colour development score), and peeled cassava scored the highest (4) and unpeeled the lowest (2). The α-glucuronidase levels increased to 5 in all fermented cassava samples after five days. The highest levels of β- glucosidase (4 – 5) were detected in all unfermented unpeeled cassava, and there was no detection in peeled cassava at initial time. After five days, the initial levels of β- glucosidase increased to 5 for all fermented cassava. This meant that the level of α-glucuronidase activity were already high at initial fermentation and a slight increase particularly in peeled cassava and substantial increase in unpeeled cassava at the end of fermentation. This suggested that they were naturally

present in cassava tissue, and further increase may have been contributed by microorganisms. In contrast, high levels of activity of β- glucosidase were detected initially in unpeeled cassava, meaning that the cassava skin naturally contained β-glucosidase. At the end of fermentation of peeled cassava, the detection of high activity was associated with microbial activities (Table 1.15). It was concluded that α-glucuronidase and β- glucosidase were partly endogenous enzymes of cassava flesh and skin respectively, and partly derived from fermenting microbial activities.

Table 1.25: Detection of cellulolytic and linamarase activities by API Zym tests

Fermentation substrate	Time	α-glucuronidase colour scoring	β- glucosidase colour scoring
Peeled cassava	Initial	4	ND
	5 days	5	5
Unpeeled cassava	Initial	3	5
	5 days	5	5
Peeled cassava cut into cylindrical pieces	Initial	4	ND
	5 days	5	5
Peeled shredded cassava	Initial	3	ND
	5 days	5	5
Unpeeled cassava cut into cylindrical pieces	Initial	2	4
	5 days	5	5
Unpeeled shredded cassava	Initial	2	5
	5 days	5	5

Enzymatic activity (average) of two cassava fermentations replicates

1.4 Conclusion

Natural cassava fermentation is associated with a complex microflora; as result the process is uncontrollable and unpredictable and as a consequence suffers from variations of all kinds. Numerous cassava fermentations gave a picture of ongoing inconsistencies of pH change, occurrence of cassava softening, microbiological status and microflora composition, growth and survival in all conditions. The duration of fermentation had a positive effect on pH value, softening and microbiological composition and development. The initial time to 72 h was the most crucial stage of fermentation where a rapid drop in pH value, rapid microbial growth and dominance of key microorganisms such as LAB occurred, and absence or detection of low numbers of undesirable organisms such as *Enterobacteriaceae* was observed. Additionally, cassava softening most of times occurs after 72 h in natural fermentation of cassava. Extending the fermentation with or without backslopping for further 4 days caused a further drop in pH value but did not initiate, enhance or improve cassava softening, and had a negative effect on the desirable microbial population such as LAB, and a positive effect on presumptive *Bacillus* species as high numbers of these could be recovered after nine days fermentation.

The temperature at which cassava was fermented influenced the pH value, as temperatures below 37°C were associated with a higher ultimate pH, and fermentations at 37 - 40°C were characterised by lower ultimate pH values. The temperature effect on softening was inconclusive, as softening was produced randomly and inconsistently at all selected temperatures of fermentation. Microbiological composition, growth and survival were affected by temperature and 37°C was generally the best temperature, most likely to favour rapid acidification due to rapid growth of LAB. The skin of cassava had no effect on pH value or on softening, while inclusion of leaves with cassava caused a minor increase of the initial pH value but had no influence on softening of cassava. The skin had an impact on microbiological composition, growth and survival, since significant numbers of non-lactic acid bacteria were recovered at initial time. Consequently, leaving the skin on cassava pieces could be one of the factors which contributed to a significant microbial load at the start and end of fermentation of unpeeled cassava. The inclusion of leaves could possibly have an undesirable influence on of LAB, as they were recovered in only low numbers, as opposed to high numbers of presumptive *Bacillus* species. Looking at the above pH, softening and microbiological profiles, it is recommended that cassava should be fermented at least for 72 h at 37°C to achieve adequate acidification to pH values under 4.2, satisfactory softening (as far as possible), rapid growth of LAB and low or no recovery of undesirable microorganisms such as *Enterobacteriaceae*. The duration of natural cassava fermentation should not exceed 5 days, and in order to effectively initiate the natural fermentation, backslopping techniques should be applied initially or at an early stage of fermentation, not at the end. High levels of *Bacillus* species were frequently detected in various fermented softened cassava samples, suggesting that these organisms may be associated with softening of cassava, and low levels of *Clostridium* species were detected only by cultural methods. Pectinolytic enzyme activity (endo-polygalacturonase and exo-polygalacturonase) were detected in fermented cassava, while cellulase seemed not to be involved in softening in this study. Both α-glucuronidase and β-glucosidase were found to be partly endogenous enzymes of cassava tissue and skin respectively, and partly contributed by fermenting microbial activities. Overall, it appears that the above fermentation parameters can be manipulated to control acidification, thus directing the succession of fermenting organisms which in turn determines the quality of the fermentation end products. Looking at the advantages and disadvantages of natural fermentation, there is a continuing need for commitment to improving fermentation systems and developing appropriate starter cultures that focus on optimisation of fermentation optimisation, resulting in improved control of the process.

CHAPTER II: SELECTION OF STARTER CULTURES OF LACTIC ACID BACTERIA FOR CASSAVA FERMENTATION

2.1 Introduction

The question is "why do we need a starter culture for cassava fermentation"? The answer consists of three vital reasons: first: it is known that cassava is mostly processed by a natural submerged fermentation before it is converted into various fermented products, and this process is normally driven by slow initiation of a complex, unpredictable microflora naturally present in the raw substrate, therefore the fermentation is reputed to be uncontrollable and frequently associated with failures or the production of attributes characterised by substantial variations and defects (Oyewole, 1997; Holzapfel, 1997; Giraud *et al.*, 1998; Sanni *et al.*, 1998; Oyewole, 2001; Holzapfel, 2002; Ogbo, 2003; Kostinek *et al.*, 2005a). The second reason is relative to the adverse intrinsic factor of toxic cyanogenic glucosides contained in the cassava; moreover, cassava contains antinutritive factors such as indigestible oligosaccharides (Vasconcelos *et al.*, 1990; Oyewole, 1990; Westby, 1994; Holzapfel, 1997; Ogbo, 2003; LeRoy and De Vuyst, 2004; Kostinek *et al.*, 2007). The third reason for consideration is that the natural fermentation is mainly a lactic fermentation characterised by dominance of lactic acid bacteria ideally giving a rapid rate of acidification (Gilliland and Walker, 1990; Oyewole, 1990; Sanni *et al.*, 1998; De Vuyst, 1999; Holzapfel *et al.*, 2001, Holzaphel, 2002; LeRoy *et al.*, 2004). During this type of fermentation, the lactic acid bacteria (LAB) produce metabolites such as organic acids which usually lower the pH to around 3.5 – 4.5, therefore, the acidification rate is one of the main desirable characteristics sought during natural fermentation in this study. The acidification plays a major safety role through control and inhibition of spoilage organisms and pathogens. This is very important as *Bacillus cereus*, *Staphylococcus* and *Clostridium* species usually considered as potential pathogens have been isolated during cassava fermentation. The acidification has also a sensory role, as it contributes to the development of flavours that improves the taste and aroma of the end product (van Kranenburg, 2002). However, previous biochemical tests had displayed differences between species and between strains, therefore, the lactic acid bacteria were further characterised in this study. They are generally considered as safe due to their long history of safe use in fermented foods and their presence in the human intestine and have been reported to possess health benefits to the host (Holzapfel and Schillinger, 1995;

Wood, 1997; Salminem *et al.*, 1998; De Vuyst, 1999, Holzaphel, 2002; Leroy and De Vuyst, 2004).

The lactic acid bacteria used in this study were previously isolated at different stages during natural submerged cassava fermentation, were phenotypically and genetically identified and the identification results used to select 13 LAB out of 32 cassava isolates, to represent microbial diversity. The selected isolates were further subjected to various biochemical characterisation tests prior to the selection of specific starter cultures for the cassava fermentation optimisation. It is anticipated that the potential future use of a starter cultures of selected LAB that possess one or more desirable biochemical and probiotic properties would be essential to address the issues associated with natural fermentation of cassava. Therefore, its application to optimise this process is a suitable approach that shortens the fermentation time, allows more control and leads to high likelihood of desirable characteristics.

The objectives of this study were to identify LAB previously isolated during natural fermentation of cassava by phenotypic characterisations based on colony and cell morphology examination as well as biochemical profiles by API 50 CHL, and by genotyping analysis of the 16S-rDNA ITS-PCR intergenic transcribed spacer – polymerase chain reaction (ITS-PCR), repetitive sequence-based PCR (rep-PCR) and 16S rRNA gene sequencing. Following genotypic identification results, the aim is to select appropriate LAB and carry out further characterisation based on ability of the isolates to ferment starch, to initiate growth at pH values of 3.5, 43.5, 5.0, 6.01, 7.0, to cause rapid acidification, to possess antimicrobial activities against four indicator bacteria (*Escherichia coli*, *Salmonella* Typhimurium, *Bacillus cereus*, *Staphylococcus aureus*) and two fungi (*Aspergillus fumugatus*, *Penicillium expansum*), to possess linamarase activity, to degrade oligosaccharides and to possess probiotic properties.

This study was one of the steps in the anticipation of development of starter cultures aimed at cassava fermentation optimisation which would guarantee reproducibility of the process.

2.2 Materials and Methods

2.2.1 Microbial isolation

Thirty two LAB (Table 2.1) previously isolated at different stages of natural fermentations were used for this study. They were purified several times by growing, subculturing and streaking in MRS broth and agar (Oxoid CM0359B, CM0361B, Basingstoke, UK) until pure colonies were obtained. For purity verification, these lactic acid isolates were phenotypically characterized by colony and cell morphology, and were subsequently given an individual identity code before maintaining them on cryobeads at -20°C. Before starting any test, one or two beads were aseptically removed from frozen storage and inoculated into 10 ml of MRS broth, then incubated anaerobically (Oxoid Gas Kits BR0038B) for 48 to 72 h at 37°C. The cultures were subcultured three times in MRS broth and agar for 48 – 72 h at 37°C anaerobically.

Table 2.1: LAB isolated at different stages of natural fermentation of cassava

Lactic acid bacteria isolate n°	The isolation at different stages	Identity code
1	at 0 h or initial fermentation	A
2	at day 1 or 24 h fermentation	AA
3	at 0 h or initial fermentation	B
4	at 0 h or initial fermentation	C
5	at day 5 or final fermentation	C1
5	at day 2 or 48 h fermentation	C2
6	at 0 h or initial fermentation	D
7	at 0 h or initial fermentation	E
8	at 0 h or initial fermentation	F
9	at day 5 or final fermentation	F1
10	at day 2 or 48 h fermentation	F2
11	at day 3 or 72 h fermentation	G
12	at day 3 or 72 h fermentation	H
13	at day 3 or 72 h fermentation	I
14	at day 3 or 72 h fermentation	J
15	at day 3 or 72 h fermentation	K
16	at 0 h or initial fermentation	L
17	at 0 h or initial fermentation	M
18	at day 5 or final fermentation	N
19	at 0 h or initial fermentation	O
20	at 0 h or initial fermentation	P
21	at 0 or initial fermentation	Q
22	at 0 h or initial fermentation	R
23	at 3 days or 72 h fermentation	R1
24	at 5 days or final fermentation	R2
25	at day 5 or final fermentation	S
26	at day 5 or final fermentation	T
27	at 0 h or initial fermentation	U
28	at 0 h or initial fermentation	V
29	at day 5 or final fermentation	W
30	at day 5 or final fermentation	X
31	at day 5 or final fermentation	Y
32	at day 1or 24 h fermentation	Z

2.2.2 Phenotypic identification

Initial phenotyping was based upon the colony and cell morphology, Gram stain, catalase and oxidase reactions after anaerobic growth on MRS agar for 48 h. Fermentation profiles of carbohydrates using API 50 CHL test strips (BioMerieux, Basingstoke, UK) were also determined. Purity of each isolate was ensured by growing and streaking several times in appropriate liquid and solid medium. A loopful of pure, freshly-prepared (48h) culture from an agar plate of each isolate was suspended in 5 ml of sterile distilled water to obtain a turbid stock inoculum. One ml of this suspension was then inoculated into API 5OCHL medium and mixed well. From the inoculated medium, 100 µl were transferred into the wells of the strips. The wells were overlaid with sterile liquid paraffin to create anaerobic conditions required for LAB, and then aerobically incubated at 37°C for 24 and 48 h. The interpretation of results was based on the scoring from 0 to 5 of the colour change reactions. A positive test corresponded to acidification revealed by an indicator contained in the medium that changes to yellow, therefore, zero was assigned to a negative reaction and five was given to a strong positive reaction, and according to the biochemical profiles, the LAB were identified with the identification BioMerieux API system Software V3.0 with database.

2.2.3 Genotypic identification

The organisms in Table 2.1 were characterized by amplification (polymerase chain reaction) of 16S-23S intergenic transcribed spacer (ITS-PCR), repetitive polymerase chain reaction (rep-PCR) followed by sequencing of the 16S-rRNA gene.

Extraction of DNA

A pure culture of each isolate was streaked on MRS media and incubated anaerobically for 48 h at 37°C. The DNA of a pure colony was extracted by suspending the colony in 1 ml of sterile distilled water. The bacterial suspension was centrifuged at 12,000 x g for 1 minute. After centrifugation, the supernatant was discarded and 100 µl of InstaGene matrix Matrix (Biorad 732-6030, Hemel Hempstead, UK) was added to the pellet, and the suspension incubated at 56°C for 30 min. After incubation, the mixture was vortexed for 10 sec, then heated at 100°C for 10 min. After heating, the suspension was again vortexed for 10 sec, followed by centrifugation at 12,000 RPM for 3 min at 4°C. The upper layer of supernatant (DNA extract) was collected into a clean sterile tube. The concentration of extracted DNA cells was checked by electrophoresis. DNA extract (10 µl) and molecular

marker (2 µl) loading dye were mixed and loaded separately into wells of 2% agarose gel (BioRad 161-310). The gel was run in 1x concentrate 100 ml Tris-Borate (TBE) buffer (89 mM Tris Borate, 2 mM EDTA (TBE), pH 8.3, Sigma) for 1 h at 120 V. After the electrophoresis, the gel was stained in a 0.5 µg/ml ethidium bromide solution for 30 min at room temperature, destained by washing in water for 30 min and then photographed using a UV transilluminator.

Typing of bacteria by 16S-23S rDNA ITS-PCR

The isolates were typed at species level by 16S-23S rDNA ITS-PCR as previously described by Daffonchio *et al.* (1998) and Ouoba *et al.* (2008). Amplification of the16S-23S ITS region was carried out in 25µl of reaction mixture containing 1 µl of DNA template, 2.5 µl of PCR buffer without $MgCl_2$ (10×; Applied Biosystems N808-0161, Warrington, UK), 4 µl of dNTP (1.25 mM), 2.5µl of $MgCl_2$ (25mM), 1µl of the forward primer S-D-Bact-1494-a-S-20 (5'-GTCGTAACAAGGTAGCCGTA-3': 10pmol μl^{-1}), 1µl of the backward primer L-D-Bact-0035-a-A-15 (5'-CAAGGCATCCACCGT-3': 10pmol μl^{-1}), 0.1µl of Taq polymerase (5U; Applied Biosystems N808-0161) and 12.9 µl of autoclaved high purity water (Sigma W4502, Gillingham, UK). Amplification consisted of 35 PCR cycles in a thermocycler (Applied Biosystems, Gene Amp PCR system 2700). The cycling program was: initial denaturation at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 minute. The PCR was ended with a final extension at 72°C for 7 min and the amplified product cooled to 4°C. At the end of PCR process, the DNA fragments were separated by applying 10 µl of each PCR product with 2 µl loading buffer to 2% gel (Biorad 161-310). DNA molecular marker (Direct Load TM Wide Range DNA Marker, Sigma) was included as standard and the gel run in 1x TBE for 1 h at 120 V. After the electrophoresis, the gel was stained in a 0.5 µg/ml ethidium bromide solution (Sigma E1510) for 20 min, destained by washing in water for 10 min and then photographed using a UV transilluminator.

The DNA profiles obtained were observed and all bacteria showing the same profile were clustered in the same group.

Typing of bacteria by rep PCR

Isolates of each group obtained by ITS-PCR were further discriminated at subspecies level by rep-PCR as previously described by Ouoba *et al.* (2008). The amplification was performed in 25 µl or reaction mixture consisting of 2 µl DNA template, 2.5 µl PCR buffer without $MgCl_2$ (10x; Applied Biosystems N808-0161), 4 µl 1.25mM dNTP (Deoxynucleotides, Promega), 2 µl 25mM $MgCL_2$, 4µl of primer GTG5 (5'-GTG GTG GTG GTG GTG-3': 5 pmol μl^{-1}), 0.25 µl of Taq polymerase (5U; Applied Biosystems N808-0161) and 10.25 µl of autoclaved high purity water (Sigma W4502). The amplification consisted of 30 PCR cycles in a DNA thermal cycler (GeneAmp PCR system 2700) under the following programmed conditions: initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 1min, elongation at 65°C for 8 min and the final extension at 65°C for 16 min and subsequent cooling of the amplified products at 4°C.

The DNA fragments were separated by electrophoresis. Each amplified product (10 µl) was mixed with 2 µl of loading dye and loaded in 1.5% agarose gel (Biorad 161-310). The DNA molecular marker (Direct Load TM Wide Range DNA Marker, Sigma) (5 µl) which was added and the gel run in 1× TBE buffer for 2 h at 120 V. The gel was stained in a 0.5 µg/ml ethidium bromide solution (Sigma E1510) for 20 min, destained in water for 10 min and photographed using a UV transilluminator.

DNA profiles obtained were observed and all bacteria showing the same profile were clustered in the same group. The DNA profiles obtained by ITS-PCR and rep-PCR were clustered using Bionumerics system (Bio-Numerics 4.50, UPGMA Dice Correlation, Applied Maths, Sint-Martens-Latem, Belgium)

Identification of bacteria by 16S rRNA gene sequencing

Lactic acid bacteria from each ITS-PCR group (33 in total) were identified by sequencing. Primers based on conserved region of the 16S rRNA gene were used to direct PCR amplification of a 940 bp portion of this gene according to Ouoba *et al.* (2008). The PCR reaction solution was prepared by mixing respectively 1µl of each extracted DNA with 5 µl 10x PCR buffer containing 15 mM $MgCL_2$ (Applied Biosystems N8080160), 0.5 µl primer pA (100 µM 5'- AGA-GTT-TGA-TCC-TGG-CTC-AG-3'), 0.5 µl 1.0 mM primer pE (100 µM 5'-CCG-TCA-ATT-CCT-TTG-AGT-TT-3') (Sigma Genosy), 5 µl 150mM dNTP, 1 µl 1U AmpliTaq DNA polymerase (Applied Biosystems, N808-0161), 37 µl of high purity water (Millipore, purification PAK). The amplification of DNA was performed

in a DNA thermal cycler under the following conditions: the first denaturations at 95°C for 5 min; then 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, final extension at 72°C for 5 min and subsequent cooling of the amplified products at 4°C.
The amplified products were purified using Qiaquick PCR purification kit (Qiagen, Dorking, England). PCR product (10 µl) was transferred into provided Qiaquick spin column tubes and 50 µl PB buffer were added. The tubes were centrifuged for 1 min at 13,000 x g. The flow-through were discarded and the products washed by adding 0.75 ml PE buffer (100% ethanol), and further centrifuged for 1 min. The flow-through was discarded to remove the ethanol, and then recentrifuged at 13,000 x g for 1 min. The DNA were eluted by carefully adding 30 µl EB buffer (10 mM Tris-HCL, pH 8.5) and left to stand for 1 min and then centrifuged for 1 min at 13,000 x g.

The purified amplicons were partially sequenced to generate 550 bp of nucleotides with primer pD: 5'- GTA-TTA-CCG-CGG-CTG-CTG-3'(Sigma-Genosys), followed by use of the ABI Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems 4337455) to stop the reaction.
The reaction mixture consisted of 4 µl of PCR product (30-90 ng), 2 µl of primer pD (20ng/ µl) and 4 µl of ABI Big Dye Reaction Mix. The amplification reaction was performed in DNA thermal cycler programmed to the following conditions: the first denaturation at 95°C for 2 min, then 35 cycles at 96°C for 15 sec, at 40°C for 1 sec and the final extension at 60°C for 4 min.
The pre-sequenced DNA were precipitated by first adding 1 µl 3M sodium acetate, pH 4.6 (Sigma) and 50 µl 100% ethanol to the amplified product (10 µl), followed by centrifugation for 20 min at 10,000 x g. The supernatants were discarded and the pellets rinsed with 250 µl of 70% ethanol, followed by centrifugation at 10,000 x g for 10 min. Finally pellets (precipitated DNA) were allowed to air dry and sent to Oxford University for sequencing. The sequencing was achieved by electrophoresis on a 3730xl DNA Analyser-Titania (Applied Biosystems). The 16S rRNA gene sequences from the 5' end of the gene of the isolates were compared to the GenBank database using BLAST programme (National Centre for Biotechnology Information, Maryland, USA).
Out of 32 identified LAB, a total of 13 isolates were selected to represent each ITS-PCR group, and were further characterised prior to the selection of starter cultures intended for fermentation of cassava.

2.2.4 Starch fermentation assessment

MRS agar plates containing starch (Sigma) were prepared by substituting the glucose with starch (20g/l). LAB incubated anaerobically at 37°C for 48 h in MRS broths were centrifuged at 5,000 x g for 10 min, and the pellets resuspended in maximum recovery diluent (Oxoid CM0325, Basingstoke, UK). To assess their growth ability in medium supplemented with starch without glucose, 100 µl aliquots of decimal dilutions (-2 to -6) were prepared from each original suspension (10^7 CFU / ml) and spread on MRS agar and incubated anaerobically for 5 days at 37°C for colony count. In addition, iodine test was done by flooding 48 h colonies grown on the surface of MRS agar with iodine solution for 5 to 10 minutes to check starch utilisation by LAB. The confirmation of starch positive reaction was indicated by a colour change to black or brown and by a presence of clear zone around colonies.

2.2.5 Initiation of growth at different pH values

The bacterial standard stock inocula (10^7 CFU / ml) were prepared as above (Section 2.2.4), and each was inoculated (10% v/v) into MRS broth adjusted using 0.1M HCl to pH values of 3.5; 4.5; 5.0; 0.1M NaOH to 7.00, and in unacidified MRS broth pH 6.01 as control. The cultures were incubated anaerobically for 5 days at 37°C and samples of each liquid culture were collected and transferred (180 µl) into microtitre plates for measurement of optical density at 600 nm (BMG LAB TECH Fluo Star Omega) to estimate the cell concentration.

2.2.6 Characterization of LAB based on pH response from glucose fermentation

Each LAB culture was prepared by growing in MRS broth followed by incubation for 5 days in anaerobic conditions at 37°C. To evaluate each LAB pH-lowering capability following utilization of glucose, samples were collected at the initial and final fermentation to determine the pH profiles, which displayed the individual acidification characteristics.

2.2.7 Rapid acidification in single culture controlled fermentation of cassava

Prior to carrying out single culture fermentations, individual selected pure cultures of LAB were subcultured over 48 h anaerobically at 37°C in MRS broth. One millilitre of each 48 h culture (10^7 CFU/ml) was inoculated onto 100 g of sterile cassava in lidded jars containing 200 ml of sterile distilled water. Inoculated and uninoculated cassava (control) samples were incubated aerobically at 37°C for 5 days. For pH analysis, samples of fermenting and control cassava were collected at 0, 24, 48, 72 and 96 h, homogenised with

distilled water (10%: w/v) and pH values measured using a pre-calibrated pH meter (Jenway, Hanna Instruments).

2.2.8 Antimicrobial activity

2.2.8.1 Inhibition of "indicator" bacteria by agar diffusion assays

The inhibitory reactions and the effect of culture supernatants of LAB on four potential pathogens: *Escherichia coli* (*Escherichia coli* NCTC 12900; a verocytotoxin-negative variant of *Escherichia coli* O157:H7), *Salmonella* Typhimurium (01037340), *Bacillus cereus* (NCFB 13507), *Staphylococcus aureus* (ATCC 29247) all provided by Microbiology Research Unit/ London Metropolitan University. In this context, these are described as indicator organisms, since during the test they indicate antibacterial activity of LAB. All were examined for antimicrobial activity using spot methods described by Navarro *et al.* (2000) and Toure *et al.* (2003). The LAB isolates (13) were grown (10%: v/v) in MRS broth for 48 h at 37°C under anaerobic conditions. The cultures were centrifuged at 10,000 x g for 10 min at 4°C. The supernatants were aseptically collected and stored at -20°C for further tests, while the pellets were resuspended in maximum recovery diluent (10 ml). For each suspension (10^7 CFU/ml), 10 µl were each spotted on the surface of MRS agar plates in duplicate. The plates were left at 4°C for one h to allow the cultures to diffuse and incubated as above for 24 h before overlaying with 20 ml of BHI soft agar (Brain Heart Infusion: Oxoid CM0225 with added 0.8% agar) containing 200 µl of overnight culture (10^9 CFU/ml) of indicator bacteria that have been grown aerobically at 37°C. Uninoculated MRS agar plates were overlaid with BHI soft agar inoculated with indicator bacteria as control. The plates were allowed to solidify for 2 h and then incubated aerobically at 37°C for 48 h. Diameters of zones of inhibition around individual colonies were measured to give an estimate of the anti-microbial activity by the LAB.

Supernatants of LAB cultures were each transferred (10%: v/v) into nutrient broth inoculated with overnight grown indicator bacteria (10^9 CFU/ml). The indicator cultures, with and without LAB supernatants (controls were those without LAB), were incubated aerobically at 37°C for 48 h. Samples were collected at 0, 24 and 48 h for enumeration by colony count based on 10 fold serial dilutions where 100 µl of appropriate dilutions of each culture were spread on (depending on indicator organism used): *Bacillus cereus* selective agar (PEMBA), Baird Parker egg yolk tellurite agar (BP), Xylose Lysine Deoxycholate agar for *Salmonella* species (XLD, Oxoid CM0469B), MacConkey agar (Mac; Oxoid

CM0007), and (VRBGA), both for *Escherichia coli.* A nutrient agar (NA) plate was also included for each indicator and all plates were incubated at 37°C for 48 h.

Additionally, cells growth of the indicator cultures with and without LAB supernatants, were measured by optical density at 405 nm (WPA biowave C08000 cell density meter).

2.2.8.2 Survival of indicator bacteria during controlled fermentation of cassava.

Indicator bacteria were grown in nutrient broth, LAB in MRS broth (10%: v/v inoculum) and incubated aerobically for 24 h and anaerobically for 48 h at 37°C respectively. These constituted the stock inocula.

Five ml of stock inocula for indicator bacteria (10^7 CFU/ml), and LAB (10^8 CFU/ml) were both inoculated into 10 g of sterile cassava submerged in 200 ml of sterile distilled water in lidded jars (test fermentations). Cassava pieces inoculated with indicator bacteria alone were prepared as controls. All cassava samples were incubated (fermented) at 37°C aerobically for 5 days. Samples of the test fermentations and controls were collected initially and at the end of fermentation for pH measurement and colony count by plate techniques (following decimal dilutions) on the following agars, as appropriate to the indicator organism used: PEMBA; *Bacillus cereus*, BP; *Staphylococcus aureus*, XLD; *Salmonella* and VRBGA and Mac; *Escherichia coli*). Nutrient agar (NA) plates were also included. This allowed estimation of survival of indicator bacteria coexisting with lactic acid bacteria during controlled fermentation of cassava.

2.2.8.3 Inhibition of indicator of filamentous fungi by agar diffusion assays

This method was described by Ouoba *et al.* (2007), and the following filamentous moulds: *Aspergillus fumigatus* (Asp1 R4681018, Oxoid, Hampshire, England), and *Penicillium expansum* (Pen1 A17772, Philip Harris, England) were used in this study.

Inocula were prepared by subculturing and growing the fungi on malt extract broth and agar (MEA) plates, Asp1 at 30°C, Pen1 at 25°C, for 7 days aerobically. After incubation, Asp1, Pen1 conidia were collected by washing the surface of MEA plates containing each fungus with 10 ml of sterile saline solution, pH 5.8. From 48 h LAB cultures (10^6 CFU/ml), 100 µl were inoculated into the molten malt extract agar. The plates were left to solidify for 2 h at 4°C, and subsequently 10 µl of the above fungal conidia suspension (10^8 cells/ml) were respectively spotted on the surface of MEA plates containing individual LAB. The plates were incubated aerobically at 30°C and 25°C for 7 days. The controls

were prepared without LAB inocula and incubated as above. After incubation, the fungal colony diameter was measured to estimate the growth.

2.2.9 Linamarase activity

The microbial linamarase enzyme was qualitatively determined as beta- glucosidase by the biochemical API Zym test (BioMerieux, Basingstoke, UK), as described in Chapter 1. Other relevant enzymatic activity profiles (Tables 2.11 and 2.14) were also investigated using the same method. Colonies of each isolate were purified several times by subculturing them on MRS broth and agar. A loopful of fresh colonies of each isolate from an MRS plate was suspended in 5 ml of sterile distilled to obtain turbid suspension for inoculation. For each suspension, 100 µl were transferred into the wells of API-Zym strips, and then aerobically incubated (as enzymatic activity is being investigated, rather than growth of the organism) at 37°C for 4 h. After incubation, reagents were added and strips were left for 5 to 10 min to allow colour development. The interpretation of results was based on 0 to 5 score of the colour change reactions. Therefore, zero was assigned to a negative reaction and three, four and five were considered as positive reaction.
The linamarase enzyme was also evaluated as beta- glucosidase activity using the substrate para-nitrophenyl-beta-D-glucoside (PNPG) modified method described by Herr *et al.* (1978b). The isolates of LAB (13) were grown (10% v/v inoculum) anaerobically in MRS broth for 48 h at 37°C. For each LAB culture (10^7 CFU/ml), 5 ml were inoculated onto 50 g sterile cassava pieces submerged in 100ml of sterile distilled water. After taking an initial sample, the cassava pieces were left to ferment aerobically at 37°C for 5 days. The fermented and unfermented cassava were homogenised in distilled water (10% w/v), and homogenates were centrifuged at 10,000 x g for 10 min and the supernatants were kept in sterile tubes and immediately used for tests. The estimation (%) of beta- glucosidase activity was determined by measuring release of a yellow product from PNPG using a spectrophotometer at 405 nm. The assay consisted of 0.05% PNPG (Sigma) in 0.05M sodium citrate phosphate buffer (pH 5.0) added to each supernatant (10% v/v), and then incubated at 30°C in a water bath for 1 h. The reaction was stopped by adding 1M sodium carbonate (25% v/v), and the absorbance measured at 405 nm (Cecil 1000 Series, CE 1020).

2.2.10 Degradation of oligosaccharides

Fermentation of raffinose and melibiose (oligosaccharides which are indigestible by humans) by the selected 13 LAB isolates was assessed using API 50 CH galleries (BioMerieux, Basingstoke, UK). Colonies of each isolate were purified several times by subculturing them into appropriate liquid and solid medium (MRS). A loopful of fresh pure culture of each isolate from an MRS agar plate was suspended in 5 ml of sterile distilled water to obtain a turbid stock inoculum. For each bacterial suspension, 1 ml was inoculated into API 5OCHL medium and mixed well. From each inoculated medium, 100 µl were transferred into the wells of the strips. The wells of the strip inoculated with the CHL medium were overlaid with sterile liquid paraffin to create anaerobic conditions required for LAB. Strips were incubated at 37°C for 24 and 48 h. The interpretation of results was based on the scoring from 0 to 5 of the colour change reactions. A positive test corresponded to acidification revealed by an indicator contained in the medium that changes to yellow, therefore, zero was assigned to a negative reaction and five was give to a strong positive reaction.

2.2.11 Probiotic activity

2.2.11.1 Acid resistance and bile tolerance

The strains were routinely subcultured three times in MRS broth and MRS agar (Oxoid) to ensure purity and incubated anaerobically at 37°C for 48 h prior to carrying out tests that evaluated their ability to survive in a medium that mimicked the acidic gastric environment of pH 2.5, as well as the intestinal tract containing levels of at least 0.3% of bile (Pereira and Gibson, 2002).

The analysis was carried out using the conventional methodology described by Jacobsen *et al.* (1999), where bacterial cells were grown, centrifuged, then resuspended in MRD before examination. In this research, the acid resistance was tested in MRS broth adjusted to pH 2.5 using hydrochloric acid (HCl), and bile tolerance in MRS broth supplemented with 0.3% ox gall bile (LP0055, Oxoid). The survival or growth following acid and bile exposure was determined by viable count on MRS agar plates.

LAB were grown in MRS broth at 37°C for 48 h under anaerobic conditions, and then centrifuged at 10,000 x g for 10 min, washed and resuspended in sterile ringer's solution. Cell suspensions (10^7 - 10^9 CFU/ml) were inoculated (10% v/v) into non-acidified MRS broth as control, acidified (pH 2.5) MRS broth and also 0.3% bile supplemented and non-supplemented (control) MRS broth. The cultures were incubated anaerobically at 37°C for

4 h and samples taken for estimation of bacterial number at 0, 1, 2, 3 and 4 h. The viability was determined by preparing serial 10-fold dilutions in MRD of each, and 100 µl volumes of appropriate dilutions were spread on MRS agar plates, followed by anaerobic incubation at 37°C for 48 - 72 h before enumeration.

2.3 Results and Discussion

Lactic acid cassava isolates (33) were identified at the species level by both phenotypic and genotypic characterisation. Table 2.1 indicates the phenotypic identification, and according to phenotypic properties of colony, cell morphology, catalase and oxidase tests, all isolates were Gram positive, catalase and oxidase negative, non motile and non spore formers. Twenty eight were rod shaped, and five were cocci. All isolates showed similar sugar fermentation profiles, as they were able to metabolise some key sugars such as D-glucose, D-fructose, D-lactose, D-mannose, D-mannitol, D-maltose and D-saccharose. The fermentation of D-arabinose, L-xylose and starch was rare in all LAB examined. L-arabinose and L-rhamnose were metabolised by 11 LAB isolates only. They were biochemically classified by API 50 CHL tests as belonging to genus *Lactobacillus,* and the majority were identified as presumptive *Lactobacillus plantarum.* This species was isolated from all stages of fermentation and dominated throughout the fermentation (Table 2.2).

Table 2.1: Phenotypic characteristics of LAB isolated from unfermented and fermented cassava

Lactic acid isolate	Colony morphology	Cell morphology	Gram reaction	Catalase reaction	Oxidase reaction
A	Small; white; translucent; slimy	Rod in chains and in clusters	+	-	-
B	Small; creamy; round	Rods in chains and clusters	+	-	-
C	Small; elevated; creamy; round, slimy	Rod in chains and clusters	+	-	-
C2	Small; creamy; smooth; round	Cocci in chains	+	-	-
E	Small t; smooth, white; slimy	Rods in clusters	+	-	-
F	Medium; creamy; smooth; elevated; slimy	Rods in chains and in clusters	+	-	-
F1	Large; white; round; elevated; compact; smooth	Rod to coccoid; in chains and clusters	+	-	-
F2	Large; white; round; compact; smooth	Rods in chains and clusters	+	-	-
G	Small; smooth, white; slimy	Rod in chains and in clusters	+	-	-
H	Small; smooth; white ; slimy	Rod in chains and in clusters	+	-	-
I	Small to medium, smooth, white ; slimy	Rod in chains and in clusters	+	-	-
J	Small to medium, smooth, white ; slimy	Rod; scattered	+	-	-
K	Medium; creamy smooth; elevated	Rod; scattered	+	-	-
L	Small; creamy white; opaque; smooth	Rod in chains	+	-	-
M	Small ;white; opaque; smooth	Rod in clusters	+	-	-
P	Pinpoint colonies; white; smooth; round	Rod in clusters	+	-	-
Q	Pinpoint colonies; white; round	Rod in clusters	+	-	-
R	White; small; round; smooth	Rod in clusters	+	-	-
R1	Small; creamy; round; smooth	Rod in chains	+	-	-
R2	Small; creamy; round; smooth	Cocci; in clusters	+	-	-
S	White; small; round; smooth	Rod in clusters	+	-	-
T	Medium; creamy; round; elevated; smooth	Rod in clusters	+	-	-
U	Large; creamy; slimy;	Rod in clusters	+	-	-
V	Small; elevated; creamy; round, slimy	Rod in chains and in clusters	+	-	-
W	Pinpoint colonies; white; round	Rod in clusters	+	-	-

Table 2.1 cont: Phenotypic characteristics of LAB isolated from unfermented and fermented cassava

Lactic acid isolate	Colony morphology	Cell morphology	Gram reactions	Catalase reactions	Oxidase reactions
X	Pinpoint colonies; white; round;	Rod in clusters	+	-	-
Y	Small; creamy; smooth; round	Short rod in clusters	+	-	
AA	Pinpoint colonies; white; round	Rod in chains; clusters	+	-	-
D	Small; creamy; opaque; round; elevated; smooth	Rod; singly; pairs; chains;	+	-	-
Z	Medium; creamy white; round; condensed	Rod in chains and clusters	+	-	-
N	Small, white, translucent; smooth	Cocci in chains	+	-	-
O	Small; white; translucent; smooth	Cocci in chains	+	-	-
C1	Small; creamy; round; smooth	Cocci in chains	+	-	-

Nine groups or species of LAB cassava isolates were obtained by the ITS-PCR which allows typing at species level, and ten groups or subspecies by rep-PCR analysis (Figures 2.1 and 2.2). For ITS-PCR results: group A comprised 23 isolates, group B comprised two isolates while groups C, D, E, F, G, H and I comprised just one isolate each (Table 2.2). The rep-PCR confirmed differences between the ITS-PCR groups and allowed differentiation of the bacteria at subspecies level. A cluster analysis of the results (Figure 2.2) allowed differentiation of two subgroups from ITS-PCR group A (subgroup A.1 included 23 isolates and subgroup A.2 one isolate), two from group B and one each from groups C, D, E, F, G, H and I.)

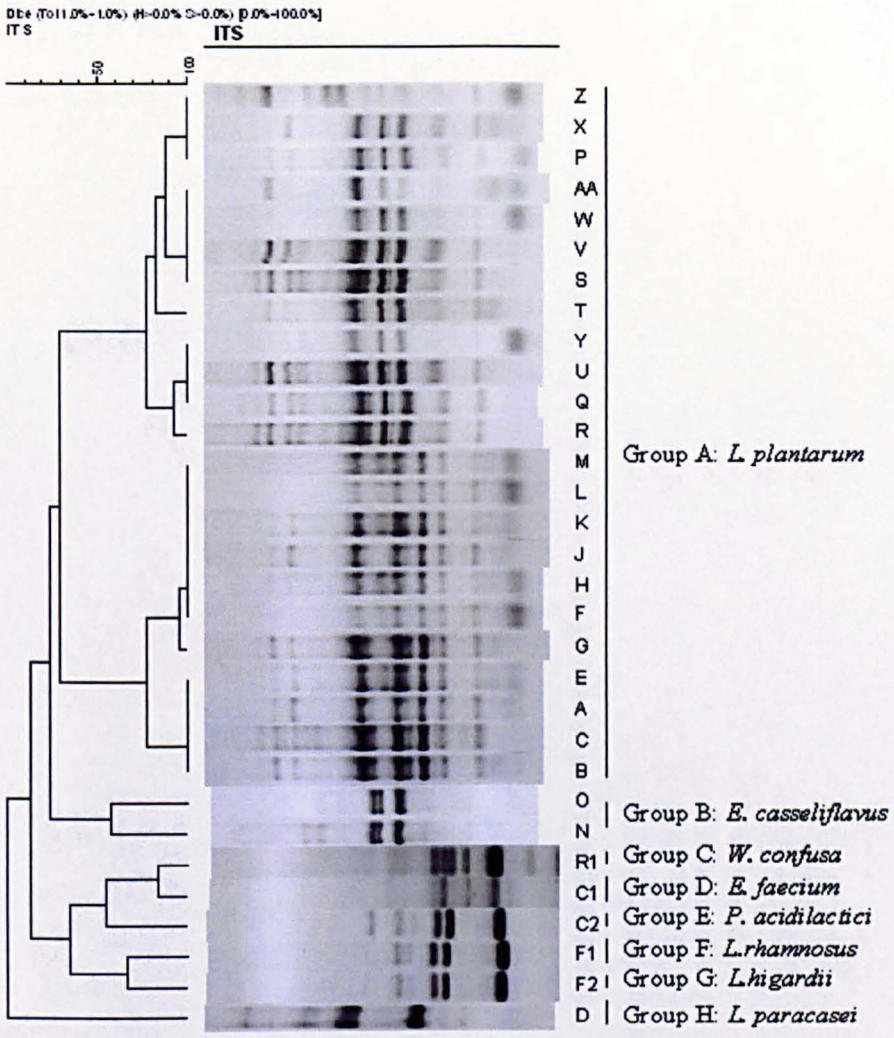

Figure 2. 1: Cluster analysis of ITS-PCR of lactic acid cassava isolates.

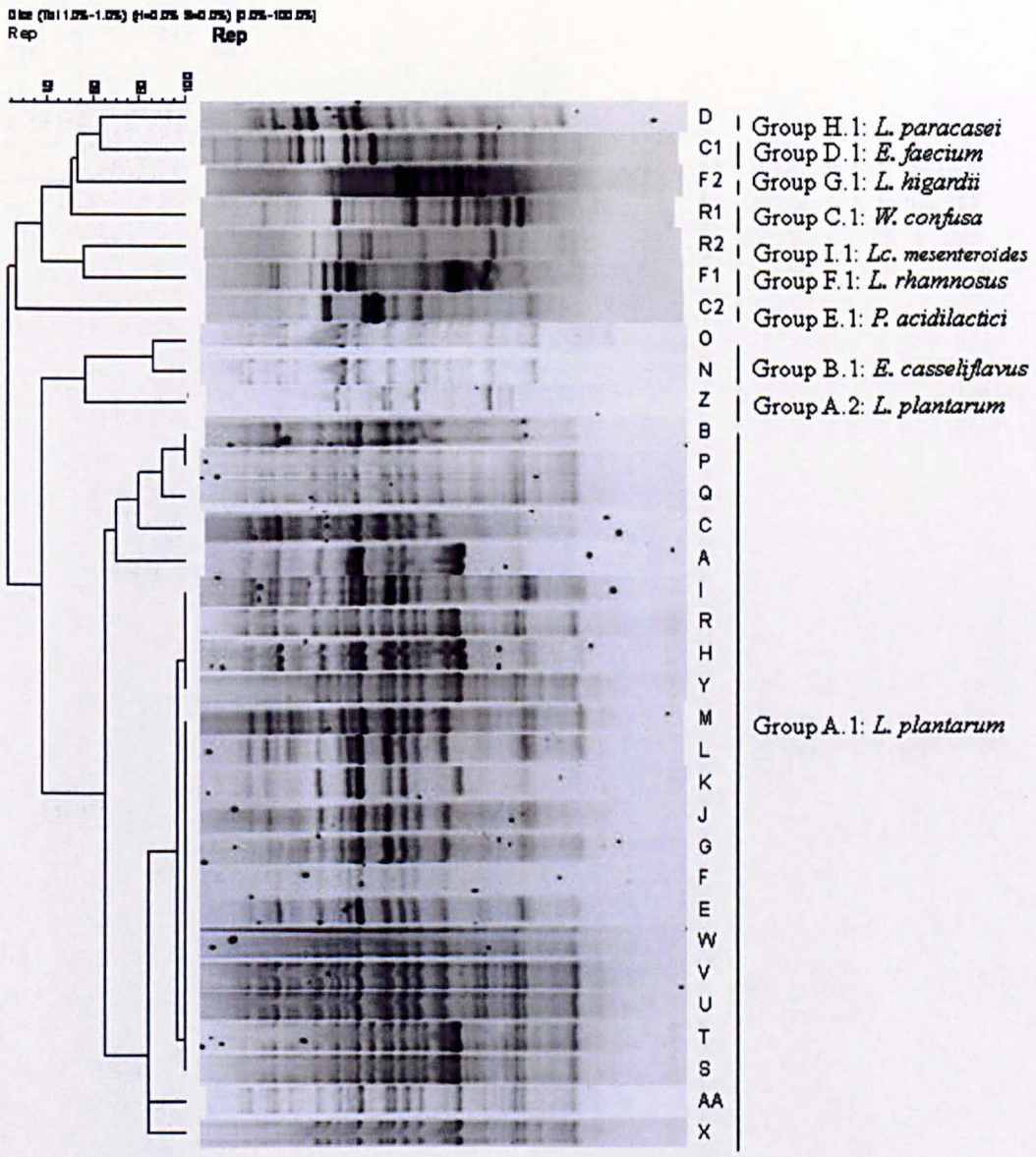

Figure 2.2: Cluster analysis of rep-PCR of lactic acid cassava isolates

Table 2.2: Identification of lactic acid bacteria

Bacteria	ITS-PCR Pattern type	rep-PCR Pattern type	Identification API 50 CHL	Identification DNA sequencing	% of identity
A	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	98
AA	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	99
B	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	98
C	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
F	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	99
G	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	98
H	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
I	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
J	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	99
K	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
L	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
M	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	98
P	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
Q	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	98
R	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
S	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	99
T	A	A.1	*Lactobacillus paracasei*	*Lactobacillus plantarum*	99
U	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	99
V	A	A.1	*Lactobacillus pentosus*	*Lactobacillus plantarum*	98
W	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	98
X	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	99
Y	A	A.1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
Z	A	A.2	*Lactobacillus plantarum*	*Lactobacillus plantarum*	97
O	B	B.1	*Lactococcus raffinolactis*	*Enterococcus casseliflavus*	99
N	B	B.1	*Lactococcus raffinolactis*	*Enterococcus casseliflavus*	99
R1	C	C.1	*Lactobacillus rhamnosus*	*Weissella confusa*	97
C1	D	D.1	*Lactococcus raffinolactis*	*Enterococcus faecium*	99
C2	E	E.1	*Lactobacillus pentosus*	*Pediococcus acidilactici*	98
F1	F	F.1	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus*	97
F2	G	G.1	*Lactobacillus rhamnosus*	*Lactobacillus hilgardii*	97
D	H	H.1	*Lactobacillus paracasei*	*Lactobacillus paracasei*	97
R2	I	I.1	*Lactobacillus rhamnosus*	*Leuconostoc mesenteroides*	99

Sequencing of 16S rDNA and API CHBL fermentation profiles allowed identification of each group of bacteria (from the ITS-PCR) at genus and species level. The DNA sequences of isolates of each ITS-PCR group were aligned with all sequences present in the GenBank database, thus identifying the microorganisms. Group A was identified as *Lactobacillus plantarum* and there were 2 subspecies: the first main subspecies included 23 isolates and the other subspecies one isolate. Both isolates in group B were identified as *Enterococcus casseliflavus* of one subspecies, group C as *Weissella confusa* of one subspecies, group D as *Enterococcus faecium*, group F as *Lactobacillus rhamnosus*, group G as *Lactobacillus hilgardii,* group H as *Lactobacillus paracasei* and group I as *Leuconostoc mesenteroides*. It was observed that the phenotypic identification did not fully agree with the genotypic identification. This was not unusual as the API

identification (relying on phenotypic characteristics) has been reported to have poor discriminatory capability and to be less accurate and less reliable (Ouoba *et al.*, 2007). The microbial identification demonstrated that a wide range of LAB species were isolated and represented the main microbial component of the complex microflora of natural fermentation of cassava. Lactobacilli, particularly *Lactobacillus plantarum* were the most predominant among LAB, and dominated throughout to the end fermentation of cassava as reported by Oyewole and Sobowale, 2008. Other predominant species belong to the genus *Enterococcus,* and to a lesser extent, to *Leuconostoc*, *Pediococcus* and *Weissella.* Similar findings were reported by Amoa-Awua *et al.* (1996), Holzapfel, (1997), Ben Omar *et al.* (2000), Sanni and Morlon-Guyot, (2002), Franz *et al.* (2003) and Kostinek *et al.* (2007, 2008).

The starch fermentation tests (results not shown) demonstrated that all 13 LAB cassava isolates were incapable of utilising the starch contained in the medium. This was shown by very limited growth, characterised by pinpoint colonies after five days incubation. Therefore, conclusive confirmation results of iodine, API Zym and API 50 CHL tests (Tables 2.11, 2.14) showed that the isolates were non amylolytic strains. This correlates with the results of most studies; few studies have reported isolation of amylolytic LAB strains, including amylolytic *Lactobacillus plantarum* strains, and *Lactobacillus manihotivorans* from cassava sourdough (Giraud *et al.*, 1993; Morlon-Guyot *et al.*, 1998). Starch contained in plants generally helps to provide suitable substrates for fermenting microorganisms, such as yeasts and *Bacillus* species. Therefore, possible starch degradation or exhaustion by fermenting microorganisms could downgrade the availability of carbohydrates contained in the product, affecting its nutritional value as a dietary energy source (Graham *et al.*, 1988; Holzapfel, 1997; Okafor *et al.*, 1998; Kostinek *et al.*, 2007). In consideration of the above observation, it seems that the observed limited starch utilisation property could be an additional desirable characteristic, appropriate for selection of starter cultures of LAB for the optimisation of cassava fermentation.

Table 2.3: Cell growth density at different pH values evaluated by optical density (OD) measurements

Growth assessment of LAB in acidified MRS broth at different pH values						
Strain	**Initial bacterial conc.**	**pH 3.5**	**pH 4.5**	**pH 5.0**	**pH 7.0**	**pH 6.01 control**
R2	0.156	0.298	0.858	1.253	1.439	1.402
T	0.277	0.902	2.808	2.776	3.313	2.729
C2	0.313	1.739	2.648	2.696	2.630	2.687
C1	0.316	2.285	3.313	3.242	3.220	2.990
D	0.294	1.190	2.727	2.176	2.557	2.639
F1	0.313	2.081	3.313	3.313	3.226	3.313
K	0.291	2.124	3.282	2.983	3.313	3.318
Z	0.285	1.376	2.452	3.018	2.468	3.107
N	0.284	2.764	3.313	2.843	3.313	3.313
F2	0.183	2.210	2.961	3.128	2.632	2.892
R1	0.313	2.888	3.313	3.313	3.906	3.313
S	0.313	2.671	3.266	2.919	3.313	3.313
F	0.313	2.081	3.313	3.313	3.226	3.313

Average OD reading values of three experiments

These LAB isolates were assessed for the ability to grow in MRS broth of different pH values by optical density (OD), and Table 2.3 indicates that isolates T, R2 and C2, respectively, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Pediococcus acidilactici* displayed poor growth performance at pH 3.5, and good growth at pH range of 4.5 – 7.0. Isolates F2, N, R1, K, F1, S, F, D, C1 and Z (*Lactobacillus rhamnosus, Enterococcus casseliflavus, Weissella confusa, Lactobacillus plantarum, Lactobacillus hilgardii, Lactobacillus plantarum, Lactobacillus plantarum, Lactobacillus paracasei, Enterococcus faecium*, and isolate Z is *Lactobacillus plantarum*) grew well at pH 3.5, and optimally at pH 4.5 – 6.0. This demonstrated that these isolates were rapid acid producers compared to isolates T, R2 and C2. This seemed to agree with previous published reports of a high percentage of the *Lactobacillus plantarum* group in particular, to be capable of lowering the pH in MRS broth to below 3.9 after 48 h, and of the high acid resistance of enterococci associated with fermented vegetables (Franz *et al.*, 1999, 2003; Kostinek *et al.*, 2007). The isolates were additionally characterised based on the pH response from their fermentation of glucose contained in synthetic medium of MRS broth and Table 2.4 shows that the cultures of isolates C1, D, F, K, N, S, R1, Z, F1 and F2 produced lower pH values than isolates C2, R2 and T. These findings were in harmony with their ability to grow at different pH values (Table 2.3), since correlation was observed between low pH values produced following glucose fermentation by single LAB cultures and profuse growth at lower pH values.

Table 2.4: pH values recorded after growth of LAB in MRS broth containing glucose

Initial pH: 6.02	
LAB strain	**pH value after 5 days incubation**
C1	3.50
C2	3.68
D	3.50
F	3.50
F1	3.54
F2	3.57
K	3.50
N	3.50
R1	3.52
R2	3.69
S	3.50
T	3.77
Z	3.53

Average pH values recorded during three experiments replicates

Lactic acid bacteria are reported to be important organisms for their fermentative ability as well as their health and nutritional benefits. They produce various compounds such as organic acids, primarily lactic acid that lowers the pH and other antagonistic agents resulting in antimicrobial activities against pathogenic and spoilage organisms, while ultimately leading to preservation of the fermented product (Gilliland and Walker, 1990; Ogunbariwo and Onilude, 2003). The changes in pH values during controlled fermentations of cassava are shown in Table 2.5, and a significant drop of almost 4 units from the initial pH value of 7.38 to 3.47 confirmed a rapid acidification rate after 24 h followed by a negligible subsequent decrease of pH values. Each LAB cassava isolate was assessed for ability to cause rapid acidification during fermentation. Table 2.5 shows that the lowest pH values were produced by individual cultures of D, N, R1, F1, K, S and particularly C1(*Lactobacillus paracasei*, *Enterococcus casseliflavus*, *Weissella confusa*, *Lactobacillus rhamnosus*, two *Lactobacillus plantarum strains*, and *Enterococcus faecium*) compared to somewhat higher pH values produced by C2, R2 *(Pediococcus acidilactici, Leuconostoc mesenteroides)* and particularly T (*Lactobacillus plantarum*). The recommended pH value of below 4.0 for cassava fermentation (Coulin *et al.*, 2006) was generally reached within 24 h, resulting in probable improved antagonism and competitive advantage against pathogens. *Lactobacillus plantarum* strains are reported to be generally fast acid producers, as these strains are able to lower the pH in MRS broth and controlled fermentation of cassava from approximately 6.60 + 0.78 to 3.63 ± 0.05 after 24 h, and 3.52 ± 0.03 after 5 days, similar results were reported by Kostinek *et al.* (2007). Two of the rapid acidifiers in this research were *Lactobacillus plantarum*, but one of the

slow acidifiers was also identified as this organism, suggesting that there is strain variation concerning this characteristic.

Table 2.5: Acidification rate by single LAB culture.

Strain	pH value recorded at 24 h intervals during fermentation			
	24 hours	48 hours	72 hours	120 hours
C1	3.47	3.52	3.58	3.50
C2	3.77	3.75	3.73	3.70
D	3.60	3.58	3.54	3.51
F	3.63	3.60	3.57	3.52
F1	3.61	3.59	3.55	3.52
F2	3.64	3.62	3.60	3.58
K	3.63	3.60	3.58	3.55
N	3.62	3.59	3.55	3.50
R1	3.60	3.58	3.57	3.55
R2	3.72	3.70	3.68	3.66
S	3.66	3.64	3.60	3.55
T	4.15	4.10	3.95	3.81
Z	3.65	3.59	3.55	3.55

Control (and initial value): 7.38

Average pH recorded values of three experiments replicates

The LAB isolates were screened for antimicrobial activity against six indicator strains, two Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*), two Gram negative bacteria (*Escherichia coli* and *Salmonella* Typhimurium) and two fungi (*Aspergillus fumigatus* and *Penicillium expansum*). The inhibition of growth of indicator bacteria, using cells or supernatants of LAB cultures, was tested by spot and optical density methods, the viable counts of both liquid cultures and in vivo cassava fermentations. The results showed discrepancies between these methods. Inhibition by LAB of potential pathogens was measured by agar diffusion assay. The spot method results (Table 2.6 and Figure 2.10 display varying levels of inhibitory activity among LAB. All isolates showed some inhibitory effects (zone diameter 0.5 – 1mm) towards *Escherichia coli*. Moderate inhibitory activities (zone diameter 1 – 2 mm) against both *Escherichia coli* and *Salmonella* Typhimurium were displayed by C1, C2, R2, T and Z (*Enterococcus faecium*, *Pediococcus acidilactici*, *Leuconostoc mesenteroides*, two *Lactobacillus plantarum* strains), while isolates D, R1 (*Lactobacillus paracasei*, *Weissella confusa*) were effective only against *Salmonella* Typhimurium. Isolate S (*Lactobacillus plantarum*) displayed strong inhibitory effects (zone diameter 2 – 4 mm) towards *Salmonella* Typhimurium. The ability of LAB to inhibit Gram negative *Escherichia coli* and *Salmonella* Typhimurium were reported by De Vuyst, (1999), Mante and Amoa-Awua, (2003) and Sanni and Edema,

(2008). All LAB isolates showed antimicrobial activities against Gram positive indicator bacteria, and specifically, displayed strong inhibitory effects towards *Bacillus cereus* (2 - 4 mm). The inhibitory effects were most pronounced (> 4 mm) with isolates C1 and D (*Enterococcus faecium*, *Lactobacillus paracasei*) and this is in accordance with previous findings (Sawadago *et al.*, 2004, 2005; Assefa *et al.*, 2008). Isolates D, R1, T and Z exhibited moderate inhibitory activities (1 – 2 mm) against *Staphylococcus aureus*.

Table 2.6: Antimicrobial activities of selected isolates measured by agar diffusion assay.

LAB Strain	Indicator bacteria			
	Escherichia coli	*Salmonella* Typhimurium	*Bacillus cereus*	*Staphylococcus aureus*
C1	++	+	++++	+
C2	++	+	++	+
D	+	++	++++	++
F	+	+	+++	-
F1	+	-	++	-
F2	+	-	++	-
K	+	+	++	+
N	+	+	+++	-
R1	++	++	+++	++
R2	++	++	+++	-
S	++	+++	+++	+
T	++	++	+++	++
Z	++	++	+++	++

Average inhibition zones of three experiments replicates.
All indicator bacteria were examined for growth inhibition determined by an area of inhibition surrounding colony.
-: no inhibition zones +: 0.5-1mm inhibition zones: weak ++: 1 – 2mm inhibition zones: moderate +++: 2- 4mm inhibition zones: strong ++++: over 4mm inhibition zones: strongest.

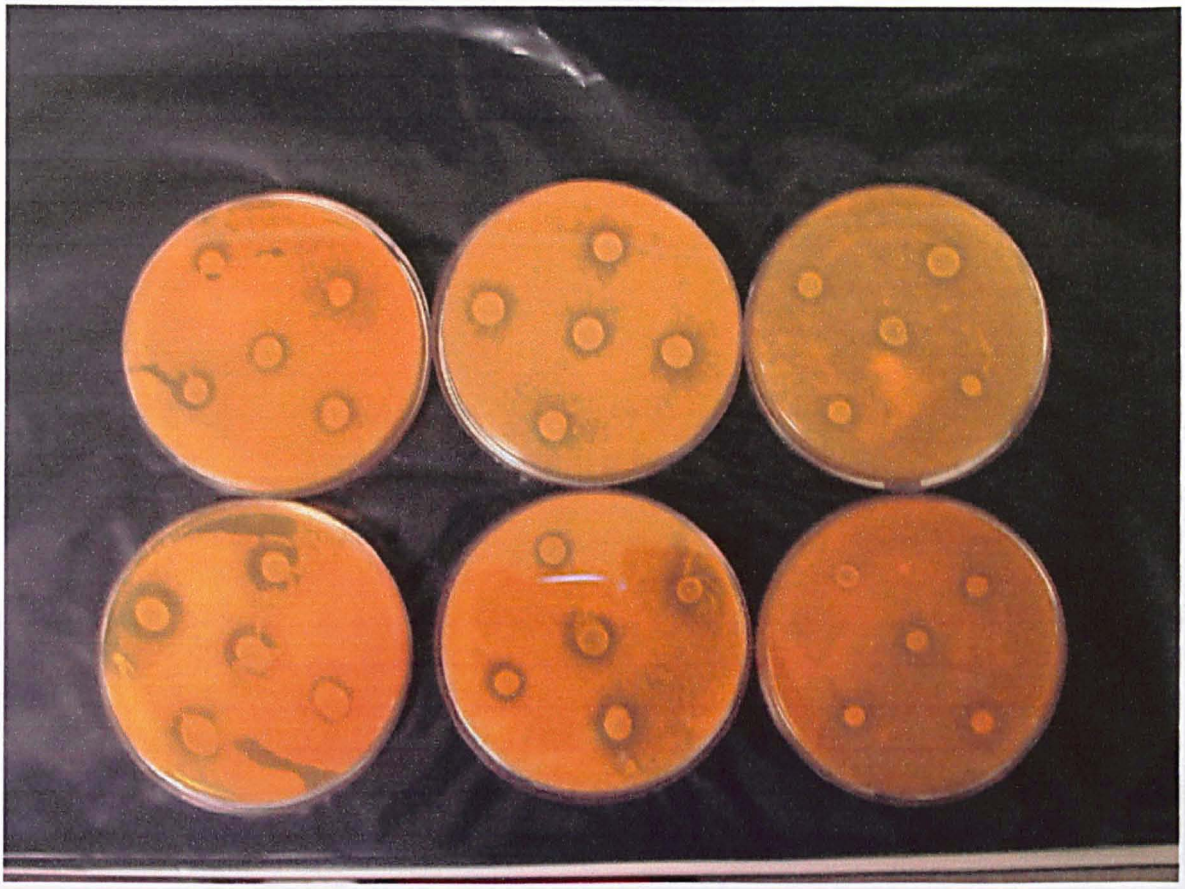

Figure 2.1: Growth inhibition zones around colonies of antimicrobial activity producers

Representative pathogens were incubated with LAB cell supernatants for 48 h, and their survival rate measured by OD. Table 2.7 displays varying levels of inhibitory activity among LAB isolates, and this seemed to be organism specific. Generally, supernatants of single culture of LAB isolates demonstrated stronger inhibitory effects towards the Gram positive indicator bacteria tested, notably *Bacillus cereus*, than the Gram negative and isolate F (*Lactobacillus plantarum*) was the best candidate to have strong dual inhibitory activities. *Escherichia coli* appeared generally less inhibited by all 13 LAB isolates using this method. *Escherichia coli* strains and other Gram negative bacteria had been reported to be less sensitive than Gram positive bacteria to inhibitory effects of LAB, and their resistance was probably conferred by the composition of the cell wall (Bhunia *et al.*, 1991; Assefa *et al.*, 2008).

Table 2.7: Survival of indicator bacteria measured by optical density (OD)

Lactic acid strain supernatant	OD reading (turbidity)			
	Escherichia coli	*Salmonella* Typhimurium	*Bacillus cereus*	*Staphylococcus aureus*
Control	0.50	0.46	0.47	0.73
C1	0.15	0.10	0.08	0.13
C2	0.26	0.26	0.25	0.25
D	0.13	0.09	0.11	0.12
F	0.26	0.23	0.22	0.26
F1	0.12	0.03	0.02	0.06
F2	0.11	0.15	0.10	0.16
K	0.18	0.15	0.10	0.18
N	0.29	0.22	0.22	0.23
R1	0.17	0.12	0.10	0.14
R2	0.22	0.19	0.13	0.17
S	0.21	0.17	0.14	0.16
T	0.25	0.17	0.18	0.20
Z	0.21	0.10	0.16	0.18

Average OD taken in three experiments replicates

Indicator bacteria initial concentration ($10^7 - 10^8$ CFU ml^{-1})

Control: representative pathogens bacteria were each grown in liquid medium without addition of LAB supernatants

The survival of potential pathogens incubated for 48 h with LAB culture supernatants was determined by viable count. In contrast to the above results, Table 2.8 indicates that LAB isolates had strongest inhibitory effects towards Gram negative bacteria and *Salmonella* Typhimurium was the most sensitive. The Gram negative indicator bacteria had survived in either low numbers between 10^2 & 10^3 CFU/ml or in undetectable numbers, while Gram positive bacteria populations survived with numbers in the range of $10^3 - 10^4$ CFU/ml.

Table 2.8: Survival of indicator bacteria shown by viable count after 48 h

LAB supernatant	Indicator bacteria viable count: log CFU/ml			
	Escherichia coli	***Salmonella* Typhimurium**	***Bacillus cereus***	***Staphylococcus aureus***
C1	$1.3x10^3$	ND	$1.2x10^4$	$0.3x10^4$
C2	$3.0x10^3$	ND	$2.1x10^4$	$1.4x10^4$
D	$1.2x10^3$	ND	$1.3x10^4$	$3.0x10^3$
F	$2.3x10^3$	ND	$2.1x10^4$	$1.2x10^4$
F1	$1.6x10^3$	ND	$1.7x10^4$	$3.0x10^3$
F2	$9.2x10^2$	ND	$1.3x10^3$	$6.0x10^3$
K	$1.5x10^3$	ND	$1.4x10^4$	$3.0x10^3$
N	$3.6x10^3$	ND	$2.1x10^4$	$1.0x10^4$
R1	$1.9x10^3$	ND	$1.4x10^4$	$5.0x10^3$
R2	$2.8x10^3$	ND	$1.5x10^4$	$1.0x10^3$
S	$2.3x10^3$	ND	$1.7x10^4$	$6.0x10^3$
T	$9.0x10^2$	ND	$2.1x10^4$	$9.0x10^3$
Z	$2.4x10^3$	ND	$1.7x10^4$	$9.0x10^3$
Control	$1.4x10^8$	$2.4x10^8$	$2.6x10^8$	$1.6x10^8$

Log CFU/ml: average of three experiments replicates **ND: not detected**

The cultures of potential pathogens were added to cassava fermentation initiated by a mixed starter culture of 13 LAB, and their survival determined by viable count. The results in Table 2.9 reveal that low viable count was an indication of strong antimicrobial activity against both Gram positive and negative organisms. Their populations dropped (respectively) from 10^7 to 10^3 CFU/ml and 10^6 to10^1 CFU/ml, after 24 h and were almost undetectable (ND) or in negligible numbers of 10^1 CFU/ml at the end of fermentation of 5 days. *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* were the most sensitive and did not survive to the end of the fermentation process.

Table 2.9: Survival of indicator bacteria during cassava controlled fermentation

		Indicator bacteria count: log CFU/ml					
	Fermentation time	**PEMBA**	**BP**	**XLD**	**VRBGA**	**MaC**	**NA**
Control: indicator alone	initial	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$
	24 hours	$3.6x10^7$	$4.8x10^7$	$5.1x10^6$	$7.0x10^6$	$6.4x10^6$	$6.5x10^8$
	48 hours	$2.8x10^7$	$3.1x10^7$	$6.8x10^6$	$7.4x10^6$	$7.1x10^6$	$3.0x10^9$
	96 hours	$7.5x10^7$	$5.0x10^7$	$8.4x10^6$	$7.0x10^6$	$6.8x10^6$	$3.8x10^9$
	120 hours	$5.0x10^8$	$1.0x10^8$	$8.1x10^6$	$7.0x10^6$	$6.8x10^6$	$3.6x10^9$
Indicator + LAB mixture							
	initial	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$
	24 hours	$5.1x10^3$	$1.1x10^4$	$1.2x10^4$	$4.2x10^1$	$3.9x10^1$	$8.8x10^8$
	48 hours	$1.0x10^3$	$1.0x10^4$	$1.0x10^4$	ND	ND	$9.4x10^8$
	96 hours	$2.0x10^2$	ND	ND	ND	ND	$4.0x10^8$
	120 hours	$1.3x10^1$	ND	ND	ND	ND	$3.0x10^8$

Average survival rate of three experiments replicates
***Bacillus cereus* on PEMBA; *Staphylococcus aureus* on BP; *Salmonella* Typhimurium on XLD; *Escherichia coli* on VRBGA, MaC; total viable count on NA**

Mbugua and Njenga (1991) found that *Salmonella* Typhimurium, *Escherichia coli* and *Staphylococcus aureus* were effectively inhibited in *uji*, a weaning food traditionally fermented. Kivanc (1990) reported antimicrobial activities against both Gram negative and Gram positive organisms by LAB isolated from cheese during a study on antibacterial properties of LAB cell free filtrates. The mixed LAB cultures used as inoculum in the experiments reported here for fermentation of cassava displayed highly effective antimicrobial activity against both Gram positive and Gram negative potential pathogens. Differences in antimicrobial activity between LAB growing in a food matrix (cassava) and when cultivated in complex media were also reported by De Vuyst (1999); LeRoy and De

Vuyst, 2004). In addition, use of LAB mixed cultures, which contains strains of more than one genus was reported to be more effective than multiple strains of the same species (Holzapfel, 2002; Timmerman *et al.*, 2004). The mechanisms underlying the enhanced effects could be attributed to a combination of synergistic effects of different strains with specific properties, or to enhanced biological activity caused by symbiosis between different strains such as exchange of different metabolites (Drago *et al.*, 1997; Timmerman *et al.*, 2004). In general, in this research, LAB isolated at the end of fermentation appeared to be more able to inhibit potential pathogens than those isolated earlier. This observation was also reported by Olsen *et al.* (1995).

It was also observed in this research that LAB antimicrobial activities correlated with the patterns of the acids they produced (Appendix 8 and 9), and the diameter of inhibitory zones tended to be larger with high-acid producing LAB. Therefore, the results suggest that the inhibitory activity was due to production of acids. For substantiation of this, other research has shown and confirmed that organic acids of pH below 4.2 were responsible for the inhibitory effects, particularly towards Gram negative bacteria including pathogens such as *Salmonella* Typhimurium (Gilliland and Walker, 1990; Svanberg *et al.*, 1992; Sanni *et al.*, 1994; Holzapfel and Schillinger, 1995, Holzaphel, 1997; Klaenhammer and Kullen, 1999 and Ouoba *et al.*, 2006). Mante and Amoa-Awua, (2003), following the study on the inhibitory effect on pathogens during fermentation to produce *agbelima*, showed that the addition of catalase and proteolytic enzymes to supernatants showed no effect on their inhibitory activity against indicator bacteria. At the same time, bacteriocinogenic LAB strains have been shown to effectively inhibit growth of pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium difficile* (Holzapfel and Schillinger, 1995). The same author also reported the inhibition of spoilage organisms by hydrogen peroxide. In the study herein reported, since LAB culture supernatants were not treated with proteinase, therefore inhibitory reactions were presumed to be attributed either to organic acids, hydrogen peroxide or bacteriocin production as reported by Kostinek *et al.* (2007).

Inhibition of indicator of filamentous fungi

Inhibition activity of cassava LAB isolates against potential spoilage and mycotoxigenic fungi were determined by colony diameter measurement. Results in Table 2.10 showed that LAB antifungal activities were displayed mainly against *Penicillium expansum* (from 1.15 – 3.25 mm) compared to *Aspergillus fumigatus* (from 0.96 – 1.24 mm). However,

isolates F2, Z, R1 (*Lactobacillus hilgardii*, *Lactobacillus plantarum*, *Weissella confusa)* caused considerable growth restriction of both fungi (Figures 2.2, 2.3). Corsetti *et al.* (1998), Gobetti *et al.*, (2000) and Magnusson and Schnurer, (2001) had discovered that LAB isolated from sourdough of wheat possessed antifungal activity and *Lactobacillus plantarum* in particular was found to have strong growth inhibitory effects towards some *Penicillium* and *Aspergillus* species.

Table 2.10: Inhibition of indicator of filamentous fungi by LAB

	Colonial diameter (cm) of fungi after 7 days incubation	
LAB	***Aspergillus fumigatus***	***Penicillium expansum***
Control	**1.25**	**3.25**
Enterococcus faecium C1	1.05	1.9
Pediococcus acidilactici C2	1.09	2.8
Lactobacillus paracasei D	1.24	2.5
Lactobacillus plantarum F	1.24	2.5
Lactobacillus rhamnosus F1	1.10	3.10
Lactobacillus hilgardii F2	0.96	1.15
Lactobacillus plantarum K	1.10	2.40
Enterococcus casseliflavus N	1.04	2.40
Weissella confusa R1	1.10	1.80
Leuconostoc mesenteroides R2	1.09	3.05
Lactobacillus plantarum S	1.04	2.25
Lactobacillus plantarum T	1.10	3.25
Lactobacillus plantarum Z	0.99	1.95

Average colonial diameter of fungi measurements of three experiments replicates

In this research, different colonial phenotypic characteristics between uninhibited control fungi, which were grown on MEA without LAB extracts and same fungi grown on MEA with individual LAB (13) extract were overt. The colony characteristics of *Penicillium expansum* as control and tested uninhibited *Penicillium expansum* were similar in most aspects. The colony (3.25 mm) was mainly white, fluffy with 3 distinctive zones within the colony: a white large outer zone, a medium and greenish central zone and a whitish

small inner zone (Table 2.10, Figure 2.2). The colony had doubled its size after 3 days incubation (results not shown). Inhibitory reactions displayed by inhibited *Penicillium expansum* demonstrated that the colony morphology characteristics were significantly different from the control and from fungi which were not affected by LAB cells or supernatants. Compared to the control (3.25 mm), the colony size of inhibited *Penicillium expansum* was halved to 1.15 + 2.1 mm diameter, the outer zone was not fluffy and was wet-looking with a faded white colour. The green central zone was absent, the outer zone decreased in size and looked like a small thin ring of fluffiness. The degree of the growth inhibition effects was characterised by the size of the outer zone: the stronger the inhibitory effects, the smaller the outer zone became or a complete disappearance in some cases, and the more the central zone become deformed, less defined and more the green colour became patchy (Figure 2.2). It was observed that the colony size of inhibited *Penicillium expansum* was not affected by any morphological change from the third day incubation and onwards.

Aspergillus fumigatus as control and tested uninhibited *Aspergillus fumigatus* displayed similar colony characteristics. The colony (1.25 mm) was white with two distinctive zones of a well defined, elevated inner zone, and an outer zone within the colony (Figure 2.3). The colony size of the uninhibited *Aspergillus expansum* doubled within three days incubation and slightly continued to grow (Results not shown). In comparison to control (1.25 mm), the colony size of inhibited *Aspergillus expansum* was reduced to 0.96 + 0.29 mm diameter, and displayed a smaller, almost absent, outer zone and a deformed, compressed and less defined inner zone (Table 2.10, Figure 2.3), and had stopped growing after three days incubation.

Figure 2.2: Inhibition of *Penicillium expansum* by lactic acid bacteria

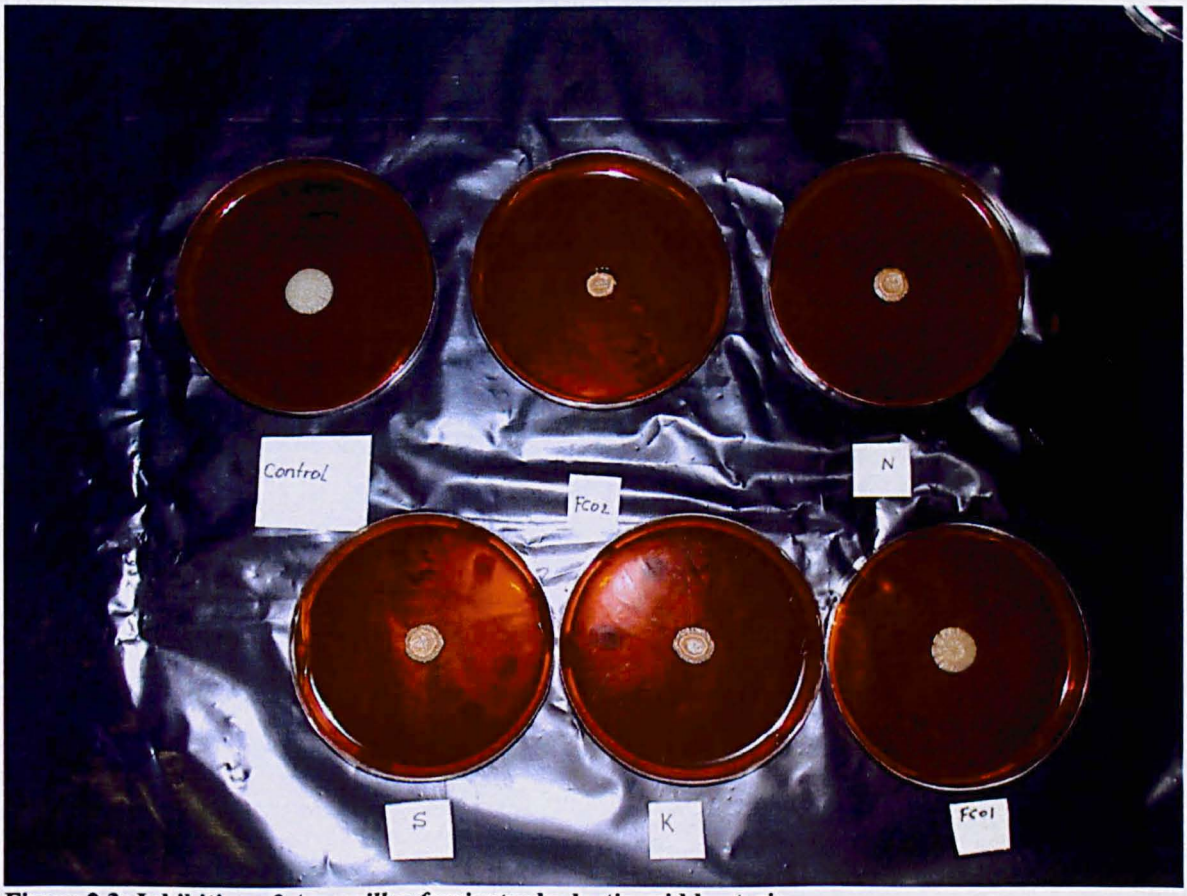

Figure 2.3: Inhibition of *Aspergillus fumigatus* by lactic acid bacteria

All 13 LAB isolates showed antimicrobial activity against one or more indicator fungi and bacteria. Isolates *Weissella confusa* R1, *Leuconostoc mesenteroides* R2, three strains of *Lactobacillus plantarum* S, T, and Z had dual activities against both Gram positive and negative indicator bacteria. Isolates C1, F2, Z and R1 were able to exert their antimicrobial activities against all tested indicator bacteria and fungi during *in vitro* laboratory cultivation and *in vivo* controlled fermentation of cassava. Mycotoxins, especially aflatoxin, were reported to be a significant risk factor for cereals as a consequence of mould growth during storage (Barrios-Gonzalez and Tomasine, 1990; Holzapfel, 1997). It has been reported that some moulds and LAB species associated with production of the fermented foods *tempeh* and *kenkey* showed the ability to reduce aflatoxin through degradation or inactivation (Steinkraus, 1983; Holzapfel, 1997).

Linamarase activity

The API Zym tests results (Table 2.11) showed that all 13 LAB isolates possessed linamarase activity, as β-glucosidase activity was observed for strains D, K, S, T, Z, N, R1, R2 (*Lactobacillus paracasei*, four strains of *Lactobacillus plantarum*, *Enterococcus casseliflavus*, *Weissella confusa*, *Leuconostoc mesenteroides*) and moderate activity for strains C1, C2, F1 (*Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus rhamnosus*), weak activity for F2 (*Lactobacillus hilgardii*) and no activity for F (*Lactobacillus plantarum).*

Table 2.11: β-glucosidase and other key enzymatic activities detected by API Zym tests

Organism	α-amylase	β-glucosidase	α-galactosidase	lypolytic	Peptidase	protease
Enterococcus faecium C1	-	+	+	-	-	-
Pediococcus acidilactici C2	-	+	+	-	-	-
Lactobacillus paracasei D	-	++	-	+	++	+
Lactobacillus plantarum F	-	++	+	+	++	+
Lactobacillus rhamnosus F1	-	+	-	-	-	-
Lactobacillus hilgardii F2	-	+	-	-	-	-
Lactobacillus plantarum K	-	++	+	+	++	-
Enterococcus casseliflavus N	-	++	+	-	++	+
Weissella confusa R1	-	++	++	+	++	+
Leuconostoc mesenteroides R2	-	++	+	+	++	+
Lactobacillus plantarum S	-	++	+	+	++	+
Lactobacillus plantarum T	-	+	+	++	++	-
Lactobacillus plantarum Z	-	++	+	+	++	+

Average of enzymatic activity of two experiments repeats

The results of the API Zym tests were compared with β-glucosidase detected in single culture fermentation of cassava assayed by para-nitrophynyl-β-D-glucoside (PNPG), which measured the release of yellow product spectrophotometrically at 405 nm, following hydrolysis. The PNPG assay demonstrated that linamarase activities were not detected in

unfermented and uninoculated cassava (Table 2.12). Comparisons with the controls showed that over 90% of linamarase were detected in 12 lactic LAB isolates except strain F (*Lactobacillus plantarum*), which exhibited no activity (Table 2.13) according to both PNPG method and API Zym test.

Table 2.12: β-glucosidase activity in 13 LAB detected by PNPG method

Organism	Blank: 0.05% - ρ- nitrophenyl -β –D-glucosidase
Enterococcus faecium C1	0.087
Pediococcus acidilactici C2	0.091
Lactobacillus paracasei D	0.091
Lactobacillus plantarum Z	0.092
Lactobacillus plantarum T	0.095
Lactobacillus hilgardii F2	0.110
Lactobacillus plantarum	0.105
Lactobacillus rhamnosus F1	0.112
Weissella confusa R1	0.129
Leuconostoc mesenteroides R2	0.163
Enterococcus casseliflavus N	0.180
Lactobacillus plantarum K	0.191
Lactobacillus plantarum F	2.969

Average β-glucosidase activity of three experiments repeats
Blank: 0.05% - ρ- nitrophenyl – β – D – glucoside (OD: 0.026)
unfermented cassava (OD: 2.999)

Table 2.13: β- glucosidase activity measured spectrophotometrically at 405 nm.

LAB strain	Fermented cassava β-glucosidase activity estimation (%)
C1	97.1
C2	97.0
D	97.0
Z	97.0
T	96.8
F2	96.3
S	96.5
F1	96.3
R1	95.7
R2	94.6
N	94.0
K	93.6
F	1.0

Average β-glucosidase activity of three experiments repeats

β -glucosidase activity estimation (%) calculation: $\frac{(A - A_0)}{A} \times 100$

Where, A= initial absorbance at 405 nm; A_0=final absorbance of test sample

The ability of isolates to hydrolyse linamarin in *in vivo* controlled fermentation of cassava by the PNPG method seemed to agree with the API Zym tests results. The β-glucosidase enzyme breaks down various compounds with β-D glucosidic linkages such as cyanogenic glucosides (Lancaster *et al.*, 1982; Mkpong *et al.*, 1990). The overall results from this study confirmed that the hydrolysis of linamarin naturally found in cassava root was caused mainly by microbial activity. Similar linamarase activity of LAB, particularly *Lactobacillus plantarum* and *Leuconostoc mesenteroides* isolated from cassava fermentation had been recorded previously by Oyewole (1990), Ciarfardini *et al.* (1994), Vasconcelos *et al.* (1990), Amoa-Awua *et al.* (1996), Holzaphel (1997), Lei *et al.* (1999, Weagent *et al.* (2001); Amoa-Awua *et al.* (2004). However, it was also reported that cassava detoxification may partly result from endogenous linamarase, when the cassava cells are disrupted by processing methods such as grating or softening, then release linamarin (Bokanga, 1989; Westby, 1994; Kimaryo *et al.*, 2000).

Other biochemical properties such as amylase activity were undetectable in all strains (Table 2.11), and this agreed with the results obtained in the study of growth assessment of LAB isolates in medium containing starch as sole energy source (Section 2.2.4). Kostinek *et al.* (2007) reported similar results. The inability of LAB to ferment starch does not explain or reconcile their detection at an early stage and subsequent exponential growth on cassava, which is a starchy food (Ketiku and Oyenuga, 1972). This surprising situation was likely to be a consequence of synergistic action between LAB and other fermenting organisms such as yeast and amylolytic *Bacillus* strains (Adams and Moss, 1995; Timmerman *et al.* 2004). These organisms are known to hydrolyse starch into simple fermentable sugars, which are subsequently used by other fermenting microorganisms such as LAB (Amoa-Awua and Jakobsen, 1995; Oyewole and Sobowale, 2008). Another possible explanation for the LAB presence at initial time could be the availability of fermentable sugars found in unfermented cassava as reported by Ketiku and Oyenuga, (1970) who identified sucrose, maltose, glucose and fructose in fresh unfermented peeled cassava root. It appears that only a few amylolytic LAB strains have been isolated from starchy African fermented foods (Sanni and Morlot-Guyot, 2002). The majority of strains possessed weak α-galactosidase and lipolytic activity with the exception of R1 and T (*Weissella confusa*, *Lactobacillus plantarum*) which displayed strong activity. All isolates possessed weak protease activities, nine strains possessed peptidase activity and no enzymatic activity was displayed by F1, F2, C1, C2 (*Lactobacillus rhamnosus*, *Lactobacillus hilgardii*, *Enterococcus faecium*, *Pediococcus acidilactici*). Most of these results agreed with the findings stated by Kostinek *et al.* (2007) in an earlier

characterisation study of LAB cassava isolates for starter culture selection. Oligosaccharides such as raffinose and stachyose are naturally present in cereals, legumes and other plants. Their chemical structure includes α-D galactosidic bonds which are usually broken down by microbial α-galactosidase enzymes such as those found in *Leuconostoc mesenteroides*, *Lactobacillus plantarum* and some moulds (Westby, 1994; Amoa-Awua, 1995; Kimaryo *et al.*, 2000). Humans are naturally unable to metabolise α-galactoside sugars, although breakdown may be possible with the help of microbial α-galactosidase activities associated with the digestive tract, and human consumption of fermented food products (Gobbetti *et al.*, 2000; Holzaphel, 2002). The fermentation of these sugars by microorganisms in the human GIT can lead to flatulence, caused by the production of gas (LeBlanc *et al.*, 2004). In this study, the sugar profiles elucidated by API 50 CHL tests and enzymatic profiles from API Zym tests results (Tables 2.11, 2.14) showed that the majority of LAB isolates possessed variable α-galactosidase enabling them to degrade these indigestible sugars, with the exception of F1 (*Lactobacillus rhamnosus*).

Table 2.14: Degradation of oligosaccharides detected by API 50 CHL tests.

Fermentation of sugar	Selected lactic acid bacteria - cassava isolates												
	C1	C2	D	F	F1	F2	K	N	R1	R2	S	T	Z
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-rhamnose	+	-	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
D-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	-	+	+	+	+	+	+	+	+
D-ribose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-xylose	-	+	-	-	-	+	-	-	+	+	-	-	-
L-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-raffinose	+	+	-	+	-	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-	-	-	-	-

Average detection of oligosaccharides of two experiments repeats

Probiotic activity

In order to exert beneficial effects on the host, probiotic bacteria must survive transit through the gastrointestinal tract (GIT) including the acidity of the stomach, must be tolerant to the bile encountered in the small intestine and must have antimicrobial activity against GIT-associated pathogens (Sanders, 1993; Hammes and Hertel, 1998; Fuller and Gibson, 1997; Gibson and Ziemer, 1998; Tuomola *et al.*, 2001; Joint FAO, WHO, 2002; Vijendra and Prasad, 2005). According to Usman (1999); Pereira and Gibson, (2002) survival at pH 3.0 for 2 h and survival in medium containing 1000 mg/l of bile are considered standard for acid and bile tolerance of probiotic cultures. All LAB isolates tested showed different acid tolerance and significant survival at pH 2.5, and among them, 10 isolates were still viable with populations between 10^3 &10^6 CFU/ml for 3 h and showed promising survival rate (10^2 – 10^5 CFU/ml) for up to 4 h of incubation (Table 2.15). A low number of 10^2 CFU/ml was presumed unsustainable for R2 (*Leuconostoc mesenteroides*). Three isolates: F1, F2, T (*Lactobacillus rhamnosus, Lactobacillus hilgardii, Lactobacillus plantarum*) had completely lost viability after 3 h. In this study, the results demonstrated that the acid tolerance was strain specific, and this agrees to some extent with the findings of Jacobsen *et al.* (1999) who stated that most of *Lactobacillus* species were unable to survive at pH 2.5 in her study. In contrast, Goldin *et al.* (1992) reported high survival of lactobacilli strains at pH 3.0.

Table 2.15: Tolerance of 13 lactic acid bacteria to MRS broth adjusted to pH

LAB	Time (h)				
	0	1	2	3	4
C1	1.0×10^8	6.0×10^7	2.5×10^7	3.6×10^6	4.0×10^5
C2	4.0×10^8	6.0×10^7	2.6×10^5	3.0×10^3	7.0×10^4
D	2.3×10^8	2.6×10^6	2.5×10^5	2.3×10^5	5.0×10^4
F	2.0×10^8	3.0×10^6	4.0×10^6	3.0×10^6	5.0×10^5
F1	3.0×10^8	3.0×10^5	2.0×10^5	ND	ND
F2	6.0×10^8	4.0×10^6	7.0×10^4	ND	ND
K	2.6×10^8	2.4×10^5	2.4×10^5	3.0×10^6	3.0×10^5
N	3.5×10^8	1.5×10^8	3.0×10^7	3.0×10^6	3.0×10^5
R1	3.6×10^8	2.5×10^8	1.5×10^7	2.0×10^6	2.0×10^5
R2	2.0×10^8	1.9×10^7	9.0×10^4	1.0×10^4	1.0×10^2
S	3.6×10^8	1.6×10^8	3.0×10^7	3.0×10^6	2.5×10^5
T	2.1×10^8	2.0×10^5	7.0×10^4	ND	ND
Z	2.5×10^8	1.0×10^5	1.1×10^5	1.0×10^4	7.0×10^4

Survival rate (average): three replicated experiments

All isolates showed good tolerance to bile (0.3%) exposure and after a slight decline in number after 2-3 h, most survived well (>10^6 CFU/ml) during 3 h exposure time (Table 2.16) was observed. More limited bile tolerance was observed with strain F1 (*Lactobacillus rhamnosus*) and its population appeared to decline to 10^1 CFU/ml at 3 h, but a number of 10^8 CFU/ml was recovered after 4h. After 4 h of bile exposure, all strains were restored to their initial concentration level of 10^8 CFU/ml.

Table 2.16: Tolerance of 13 lactic acid bacteria to MRS broth supplemented with 0.03% bile

LAB	Time(h)				
	0	1	2	3	4
C1	4.4×10^8	2.6×10^7	3.1×10^7	3.8×10^7	2.4×10^9
C2	4.5×10^8	1.4×10^7	1.6×10^7	2.0×10^7	6.1×10^7
D	2.3×10^8	1.6×10^7	1.5×10^6	3.8×10^6	2.0×10^8
F	2.0×10^8	1.9×10^7	3.4×10^6	4.3×10^6	1.2×10^7
F1	3.0×10^8	2.0×10^5	2.0×10^3	6.0×10^1	7.1×10^8
F2	6.0×10^8	2.2×10^6	4.5×10^6	6.5×10^6	1.8×10^8
K	2.6×10^8	3.6×10^7	2.4×10^7	3.5×10^7	2.6×10^8
N	3.5×10^8	2.0×10^7	3.3×10^7	3.5×10^7	2.8×10^8
R1	3.6×10^8	6.0×10^5	8.0×10^5	2.0×10^6	2.5×10^8
R2	2.0×10^8	4.3×10^8	3.0×10^7	5.3×10^7	5.0×10^8
S	3.6×10^8	1.9×10^8	3.0×10^7	4.0×10^7	5.8×10^8
T	2.1×10^8	2.0×10^8	7.0×10^7	1.2×10^8	1.3×10^8
Z	2.5×10^8	8.3×10^7	5.1×10^7	1.4×10^7	1.8×10^8

Survival rate (average): three replicated experiments

Antimicrobial activity has been stated to be one of the selection criteria for a successful probiotic, and potentially probiotic strains should have the ability to produce metabolites such as organic acids of pH below 4.0, and exert other inhibitory effects towards enteropathogens (Oyewole, 1997; Jacobsen *et al.* 1999; Vijendra and Prasad, 2005). The overall results revealed that all 13 LAB isolates showed antimicrobial activity against one or more indicator fungi and bacteria, and 10 out of 13 exhibited potential probiotic properties. As a matter of interest, two distinctive results patterns were observed: one group of LAB isolates R2, and S, T, Z (*Leuconostoc mesenteroides,* three *Lactobacillus plantarum* strains) displayed dual activities against both Gram positive and negative indicator bacteria as well as potential probiotic activity except T, while the other group C1, F1, F2, Z, R1(*Enterococcus faecium*, *Lactobacillus rhamnosus*, *Lactobacillus hilgardii*, *Lactobacillus plantarum*, *Weissella confusa*) showed the ability to exert antimicrobial activity against Gram positive and negative and filamentous fungi in vitro laboratory cultivation and *in vivo* controlled fermentation of cassava as well as being potential probiotic, apart from isolate F1. Therefore, it was concluded that the majority of all

selected LAB cassava isolates possessed potential probiotic properties. Lactic acid bacteria from African fermented foods, particularly lactobacilli, were found to be able to demonstrate the characteristic of inhibition of intestinal pathogens, as well showing probiotic activity in terms of acid and bile tolerance (Olukoya, 1994; Holzapfel, 1997).

2.4 Conclusion

Thirty three LAB were isolated at different stages of spontaneous cassava fermentation and phenotypic and genotypic characterisations showed species diversity. All were Gram positive, catalase and oxidase negative and non spore formers, 28 were rod shaped, five were coccoid and all seemed to have the same sugar fermentations patterns. A wide range of indigenous LAB of nine species, were isolated and represented the main microbial component of the complex microflora of natural fermentation of cassava. Among 33 isolates, 24 were *Lactobacillus plantarum*, two *Enterococcus casseliflavus*, one *Enterococcus faecium*, one *Lactobacillus paracasei*, one *Lactobacillus rhamnosus*, one *Lactobacillus hilgardii*, one *Leuconostoc mesenteroides*, one *Pediococcus acidilactici* and one *Weissella confusa*. The *Lactobacillus* group, particularly *Lactobacillus plantarum,* was the most predominant among LAB, and dominated throughout to the end of the cassava fermentation. Different subgroups within the species were identified. Thirteen representative lactic acid cassava isolates: *Enterococcus faecium* (C1), *Enterococcus casseliflavus* (N), *Lactobacillus* rhamnosus (F1), *Lactobacillus higardii* (F2), *Leuconostoc* mesenteroides (R2), *Pediococcus acidilactici* (C2), one *Weissella confusa* (R1, *Lactobacillus paracasei* (D) and five *Lactobacillus plantarum* strains (F, K, S, T, Z) were selected and subjected to further characterisation tests. The strain F seemed to be different from the others in terms of its low level of acid production, bile tolerance, linamarase and low antifungal activity.

All 13 isolates were found to be non amylolytic strains as they were unable to ferment starch. They were all able to grow at pH 3.5, 4.5, 5.0, 6.01, 7.0, but displayed different acid sensitivity. A rapid acidification rate during the first 24 h of fermentation was achieved by 10 isolates (C1, N, F1, F2, R1, D, F, K, S, and Z). These isolates appeared to be high acid producers following glucose fermentation as they lowered the pH from 7.38 to 3.41+0.19. Isolates C2, R2 and T were low acid producing strains. In fermenting cassava, potential pathogens survived to different degrees depending on pH and their sensitivity to acids, therefore, the LAB antimicrobial activity was attributed generally to production of acids. In spite of a display of varying degrees of inhibition effects among selected LAB, they showed generally good antimicrobial activities against one or more indicator bacteria

and fungi, and 10 out of 13 showed potential probiotic properties. The majority of LAB isolates possessed linamarase activity and ability to degrade indigestible sugars. On basis of possessing one or more of the above desirable properties, careful combinations among selected LAB would be potential selection for starter cultures development.

CHAPTER III: DETERMINATION AND SELECTION OF STARTER CULTURES OF BACTERIA CAUSING CASSAVA SOFTENING

3.1 Introduction

One of the reasons cassava is fermented in many parts of Africa is to obtain desirable characteristics such as texture modification through the softening of cassava. Softening of cassava is a desirable attribute that contributes to the detoxification of cassava as well as to the organoleptic quality of colour and taste (Oyewole, 1990, 1991; Amoa-Awua *et al.*, 1996, 1997; Kimaryo *et al.*, 2000; Oyewole, 2001). Through leeching into the fermenting medium, the softening helps to reduce cyanogenic glucosides to low levels, which had been reported to be directly proportional to the degree of softening (O'Brien *et al.*, 1991; Mathew and Moorthy, 1991; Giraud and Raimbault, 1994; Essers, 1995).

Natural fermentation is reported to result from competitive activities of microorganisms of the complex microflora (Holzapfel, 2002) and in this study, the major concern is that cassava softening was found to be extremely haphazard and unpredictable, as eight out of 10 previous *in vitro* cassava fermentations carried out during preliminary tests failed to produce cassava softening. This highlighted how natural fermentation of cassava are frequently characterised by many softening failures (Chapter I), and represents a major concern, since it limits attempts to develop a suitable process for consistent fermentation. Current published reports that associate softening of cassava with organisms such as *Bacillus* and *Clostridium* species, yeasts and fungi can be contradictory, vague and ambiguous (Ogbo, 2003; Kimaryo *et al.*, 2000; Oyewole, 2000; Okafor *et al.*, 1984, 1998; Brauman *et al.*, 1996; Keleke *et al.*, 1996; Amoa-Awua, 1995; Amoa-Awua *et al.*, 1997; Coulin *et al.*, 2006). These facts highlight the serious lack of understanding of the way in which cassava softening occurs; therefore identification of specific organisms responsible for cassava softening, and a better understanding of its mechanism, would contribute to optimisation of the process.

In an attempt to detect reported fermenting softening organisms such as *Bacillus* and *Clostridium* species, yeasts and fungi (Okafor *et al.*, 1984, 1998; Brauman *et al.*, 1996; Keleke *et al.*, 1996; Amoa-Awua, 1995; Amoa-Awua *et al.*, 1997; Oyewole, 2000; Ogbo, 2003; Kimaryo *et al.*, 2000; Oyewole, 2000), several natural fermentation batches of cassava were set up to isolate non-lactic acid-producing microorganisms and establish the

patterns of softening occurrences (Chapter I, sections 1.27, 1.28). Preliminary results from those experiments suggested that temperature and time were likely to be the most influential factors. Softening seemed most likely to occur at 37°C over 72 h and variations in the degree of softening were characterised either by completely satisfactory softening, or uneven or pockets of softening, or both, between fermentation batches and within a single fermentation of cassava. Intensive cassava softening was distinguished by complete tissue disintegration of all cassava pieces accompanied by starch sedimentation. This degree of softening is also undesirable, since it leads to a visually unappealing product of poor colour that is difficult to handle. Consequently, it was necessary to characterise and identify the organisms associated with a successful fermentation which are responsible for cassava softening, with a view to developing a well defined, multiple strain starter culture for optimisation of the fermentation process.

Non-lactic acid-producing organisms isolated previously during natural fermentations of cassava and from various fermented cassava samples (Chapter I, sections 1.27, 1.28) were used in this study. During preliminary tests, alcohol (100%) treated (for sterilisation) cassava was used in conjunction with the method described by Obillie *et al.*, (2003). The sterilisation method was subsequently modified such that use of only irradiated (18 kG of ionisation radiation; Isotron Ltd., Swindon, UK) sterile cassava was used with a liquid inoculum to give reliable and more predictable results. Fifty five previously isolated organisms were individually screened for ability to soften alcohol-sterilised cassava, and 17 organisms did not meet the softening test requirement and were eliminated. The test requirement for each organism was to consistently cause reproducible cassava following inoculation onto the surface of both alcohol-sterilised and (later) irradiated cassava pieces. Thirty eight isolates produced softening in an irregular manner using alcohol sterilised cassava, and 33 out of those 38 produced a consistent, reproducible softening using irradiated cassava. Three organisms were found not to cause softening but were responsible for production of the characteristic odour associated with fermented cassava. This characteristic odour was reported by Okafor *et al.* (1984) and Kimaryo *et al.* (2000) to be disliked by many consumers, and this factor had adversely limited its acceptance in mostly urban regions of Africa. Thirty one successful softening organisms and three odour producers were phenotypically and genotypically identified. Following identification, 13 out of 34 bacteria were selected for further research. The selected isolates were further subjected to biochemical characterisation. This study was one of the steps in the intention to develop starter cultures aimed at optimisation of cassava fermentation to guarantee

reproducibility of softening and thus an overall improvement of organoleptic quality of the product.

The specific objectives of this aspect of the research were to individually assess non- LAB isolates for ability to cause cassava softening using discriminatory tests, to evaluate and validate cassava softening reproducibility, to identify successful isolates by phenotypic and genotypic characterisation, and based on genetic identification results, to select representative bacteria and carry out further biochemical characterisation to determine their sugars fermentation patterns, pectinolytic activity and compounds responsible for the characteristic odour using gas chromatography.

3.2 Materials and methods

3.21 Isolation of Microorganisms

Non-LAB isolates from fermentations that produced cassava softening, including from backslopped fermentations and pasteurised cassava samples (Chapter I, sections 1.27, 1.28) were used in this study. The isolation of microorganisms was made on various selective and non-selective media and 55 non-LAB were isolated, purified several times and were each given an individual identity code before maintenance at -20°C awaiting further tests (Appendix: table 10). Before the tests, all isolates were grown and subcultured three times: 52 in nutrient broth and agar (NB and NA) under aerobic incubation at 37°C for 24 h, three in reinforced clostridial broth and agar (RCM and RCA) in anaerobic conditions at 37°C for 24 h.

3.2.2 Assessment of cassava softening by discriminatory tests

Non-LAB organisms previously isolated during natural fermentations of cassava and from various fermented cassava samples (Chapter I, Sections 1.27, 1.28) were used in this study. Two methods for assessment of cassava softening were examined, that of Obilie and Amoa-Awua, 2003 and a modified version of the same method were used. Fifty two isolates from earlier fermentation experiments were grown and subcultured three times in NB, with corresponding streaking on NA as a purity check at each stage of subculturing, and three were subcultured in and streaked on RCM and RCA respectively. For the non-modified method, a loopful of 24 h colonies of each isolate directly from agar was inoculated over the surface of alcohol sterilised and irradiated cassava pieces, and then

submerged (1:2: w/v) in sterile distilled water and a control of uninoculated cassava was also included. For the modified method, pure cultures of 52 aerobic isolates and 3 anaerobic isolates, were respectively subcultured in NB and RCM, and incubated respectively aerobically and anaerobically at 37°C for 24 hours. For each liquid culture (10^6 CFU/ml), 500 µl were inoculated into the surface of alcohol-sterilised and irradiated cassava pieces, and then the cassava was covered (1:2: w/v) with sterile distilled water in sterile lidded jars. The cassava pieces were fermented aerobically at 37°C for five days. The degree of softening was assessed manually at the end of fermentation by pressing each cassava piece with a sterile metal spatula. The colour of the cassava and the fermenting medium were visually assessed at the end of fermentation. The pH values of the cassava (10% homogenate in distilled water) and the fermenting liquid (direct measurement) were measured after 5 days.

From these experiments, 34 bacteria were selected: 31 were successful in softening the cassava and three responsible for the odour, and the next step was to identify them by phenotyping and genotyping.

3.2.3 Phenotypic identification

Initial phenotyping of 34 isolates was based upon the colony and cell morphology, Gram stain, catalase and oxidase reactions after aerobic growth on NA and anaerobic RCA for 24 h. Carbohydrate fermentation profiles using API 50 CHB strips tests (BioMerieux, Basingstoke, UK) were also determined as described in Chapter II, Section 2.2.2).

3.2.4 Genotypic identification

A total of 34 organisms were characterized by amplification (polymerase chain reaction) of the 16S-23S intergenic transcribed spacer (ITS-PCR), repetitive polymerase chain reaction (rep-PCR) followed by the sequencing of the 16S-rRNA gene.

Extraction of DNA

Pure cultures of each isolate were streaked on NA or RCA and incubated under aerobic and anaerobic conditions, as appropriate, for 24 h at 37°C. The DNA of a pure colony was extracted using InstaGene Matrix as described in Chapter II, Section 2.2.3.

Typing of bacteria by 16S-23S rDNA ITS-PCR

The isolates (34) were typed at species level by 16S-23rDNA ITS-PCR as previously described in Chapter II, Section 2.2.3.

Typing of bacteria by rep-PCR

A total of 34 isolates were further typed at subspecies level by rep-PCR as previously described in Chapter II, Section 2.2.3.

Identification of bacteria by 16S rRNA gene sequencing

The 34 isolates were also identified at genus and species level by DNA sequencing using the procedure described in Chapter II, Section 2.2.3.
After identification, 12 softening bacteria, and one odour producing bacterium were selected and further characterised.

3.2.5 Pectinolytic activity

For each representative softening bacterium (12) and the single odour-producer, pectinolytic activity was determined using the DNS method described in Chapter I, Section 1.2.9.2.

3.2.6 Sugar fermentation profiles

Carbohydrates fermentation profiles were determined using API 50CHB strips by the technique described in Chapter II, Section 2.2.2.

3.2.7 Metabolites produced by softening, and odour-producing strains

Experiments to determine softening using the 12 softening isolates individually were carried out in order to investigate the ability of each softening bacteria and also the odour producer isolate to utilise available carbohydrate and produce organic acids and volatile compounds. Individual pure cultures of softening bacteria and odour producer were grown and subcultured for 24 h, aerobically and anaerobically at 37°C in NB, RCM (Oxoid) respectively. One ml of each culture (10^6 CFU/ml) was inoculated into 100 g of sterile cassava in lidded jars containing 200 ml of sterile distilled water. Uninoculated cassava was included as control. The control and inoculated cassava were incubated aerobically at 37°C for 5 days. Cassava samples of 10 g each were collected initially and at the end of

fermentation, centrifuged at 15,000 x g for 15 min, and supernatants filtered (Millipore 0.2 μm). Six organic acids and four alcohol standards were prepared: one standard containing 10 μl each of formic, lactic, acetic, butyric and propionic acids mixed in 10 ml of deionised water, one standard of each individual acid (10μl) mixed with 10 ml of deionised water and one standard containing 100 μl each of ethanol, methanol and butanol, and one standard of each individual alcohol (100 μl). For each organic acid standard and supernatant, 10 μl were injected into High Performance Liquid Chromatography (HPLC) Perkin Elmer: Series 200; 99:1 ACN, 25 mM potassium phosphate (pH 2.25), UV: 210, Flow rate: 1 ml/min, HPLC columns: SN0170602934, PN: 5138810, GraceSmart RP18 column 250 x 4.6 mm 5u 120A. For separation and detection of volatile compounds, 1μl of each sample was injected into a gas liquid chromatography (GC, Varian 3900, Saturn 2100T, Holland) in the following operating conditions: Oven power: 25°C, split ratio 1 : 200, Column oven: 50°C increased to 100°C at 10°C/min, hold: 0°C/min, total: 5°C/min, FID detector: 280°C, make up flow: 25 ml/min, H_2 flow rate: 30 ml/ min, air flow rate: 300 ml/min, column: 30 M, BPX70-0.25 MM internal diameter, thickness x 1.0 μM film.

3.3 Results and Discussion

Cassava softening, or tissue texture modification during fermentation, is attributable to breakdown of the materials that give plant cells their integrity. The cassava tuber cell wall is made up of cellulose held together by pectin, which is naturally hydrolysed by pectinolytic action (Downie *et al.*, 1998; Bekri *et al.*, 1999; Kapoor, 2002; Dartora *et al.*, 2002).

In this study, organoleptic properties of fermented cassava were assessed and the most desirable properties were the softening and to a lesser extent, the colour. Tables 3.1, 3.2 and 3.3 show the results for the 38 out of the total of 55 isolates that caused some degree of softening of cassava. The remaining 17 were eliminated. The results for the softening assessment indicated that only 38 isolates were able to produce softening in alcohol-sterilised cassava using both liquid culture and direct application of microbial colonies for inoculation, but not in a consistent manner (Table 3.1). The softening profiles exhibited varying degrees of softening, which were described as: (i) normal satisfactory softening, when all cassava pieces underwent an even and smooth tissue modification; (ii) intensive softening, characterised by complete tissue disintegration accompanied by starch sedimentation and presence of fibrous residues; (iii) partial softening, characterised by a mixture of normal softening and localised, patchy or uneven softening of cassava. a

consistent and reproducible intensive softening was achieved over 48 h when irradiated cassava was used with the exception of isolates 43, 412 and 413, which displayed consistent patterns of inability to cause softening using irradiated cassava.

Table 3.1: Assessment of ability of 38 organisms to cause softening of alcohol-sterilised cassava during incubation at 37°C for 48 h

Organism		Organism		Organism		Organism		Organism	
11	+- - -	1A1	- - - -	7A1	+ + + +	7A10	+ + + +	411	+ + + +
12	+ + + +	1A2	+ - + +	7A2	+ + + +	7A11	+ + + +	412	+ + + +
13	+ + + +	1A3	+ + + -	7A3	+ + + +	7A12	+ + + +	413	+ + - -
14	+ + + +	1A4	+ + + +	7A4	+ + + +	7A13	+ + + +	42	+ + + +
15	+ + + +	1A5	- - + +	7A5	+ + + +	7A14	+ + + +	421	+ + + +
16	+ + + +	1A6	+ + + +	7A6	+ - + +	7A15	+ + + +	43	+ + + +
17	+ + + +	1A7	- - + +	7A7	+ + + +				
18	+ + + +	1A8	+ + + +	7A8	+ + + +				
		1A9	+ + + -	7A9	+ + + +				

+: softening -: no softening
Each symbol (+ or -) represents a separate experiment, i.e. there were 4 experiments from each organism.

Contrasting results were obtained using irradiated cassava (Table 3.2). No softening was observed in isolates 411, 412, 413, 7A11, 11 and 43 in irradiated cassava, whereas in alcohol-sterilised cassava, they caused some degree of softening.

Table 3.2: Assessment of ability of 38 organisms to cause softening of irradiated cassava during incubation at 37°C for 48 h

Organism		Organism		Organism		Organism		Organism	
11	- - - -	1A1	+ + + +	7A1	+ + + +	7A10	+ + + +	411	+ + + +
12	+ + + +	1A2	+ + + +	7A2	+ + + +	7A11	- - - -	412	- - - -
13	+ + + +	1A3	+ + + +	7A3	+ + + +	7A12	+ + + +	413	- - - -
14	+ + + +	1A4	+ + + +	7A4	+ + + +	7A13	+ + + +	42	+ + + +
15	+ + + +	1A5	+ + + +	7A5	+ + + +	7A14	+ + + +	421	+ + + +
16	+ + + +	1A6	+ + + +	7A6	+ + + +	7A15	+ + + +	43	- - - - -
17	+ + + +	1A7	+ + + +	7A7	+ + + +				
18	+ + + +	1A8	+ + + +	7A8	+ + + +				
		1A9	+ + + +	7A9	+ + + +				

+: softening -: no softening
Each symbol (+ or -) represents a separate experiment, i.e. there were 4 experiments from each organism

Following these results, it was concluded that use of irradiated cassava inoculated with a liquid culture inoculum (modified method) led to a high degree of softening with reproducible results, compared with alcohol-sterilised cassava using a direct inoculum of colonies, unsatisfactory and irregular softening. Consequently, the modified method, the results of which are displayed in Table 3.3, shows that 31 individual isolates were able to cause reproducible softening of irradiated cassava over 48 h. Some studies showed that cultures of softening bacteria were not effective in causing softening as individual cultures, only when applied in mixed cultures (Oyewole, 1990). Another significant observation in this research was that the occurrence of both a normal satisfactory and an intensive softening was generally characterised by high pH values, up to 11.55 of the liquid medium. Lower pH values (up to 7.42, with a minimum of 4.72) were recorded in the softened cassava itself (Table 3.3).

Three isolates (43, 412, 413) were not able to cause softening, but were responsible for production of the characteristic odour. Moreover, these three isolates resulted in pH values

below 5.0 in the medium and the cassava itself. The observation of a butyric odour suggests acid formation which explains the lower pH values. However, this was not the case with the finding of Okafor *et al.* (1984) who stated that LAB were responsible for the development of the odour.

The colour of the fermented cassava was organoleptically associated with the softening strains and the degree of softening: white and brownish cassava pieces were generally produced by slimy softening bacteria, and brown by non slime-forming strains, accompanied by higher pH values (Appendix 12, 13 and 14). Cassava pieces were white each time there was no softening and lower pH values were induced. Other influential factors of colour such as the duration had been reported, and Oyewole *et al.* (2001) stated that there was no significant colour differences in cassava fermented within 24 – 96 h. The same authors also reported that the intensified white colour was brought out mainly by a prolonged fermentation. Cassava colour profiles produced by controlled fermentations were different and opposite to profiles obtained at the end of natural fermentation (Chapter I, Section 1.3).

Table 3.3: Degree of softening, odour production and pH values induced by inoculation of irradiated cassava with individual cultures during 48 hours at 37°C

Softening/odour producing strain	Softening occurrence or odour production	pH fermented cassava	pH fermenting medium	Colour visual attributes cassava	medium
1A1 (slimy)	+, normal	6.58	6.90	White	clear
1A2	+ with tissue disintegration	6.46	10.30	brown	brown
1A3 (slimy)	+ with tissue disintegration	5.75	10.30	brownish	brownish
1A4	+ with tissue disintegration	6.36	10.30	brown	brown
1A5	+ with tissue disintegration	6.01	10.33	brown	brown
1A6	+ with tissue disintegration	5.65	10.45	white	clear
1A7 (slimy)	+ with tissue disintegration	5.98	10.20	brownish	brownish
1A8	+ with tissue disintegration	5.85	9.67	brown	brown
1A9	+ with tissue disintegration	6.35	9.84	brown	brown
12	+ with tissue disintegration	6.80	10.45	extra brown	extra brown
13	+ with tissue disintegration	6.84	10.15	brownish	brownish
14	+ with tissue disintegration	6.83	11.16	brownish	brownish
15	+ with tissue disintegration	6.81	11.05	brownish	brownish
16	+ with tissue disintegration	6.71	10.39	brownish	brownish
17	+ with tissue disintegration	7.33	9.79	brownish	brownish
18	+ with tissue disintegration	6.25	8.47	brownish	brownish
7A1 (slimy)	+ with tissue disintegration	6.96	8.62	brownish	brown
7A2	+ with tissue disintegration	6.84	8.83	brown	brown
7A3 (slimy)	+ with tissue disintegration	4.72	10.53	white	yellowish
7A4	+ with tissue disintegration	6.91	9.99	brown	brown
7A5	+ with tissue disintegration	6.70	9.46	brown	brown
7A6	+ with tissue disintegration	7.15	8.34	brown	brown
7A7	+ with tissue disintegration	6.58	8.80	white	yellowish
7A8	+ with tissue disintegration	7.42	9.04	brown	brown
7A9	+ normal	6.11	6.75	white	yellowish
7A10	+ with tissue disintegration	6.39	9.35	brown	brown
7A12	+ with tissue disintegration	6.41	10.78	brown	brown
7A13	+ with tissue disintegration	6.63	10.97	brown	brown
7A14	+ with tissue disintegration	6.84	10.77	brown	brown
7A15	+ with tissue disintegration	6.88	11.06	brownish	brownish
411	+ with tissue disintegration	6.29	11.55	white	clear
42 (slimy)	+ with tissue disintegration	6.29	11.55	white	clear
421	+ with tissue disintegration	6.67	11.50	white	clear
412	-, butyric odour formation	4.42	4.80	white	clear
413	-, butyric odour formation	4.54	3.61	white	clear
43	-, butyric odour formation	4.32	4.95	white	clear

Average results of three replicated experiments **+: softening** **-: no softening**

It had been thought that the odour was obligatorily formed whenever softening occurred during natural fermentation (Chapter I). However, that expectation gave an incorrect impression of an association between softening occurrence and production of the characteristic odour. Besides this, overall results revealed organoleptic attributes were indirectly dependent on time and temperature factors. This agreed with previous observations by Ampe *et al.* (1994) who stated that temperature between 28° C and 37°C produced the most favourable organoleptic quality of fermented cassava.

Genotypic identification

After determining and selecting bacteria (31) responsible for softening, and odour formation (3), the next stage was to identify them to species level by phenotypic and genotypic characterisation. Table 3.4 indicates the phenotypic characteristics of the isolates: variable colony and cell morphologies were observed; all were Gram and catalase positive rods and spore formers except isolate 7A9, which was coccoid and a non spore former. Twenty nine of the 31 softening bacteria were biochemically classified by API 50 CHB tests as belonging to the genus *Bacillus* and the remaining two belonged to *Brevibacillus.* The majority were generally identified as presumptive *Bacillus subtilis* (Table 3.5).

Table 3.4: Phenotypic identification of softening organisms and odour-producing isolates

Organism	Colony morphology	Cell morphology	Catalase	Spore	Gram reaction
12	Large; creamy; flat, rough; irregular edge	Rod in chains and clusters	Positive	Positive	Positive
13	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
14	Large; cream; flat; rough; irregular edge	Rod in chains and clusters	Positive	Positive	Positive
16	Large; creamy; rough; flat; irregular edge	Rod in chains and clusters	Positive	Positive	Positive
18	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
1A1	Medium; creamy; elevated; irregular edge	Rod; in chains and clusters	Positive	Positive	Positive
1A2	Large; creamy; rough; flat; irregular edge	Rod in chains and clusters	Positive	Positive	Positive
1A3	Medium; creamy; elevated; slimy; glossy; irregular edge	Elongated rods; singly, in long chains	Positive	Positive	Positive
1A4	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
1A6	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
1A7	Medium; creamy; elevated; irregular edge	Rod; in chains and clusters	Positive	Positive	Positive
1A8	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
1A9	Medium; creamy white; rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A1	Medium; creamy; flat; elevated centre ; slimy; glossy	Short rod in chains and clusters	Positive	Positive	Positive
7A2	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A3	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters Positive		Positive	Positive
7A4	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive

Table 3.4 cont: Phenotypic identification of softening organisms and odour producer isolates

Organism	Colony morphology	Cell morphology	Catalase	Spore	Gram reaction
7A5	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A6	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A7	Medium; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A8	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A9	Small ; creamy white; smooth edge	Short cocci in chains and clusters	Negative	Positive	Positive
7A10	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A12	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A13	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A14	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
7A15	Large; creamy; flat, rough; irregular edge	Rods in chains and clusters	Positive	Positive	Positive
411	Large; creamy; flat; rough; fluffy; irregular edge	Rods in clusters	Positive	Positive	Positive
42	Medium; creamy; elevated; slimy; glossy; irregular edge	Elongated rods in long chains	Positive	Positive	Positive
421	Medium; creamy; elevated; slimy; glossy; irregular edge	Elongated rods in long chains	Positive	Positive	Positive
422	Medium; creamy; elevated; slimy; glossy; irregular edge	Elongated rods in long chains	Positive	Positive	Positive
412	Small ; creamy white; dense and opaque; elevated; smooth	Elongated rod singly; in chains and clusters	Positive	Positive	Negative
413	Small ; creamy white; dense and opaque; elevated; smooth	Elongated rod singly; in chains and clusters	Positive	Positive	Negative
43	Small ; creamy white; dense and opaque; elevated; smooth	Elongated rod singly; in chains and clusters	Positive	Positive	Negative

Average results of two replicated experiments

ITS-PCR allowed typing at species level and five groups of bacteria were observed (Figure 3.1 and Table 3.5). Group 1 comprised 25 isolates, group 2 comprised three isolates, group 3 comprised two isolates, group 4 comprised three isolates and group 5 comprised one isolate.

The rep-PCR confirmed differences between the ITS-PCR groups and allowed differentiation of the bacteria at subspecies level. A cluster analysis of the results (Figure

3.2) allowed differentiation of three subgroups from group 1, one from group 2, one from group 3 and one from group 4.

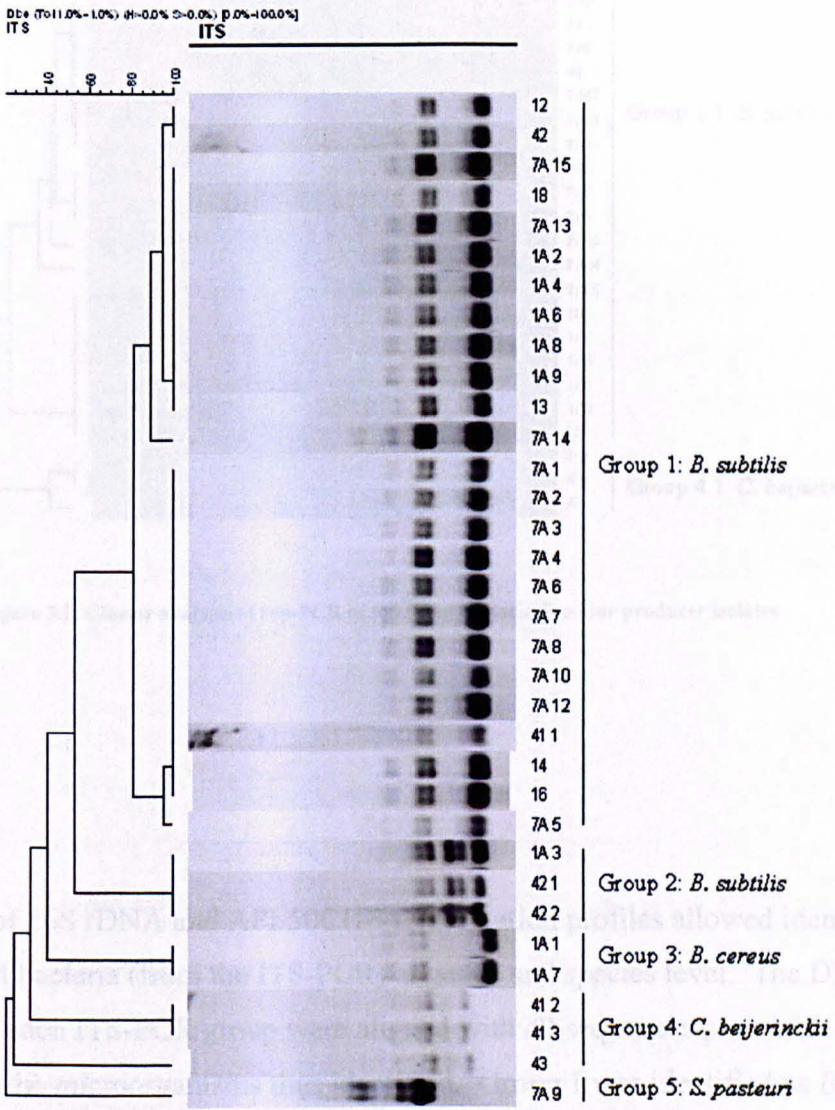

Figure 3. 1: Cluster analysis of ITS-PCR of softening bacteria & odour producer isolates

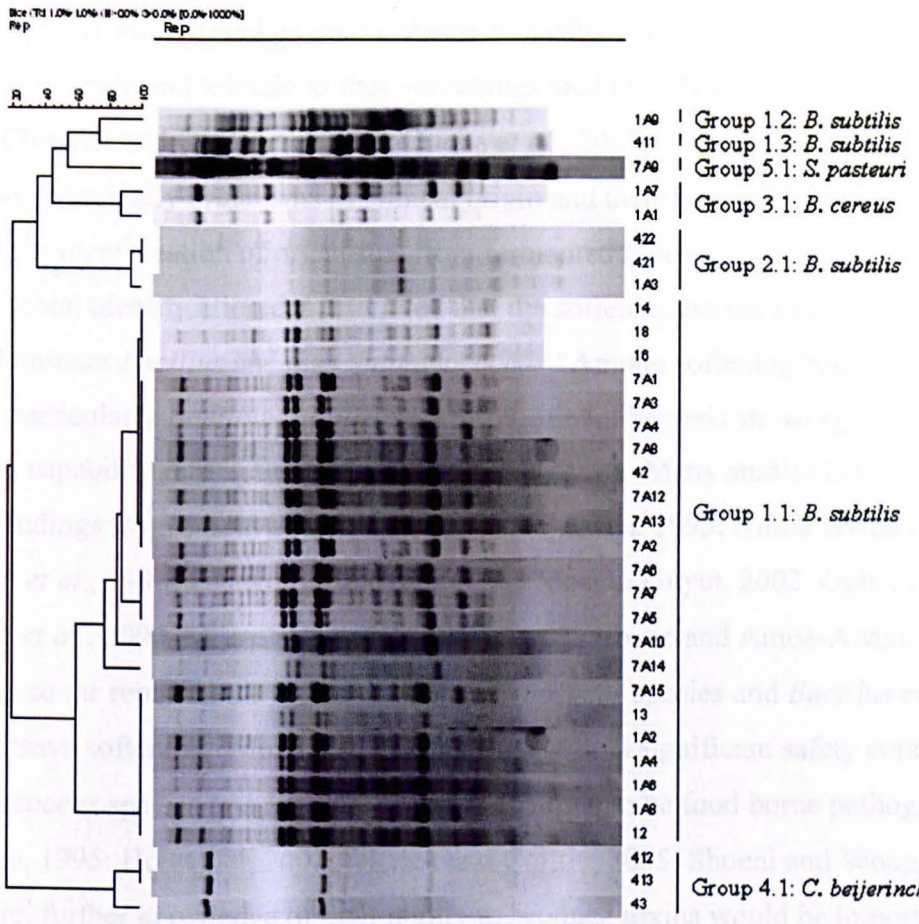

Figure 3.2: Cluster analysis of rep-PCR of softening bacteria & odour producer isolates

Sequencing of 16S rDNA and API 50CHB fermentation profiles allowed identification of each group of bacteria (from the ITS-PCR) at genus and species level. The DNA sequences of isolates of each ITS-PCR group were aligned with all sequences present in the GenBank database and the microorganisms thus identified. Group 1 was identified as *Bacillus subtilis* and comprised three subspecies: the first main subspecies included 23 isolates, the second and the third, one subspecies each. Group 2 was identified as *Bacillus subtilis* and comprised one subspecies. Group 3 was identified as *Bacillus cereus* and comprised one subspecies. Group 4 was identified as *Clostridium beijerinckii* and comprised one

subspecies. Finally group 5 was identified as *Staphylococcus pasteuri* and comprised one subspecies. The phenotypic identification indicated the genus and species; however it was observed that it did not fully agree with the genotypic identification. This is not unusual as the API identification based on the biochemical profiles had been reported by other studies to be less accurate and reliable as they sometimes unable to distinguish between bacterial species (Towner and Cockayne, 1993; Ouoba *et al.*, 2007). Moreover, the API database comprises principally organisms of clinical origin and therefore may not be particularly relevant for identification of organisms from fermented foods.

The microbial identification demonstrated that the softening bacteria belong to two genera: the predominant *Bacillus* and also *Staphylococcus*. Among softening bacteria, *Bacillus* species, particularly *Bacillus subtilis* were the dominant bacteria showing the highest softening capability during the fermentations of cassava. Many studies have reported similar findings from cassava fermentation (Amoa-Awua, 1995; Amoa-Awua *et al.*, 1996; Brauman *et al.*, 1996; Oyewole, 2000, Sanni and Morlon-Guyot, 2002, Ogbo, 2003, Kimaryo *et al.*, 2000, Mante and Amoa-Awua, 2003; Obelie and Amoa-Awua, 2003) but no study has so far reported the isolation of *Staphylococcus* species and *Bacillus cereus* able to cause cassava softening. Moreover, this finding is also a significant safety concern as some *Staphylococcus* species and *Bacillus cereus* are known to be food borne pathogens (Adams and Moss, 1995; Holzapfel, 2002; Dicrick and Coillie, 2005; Shoeni and Wong, 2005). Therefore, further knowledge of their ability to produce toxins would be important. In this study, the odour-producing bacteria were identified with the genus *Clostridium,* and were found not to be involved in softening of cassava. However, these species had been reported to be associated with softening as well as having a role in the production of odour (Keleke *et al.*, 1996). Other fermenting microorganisms such as *Candida krusei*, *Lactobacillus plantarum* and other LAB had been reported to cause the characteristic odour, which is considered offensive by many consumers, and limits marketability of fermented cassava products (Okafor *et al.*, 1984; Oyewole, 1990; Kimaryo *et al.*, 2000; Oyewole and Sobowale, 2008).

Table 3.5: Identification of softening bacteria and odour-producing isolates

Isolate	ITS-PCR pattern type	rep-PCR pattern type	Identification API 50CH	Identification 16S sequencing + ITS-PCR similarity	Similarity %
	Group	Group			
12	1	1.1	*Brevibacillus brevis*	*Bacillus subtilis*	98
42	1	1.1	*Bacillus coagulans*	*Bacillus subtilis*	98
7A15	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	98
18	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
7A13	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
1A2	1	1.1	*Bacillus coagulans*	*Bacillus subtilis*	99
1A4	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
1A6	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
1A8	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
1A9	1	1.2	*Bacillus coagulans*	*Bacillus subtilis*	99
13	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
7A14	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
7A1	1	1.1	*Bacillus coagulans*	*Bacillus subtilis*	97
7A2	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	97
7A3	1	1.1	*Bacillus licheniformis*	*Bacillus subtilis*	99
7A4	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	97
7A6	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
7A7	1	1.1	*Bacillus licheniformis*	*Bacillus subtilis*	99
7A8	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
7A10	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
7A12	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
411	1	1.3	*Bacillus subtilis*	*Bacillus subtilis*	98
14	1	1.1	*Bacillus coagulans*	*Bacillus subtilis*	99
16	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
7A5	1	1.1	*Bacillus subtilis*	*Bacillus subtilis*	99
1A3	2	2.1	*Bacillus subtilis*	*Bacillus subtilis*	99
421	2	2.1	*Bacillus firmus*	*Bacillus subtilis*	97
422	2	2.1	*Bacillus polyfermenticus*	*Bacillus subtilis*	97
1A1	3	3.1	*Bacillus cereus*	*Bacillus cereus*	97
1A7	3	3.1	*Bacillus cereus*	*Bacillus cereus*	97
412	4	4.1	*Bacillus sphaericus*	*Clostridium beijerinckii*	97
413	4	4.1	*Brevibacillus brevis*	*Clostridium beijerinckii*	97
43	4	4.1	*Bacillus sphaericus*	*Clostridium beijerinckii*	97
7A9	5	5.1	*Bacillus firmus*	*Staphylococcus pasteuri*	

Twelve out of 31 identified softening bacteria, and one of three odour producing bacteria were selected and were further characterised for sugar fermentation profiles. Table 3.6 shows little variation of sugar fermentation profiles among softening bacteria. Glucose and fructose were the most frequently fermented sugars, achieved by 100% of isolates. The majority were able to ferment sucrose, ribose and raffinose, and all softening isolates exhibited a common trait of not being able to ferment galactose or starch, and only two degraded lactose and three degraded arabinose. None of the appropriate sugars were metabolised by isolate 43, a strict anaerobe responsible for the production of the characteristic odour during fermentation. Similar phenotypic characteristics of inability or

limited ability to ferment galactose and lactose were also reported by Okafor *et al.* (1984), Mante and Amoa-Awua (2003) and Ouoba *et al.* (2004) following their studies on microbiology of (respectively) cassava retting for *foo-foo* production, antimicrobial interactions during the fermentation of cassava to produce *agbelima*, and characterisation of starter cultures for the fermentation of African locust bean for *soumbala* production. The absence of α-amylase activities displayed by *Bacillus* species in this research could be described as an atypical feature, as these organisms have been reported to be amylolytic strains (Amoa-Awua and Jakobsen, 1995, Amoa-Awua *et al.*, 1996; Jakobsen and Olsen, 1995). However, the inability to ferment starch by some *Bacillus* species such as *Bacillus subtilis* was reported by Okafor *et al.* (1984) and Ouoba *et al.* (2004). Besides, the API Zym test does not always reflect the real enzymatic activity of bacteria, which depends on the bacterial strain and type of substrate. In fact, the paper by Ouoba *et al.* 2003 reported strong lypolytic activity of isolates of *Bacillus pumilus* and *Bacillus subtilis* from *soumbala* using African locust bean oil as substrate; however these isolates did not show ability to produce lipases using API Zym.

Table 3.6: Sugar fermentation profiles of selected cassava softening strains and one odour-producing strain by API 50CHB test.

Strain	Sugar fermentation								
	glucose	sucrose	fructose	ribose	arabinose	raffinose	galactose	lactose	starch
1A5	+	+	+	+	+	+	-	-	-
1A7	+	+	+	+	+	+	-	-	-
1A9	+	+	+	+	-	-	-	-	-
12	+	-	+	+	-	-	-	-	-
13	+	-	+	+	-	-	-	-	-
18	+	+	+	+	-	+	-	-	-
7A1	+	+	+	-	-	+	-	-	-
7A7	+	+	+	-	-	+	-	-	-
7A14	+	+	+	+	-	+	-	-	-
411	+	+	+	+	-	+	-	-	-
42	+	+	+	+	-	+	-	+	-
421	+	+	+	+	+	+	-	+	-
43	-	-	-	-	-	-	-	-	-

Pectinolytic activities of each isolate were detected and Table 3.7 shows comparative absorbance readings relative to various galacturonic acid released following the hydrolytic effects of crude enzymes contained in the fermented cassava filtrates after the reaction. This indicated that polygalacturonase activity-polygalacturonic acid in the range 3.0 – 8.0 µmol was detected in the filtrate containing crude pectinolytic enzymes produced by the softening bacteria tested. One unit of polygalacturonase is defined as the amount of enzyme required to release 1 µmol of reducing sugar group (D-galacturonic acid) from substrate. The dinitrosalicylic acid (DNS) reagent method was used for the detection of polygalacturonase activity. The method is based on hydrolytic release of reducing groups from polygalacturonic acid by measuring the residues produced during the breakdown of pectin following the hydrolysis by polygalacturonase enzymes. The polygalacturonases (endopolygalacturonase and exopolygalacturonase) act on α- glycosidic linkages of pectin chains resulting in the generation of oligogalacturonic acid (Wang *et al.*, 1997; Madigan *et al.*, 2000). Detection of abundant pectinolytic activities of yeasts such as *Candida krusei*, *Candida tropicalis*, *Zygosaccharomyces* and *Penicillium anomala* suggested that these microorganisms are associated with the softening of cassava (Amoa-Awua *et al.*, 1997; Oyewole, 2001). *Clostridium* species were implicated in the cassava softening process (Keleke *et al.*, 1996), and among these, a versatile *Clostridium cellulovorans* with cellulolytic and pectinolytic activities were reported to exhibit exceptional abilities to degrade plant cell wall materials (Tamaru and Doi, 2001).

Table 3.7: Detection of polygalacturonase activity by DNS method

Substrate	Absorbance Reading
7A5	0.156
7A7	0.154
7A14	0.150
1A5	0.158
1A7	0.160
1A9	0.155
12	0.227
13	0.230
18	0.231
411	0.170
42	0155
421	0.155
43	1.996

Averaged PGA activity of two replicated experiments
PGA standard (0.8 ml): 2.00 (OD), PGA standard (1.6 ml): 2.51 (OD)

Detection of metabolites

The results by chromatographic analysis demonstrated that three organic acids: acetic, formic and propionic were detected in uninoculated unsoftened cassava, and in cassava inoculated with softening bacteria and also odour producer bacteria. Lactic acid was detected only in uninoculated, unsoftened (control) cassava. Butyric acid, ethanol, butanol and methanol were detected only in cassava inoculated with odour producing bacteria (43, 412 and 413). It had been reported that strict anaerobes such as *Clostridium* species usually ferment glucose to yield butyric acid and alcohols such as butanol. The fermentation associated with these metabolic pathways was reported to be a butyric or butanol fermentation where sugars are converted into pyruvate, then into acetyl-Coenzyme A, then to aceto-acetyl-CoA, then to butyryl-CoA and finally into butyric acid and butanol (Stryer, 1995; Madigan *et al.*, 2000). Therefore, based on the above facts, the particular presence of butyric acid and butanol appeared to have contributed to the characteristic odour as previously reported by Keleke *et al.* (1996).

3.4 Conclusion

Organoleptic properties of fermented cassava were assessed and the most desirable properties were softening or texture modification and pale colour. The cassava softening was found to be unpredicable and characterised by variations between batches and within batches of cassava during natural fermentations.

Among 55 non-LAB isolates tested for ability to cause cassava softening in controlled fermentations, individually-inoculated cultures of 31 isolates caused a consistent, reproducible softening of irradiated cassava, but not in alcohol-sterilised cassava pieces. These isolates belong to two genera: the more predominant *Bacillus* and *Staphylococcus*. Among the *Bacillus* isolates, 28 were identified as *Bacillus subtilis* and showed the highest capability for softening cassava; two were identified as *Bacillus cereus*. One softening bacterium was identified as *Staphylococcus pasteurii*. The characteristic odour was produced by *Clostridium beijerinckii* during controlled fermentation of cassava, and butyric acid and butanol produced by this isolate could have contributed to the odour. There were two degrees of softening: a normal satisfactory softening and an intensive softening accompanied by complete tissue disintegration, and both were characterised by pH values in the range 5.65 – 7.42. The liquid surrounding the cassava was characterised

by extraordinarily high pH values of up to 11.55. Use of softening cultures, degree of cassava softening and induced pH influenced the colour of cassava in controlled fermentation but not natural fermentation. Brown, brownish and white softened cassava were characterised by near neutral pH values in the range 5.75 – 7.42, and white unsoftened cassava by lower pH values (4.32 – 6.58).

All 12 selected softening bacteria displayed a similar sugar fermentation profile but the odour-producer (*Clostridium beijerinkii*) was not saccharolytic. Pectinolytic activity was detected in softened cassava produced by individual cultures of softening strains.

This study demonstrated that softened cassava could be produced with consequent benefits of consistent softening and shortened time, from 72 to 48 h for a quicker softening for optimisation of natural fermentation of cassava. It indicated opportunities to develop starter cultures that can include or exclude odour-producing bacteria to cater for consumer preference.

CHAPTER IV: Controlled fermentations of cassava using selected cultures to identify combinations of organisms suitable for use as starter cultures

4.1 Introduction

The natural fermentation of cassava is triggered by a complex microflora present in the raw substrate and the environment leading to an uncontrollable and unpredicable process in relation to microbiological stability and the quality of the end product. Therefore, the application of a starter culture is the way forward to improve the safety of the end product and guarantee consistency of fermentation and ultimate quality (Mbugwa and Njenga, 1991; Nout *et al.*, 1989; Odunfa and Adewuyi, 1985; Oyewole, 1997; Giraud *et al.*, 1998; Holzapfel, 2002; Miambi *et al.*, 2003; Kostinek *et al.*, 2008).

A starter culture may be described as a preparation containing a high population of viable microorganisms to initiate and accelerate the initial phase of a fermentation process (Holzapfel, 2002). Starter cultures have been found to shorten fermentation time and make the process more predicable by allowing better control of development of desirable characteristics. In this study, these characteristics can be summarised as rapid acidification, inhibitory effects against pathogens, improved toxicological safety and food digestibility (through degradation of toxic components such as linamarin and indigestible oligosaccharides), beneficial activity of potential probiotic properties, and improved organoleptic attributes such as tissue softening, colour appearances and control of odour. The characteristic odour associated with fermented cassava may possibly be linked to butyric acid, a metabolite produced by some fermenting strains, and the odour can be a desirable as well undesirable attribute, depending on consumer preference (Oyewole, 1990, 1992, 2001); Mathew and Moorthy, 1991; Amoa-Awua and Jakobsen, 1995; Brauman *et al.*, 1996; Ogbo, 2003). Most of these desirable attributes are in a way associated with natural fermentation of cassava, but with unpredictable features, therefore it is imperative that potential starter cultures must be compatible with what is expected from a natural or traditional fermentation (for acceptance by indigenous African population) but produce the more desirable properties (Sanni *et al.*, 1994; Holzapfel and Schillinger, 1995; Holzaphel, 1997; Wood, 1997; Harris, 1998; Kostinek *et al.*, 2008). Several criteria applicable to bacteria used as starter cultures include technological suitability, viability, competitive behaviour and stability in mixed cultures. These are important when selecting appropriate starter cultures for fermented products (Klaenhammer and Kullen, 1999; Kostinek *et al.*,

2008). Modern starter cultures are selected either as single or multiple strains belonging to one or more species/genera and specifically adapted to a substrate. A mono-strain culture is defined as a culture containing one strain of a certain species, and multi-strains cultures contain more than one strain of same species or, at least of the same genus (Mulder *et al.*, 2004).

Thirteen LAB having one or more of the above desirable characteristics included the 12 strains found able to cause cassava softening, and one odour-producing bacterium were selected for development of starter cultures in controlled and uncontrolled fermentation of cassava. Starter cultures containing mono cultures and different combinations of cultures of LAB, *Bacillus*, *Staphylococcus* and *Clostridium* strains were developed to study their interactive impact on pH, organoleptic attributes of softening, colour and odour of fermented cassava in controlled fermentation using sterile and non-sterile cassava. Not all controlled fermentations produced the softening of cassava. Following several fermentation experiments, the cassava softening profiles displayed one constant common characteristic: successful softening induced high pH values or alkaline conditions. Consistent complete cassava softening was achieved in a short period of time whenever softening bacteria were applied as sole starter cultures without LAB cultures. In contrast, cassava softening was significantly lacking or limited, or patchy, when LAB were incorporated in the starter cultures. Due to this technological setback, strategies to improve cassava softening were implemented; subsequently five hypotheses were proposed to evaluate whether these would help to enhance or stabilise bacterial softening activity. The first hypothesis was adjustment of the soaking medium to alkaline conditions before using the starter cultures of softening strains and LAB in combination with use of various inoculum concentrations and volumes.

The second hypothesis was based on supplementation of the medium by inclusion of a rapidly-metabolised energy source (glucose) and Lab – Lemco (Oxoid L29) as a source of nitrogen and amino acids. This approach would provide readily metabolisable simple sugars and other nutrients required to achieve rapid robust growth and stabilise metabolic activities (Salminen and Lee, 1995).

The third hypothesis was application of starter cultures containing mono or mixed softening strains and co-cultures of selected predominant *Lactobacillus plantarum* strains mixed with non-*Lactobacillus plantarum* strains.

The fourth hypothesis was a two stage inoculation that requires initiating the fermentation using only softening strains cultures for 24 h, before adding an inoculum of LAB and

leaving the fermentation to continue for an additional 5 days. This action was expected to allow softening bacteria to achieve optimum growth before exposure to nutrient competition and inhibitory effects of LAB.

The fifth hypothesis was to optimise the natural fermentation by backslopping techniques using mono or mixed starter cultures of LAB and softening bacteria to help initiation of fermentation with an opportunity to produce softening of cassava.

The main objective of this study (and culmination of the project) was to develop a starter culture for optimisation of cassava fermentation, consisting of combinations of organisms, selected from among the 13 LAB and 12 softening bacteria that had shown to induce one or more desirable properties, and to a lesser extent, the one odour-producing bacterium. The ultimate choice of cultures would be based on the effect of their interactions assessed on a basis of pH, softening, colour and odour of fermented cassava using the combined cultures.

4.2 Materials and methods

These experiments were aimed at verifying the third hypothesis (above)

The following table lists various combinations of LAB and softening bacteria, bacterial concentration and inoculation volume used in controlled fermentations of cassava for trials for development of starter cultures.

Table 4.3.1: Combinations of starter cultures, inoculation concentrations and volumes ratios

Starter cultures Combination	Initial bacterial Concentration /log	Inoculation volume	Ratio of LAB & softening bacteria
1.Mono cultures of softening bacteria	10^6	10 ml	
2. Mono cultures of LAB + mono cultures of softening bacteria	10^7 10^7	2 ml 2 ml	1:1
3. Two mixed cultures of six and seven LAB + groups of 3 softening bacteria	10^7 10^7	2 ml 10 ml	1:5
4. Mono and mixed cultures of LAB and softening bacteria	10^7 10^7	2 ml 2 ml	1:1
5. Supplementation and pH adjustment + LAB Co-culture and mono-culture of softening strain	LAB: 10^4 (½ concentrated) Soft. strain: 10^7	LAB: 0 -500 µl Soft. strains: 2 ml	0:1 - 4
6. *Lactobacillus plantarum* co-cultures with other LAB strains + mono-culture of softening strain	10^4 10^7	LAB: 100 µl Soft. Strain: 2.5 ml	1:25
7. Backslopping using LAB co-cultures and mono-culture of softening strain	10^4 10^7	LAB: 0 – 500 µl Soft. Strain: 4 ml	

4.2.1 Starter culture combination 1: Mono-cultures of softening

Prior to the investigations using combined cultures, controlled fermentation of cassava using mono cultures of softening bacteria and an odour-producing strain were carried out to reassess and revalidate the reproducibility of the results of cassava softening and production of odour obtained in Chapter III. Therefore the 12 softening strains were each grown and subcultured (10% inoculum) three times for 24 h at 37°C aerobically in NB and NA and the odour-producer was subcultured three times for 24 h anaerobically at 37°C in RCB and RCA. After incubation, each culture was centrifuged at 10,000 x g for 10 min and resuspended in 10 ml ringer's solution volume. Suspensions (10^7 CFU/ml) of 10 ml were each individually inoculated onto 50 g of sterile (irradiated) cassava in sterile lidded jars containing 100 ml of sterile distilled water. Uninoculated cassava was prepared in the same way to act as control. The cassava pieces were incubated aerobically at 37°C for 5 days.

After incubation for 5 days, the softening was assessed by manually pressing a sterile metal spatula against fermented cassava pieces. The pH values of the cassava (10% homogenate in distilled water) and the liquid medium were measured. The colour of cassava was visually assessed and the odour production was evaluated.

4.2.2 Starter culture combination 2: Mono cultures of LAB with mono cultures of softening bacteria

Softening bacteria were each grown overnight aerobically in nutrient broth, and the 13 selected LAB in MRS broth (10% v/v inoculum) anaerobically for 48 h, all at 37°C. For the stock inocula, each culture was centrifuged and at 10,000 x g for 10 min and resuspended in 10 ml ringers solution. Two ml of individual softening inoculum (10^7 CFU/ml) and two ml of LAB (10^7 CFU/ml) cultures were inoculated onto 50 g of sterile cassava submerged in 100 ml sterile distilled water. For the control experiments, cassava pieces were solely inoculated with 2 ml of softening strains suspended in ringers solution. All cassava pieces were incubated aerobically at 37°C for 5 days. Initial and final pH values were measured on cassava samples prepared as in section 4.2.1 (but not the liquid medium) and the degree of softening and the colour were also assessed at the end of fermentation.

To sumarise, the 13 individual LAB were inoculated onto cassava and each one was combined with the 12 softening stains giving a total of 156 evaluations.

4.2.3 Starter culture combination 3: Two mixed cultures of six and seven LAB with groups of three softening bacteria with and without odour-producing culture.

Isolates of LAB were divided into groups for the preparation of two inocula: group 1 was composed of *Lactobacillus paracasei* (D), *Lactobacillus plantarum* (F), *Lactobacillus plantarum* (K), *Lactobacillus plantarum* (S), *Lactobacillus plantarum* (T), *Lactobacillus plantarum* (Z), *Enterococcus casseliflavus* (N), and group 2 contained: *Enterococcus faecium* (C1), *Pediococcus acidilactici* (C2), *Weissella confusa* (R1), *Leuconostoc mesenteroides* (R2), *Lactobacillus rhamnosus* (F1), *Lactobacillus hilgardii* (F2). Each LAB was grown in MRS broth (10/%: v/v inoculum), anaerobically at 37°C for 48 h. Ten ml of each culture were used to prepare the inoculum for the corresponding group. At the same time, mixed cultures each composed of three softening strains were prepared by growing each in nutrient broth (10/%: v/v inoculum) at 37°C for 24 h under aerobic conditions and 10 ml of each were mixed to form a stock inoculum. Two ml of each mixed stock culture inoculum of LAB (10^7 CFU/ml) and 10 ml softening strain stock inoculum (10^7 CFU/ml) were added to 50 g of sterile cassava submerged in 100 ml of sterile distilled water.

In order to evaluate the production of characteristic odour, this experiment was repeated identically, but with following modification: *Clostridium beijerinckii* (43) was grown in RCM (10/%: v/v inoculum) anaerobically at 37°C for 24 h, and 100 µl of the culture (10^6 CFU/ml) were added to each cassava fermentation prepared as above. Uninoculated cassava pieces were prepared as control.

All cassava pieces were fermented aerobically at 37°C for 5 days. The colour of cassava, and liquid medium, were visually assessed, the pH values of the cassava were determined and the production of the characteristic odour was evaluated as described in Section 4.2.1.

4.2.4. Starter culture combination 4: Mono and mixed cultures of LAB and softening bacteria with and without odour-producing culture

LAB and softening bacteria were grown in MRS broth, and NB (10/%: v/v inoculum), anaerobically and aerobically at 37°C for 48 h, and 24h respectively. Two ml of LAB inoculum (10^7 CFU/ml) and two ml of softening inoculum (10^7 CFU/ml) were added to 50 g of sterile cassava submerged in 100 ml of sterile distilled water.

In order to evaluate the production of characteristic odour, this experiment was repeated identically, but with following modification: *Clostridium beijerinckii* (43) was grown in RCM (10/%: v/v inoculum) anaerobically at 37°C for 24 h, and 100 µl of the culture (10^6

CFU/ml) were added to each cassava fermentation prepared as above. Uninoculated cassava pieces were prepared as control.

4.2.5 Starter culture combination 5: Supplementation and pH adjustment of liquid medium, and use of LAB co-cultures and mono-culture of softening bacteria

To enhance growth of softening bacteria, sterile cassava aliquots (50 g) were respectively submerged in the following sterile liquid environments: 100 ml sterile distilled water at pH 6.0, 7.0, 8.0, 9.0, 10.0 adjusted with 0.5M HCL and NaOH; 100 ml sterile distilled water with glucose (2% w/v; G8770, Sigma); 100 ml sterile distilled water with Lab – Lemco (0.2% w/v total nitrogen; 12.4%, amino acid: 2.5%); unsupplemented water was prepared as control. Inoculation volumes of the two stock suspensions of LAB and the suspension of softening strain used to initiate the fermentation are shown in Table 4.3.1. The pH value of cassava, cassava softening and the colour and visual appearance were analysed at the end of fermentation as in Section 4.2.2.

4.2.6 Starter culture combination 6: Selected *Lactobacillus plantarum* co-cultures with other LAB strains, plus mono culture of softening bacteria

Co-cultures of *Lactobacillus plantarum* strains (K and S) with selected individual non *plantarum* LAB were grown in MRS broth (10%: v/v) at 37°C anaerobically for 48 h to determine their preliminary pH values after 5 days. Following the pH results, co-cultures (Ratio 1:1, v/v) of LAB were prepared by mixing 48 h culture of each of the two selected single *Lactobacillus plantarum* cultures with each selected non LAB plantarum. Twenty four hours mono cultures of selected softening bacteria in nutrient broth were prepared as well. Then LAB co cultures (100 µl), and 2.5 ml of softening strain monoculture were inoculated into 50 g of sterile cassava submerged in 100 ml of sterile distilled water. Controls were prepared by inoculating cassava with two no plantarum LAB cultures. The pH values, cassava softening and the colour visual appearance were analysed at the end of fermentation as in Section 4.2.2.

4.2.7 Starter culture combination 7: Backslopping natural fermentation using LAB co-cultures and mono-culture of softening strain

Natural cassava fermentations were set up in combination with backslopping using various volumes ratio of softening and LAB inocula (Table 4.3.1). Therefore, various fermentation batches containing each 50 g of non-sterile cassava pieces submerged into 100 ml of

distilled water containing glucose (2%: w/v) were prepared. Each fermentation batch was inoculated with various volumes between 0 – 500 µl of 48 h selected mixed LAB cultures (10^4 CFU/ml) and 4 ml of 24 h of selected softening strains cultures (10^7CFU/ml). The LAB and softening cultures were obtained by growing (10%; v/v) in MRS and NB broth anaerobically and aerobically at 37C°. Controls were made by fermenting cassava in unsupplemented fermenting medium without backslopping. All cassava were incubated aerobically at 37°C for 5 days before assessing cassava for the softening occurrence and colour appearance, and measuring the pH values as in Section 4.2.2.

4.2.8 Evaluation of controlled fermentation of cassava using a two stage inoculation of softening strains and LAB cultures.

The controlled fermentation models were prepared as in section 4.2.4 with the following modification: first, the cassava pieces were inoculated with selected mono cultures of individual softening strains (12) and left for 24 h at 37°C before adding mixed cultures of selected 48 h LAB. The fermentation was left to continue for a further 5 days. The pH and the softening of cassava were assessed as in section 4.2.2 at the end of fermentation.

4.3 Results and discussion

The results in Table 4.3.2 reconfirms the previous reproducibility of softening of cassava and the ultimate pH values achieved for the softening bacteria: 6.01 – 6.96 for cassava and 8.47 - 11.55 for the liquid medium at the end of fermentation (Chapter III, Section: 3.3, Tables 3.3 and 3.4). A reproducible result for the odour-producer also was obtained. The results conformed to previous reports of high pH induced during alkaline fermentation involving *Bacillus* species in particular *Bacillus subtilis* to produce *Soumbala*, a locust bean product consumed in Burkina Faso (Ouoba *et al.*, 2004). The visual appearance (colour) was affected by the degree of cassava softening and also seemed to be strain dependant in this fermentation model. Well softened cassava pieces were generally characterised by brown colour, partially softened cassava pieces were brownish and fermented but unsoftened cassava were associated with white colour. The brown colouration of fermented cassava has been reported to be undesirable and unappealing for the production of *foo-foo* (Oyewole, 2001). However, these colour observations were different in natural fermentations, where unsoftened cassava was accompanied by a brown

colour and softened cassava by a white colour (Chapter I, Section 1.3) which produced similar colour profiles to those described by Oyewole, (1990, 1991, 2001).

The colour trends demonstrated that brown cassava pieces were produced following inoculation of non-slimy softening bacteria (1A5, 1A9, 12, 13, 18, 7A7, 7A14, 411, 421). White and brownish cassava resulted from slimy softening cultures (1A7, 7A1, 42). The odour producing bacteria (43) induced lower pH, the characteristic odour and white cassava, but did not cause softening. Similar findings were reported by Okafor *et al.* (1984, 1998) who stated that organisms that lowered pH were unable to produce softening in his study of organisms responsible for cassava tissue texture modification.

Table 4.3.2: Starter culture combination 1: Evaluation of pH, degree of softening, colour and odour during controlled fermentation of cassava using 13 individual strains

Softening bacteria	Softening occurrence	pH	pH fermenting	Colour visual attributes	
		cassava	medium	Cassava	medium
1A5	+, tissue disintegration	6.01	10.33	brown	brown
1A7 (slimy)	+, tissue disintegration	5.98	10.20	brownish	brownish
1A9	+, tissue disintegration	6.35	9.84	brown	brown
12	+, tissue disintegration	6.67	11.04	strong brown	strong brown
13	+, tissue disintegration	6.84	10.15	brown	brownish
18	+, tissue disintegration	6.25	8.47	brown	brownish
7A1 (slimy)	+, tissue disintegration	6.96	8.62	brownish	brownish
7A7	+, tissue disintegration	6.58	8.80	white	yellowish
7A14	+, tissue disintegration	6.84	10.77	brown	brown
411	+, tissue disintegration	6.88	11.06	brown	brown
42 (slimy)	+, tissue disintegration	6.29	11.55	white	clear
421	+, tissue disintegration	6.54	9.61	white	clear
***43**	-, butyric like odour	4.32	4.95	white	clear

Average results of three experiments
Intensive softening: +; no softening: - *odour-producer

In this study, the softening bacteria were characterised by loss of stability and failed to express the biochemical properties of tissue degrading or softening displayed previously and discussed in Chapter III. Using equal concentrations of inocula of mono cultures of LAB with mono cultures of softening bacteria had a tendency to cause low pH 2.87 – 4.31, not to produce softening, to yield white cassava, or to produce partial and irregular softening, brownish cassava and pH range of 3.24 – 4.72, higher than the unsoftened fermented cassava. Similar results were reported by Okafor *et al.*, (1984) who confirmed lack of cassava softening in controlled fermentation that induced low pH values following inoculation of *Bacillus* and LAB cultures. All monostrain cultures of 13 LAB and the following mono

cultures of softening strains: 1A5, 1A9, 12, 7A1, 42, 421 failed to produce cassava softening (Tables 4.3.2.1, 4.3.2.3, 4.3.2.4, 4.3.2.7, 4.3.2.10 and 4.3.2.11); this was also observed by Okafor *et al.*, (1984). All monostrain cultures of 13 LAB and softening strains: 1A7, 13, 18, 7A7, 7A14, 411 produced both partial, uneven softening, and no softening at all in most cases (Tables 4.3.2.2, 4.3.2.5, 4.3.2.6, 4.3.2.8, 4.3.2.9 and 4.3.2.10).

It was found that monostrain or multispecies cultures of LAB provided satisfactory fermentation of cassava, with fast acid production and rapid pH fall, as reported by Gilliland and Walker, (1990) and De Vuyst, (1999), and displayed good stability traits compared to results for individual softening cultures. The accelerated acidification achieved by this type of starter culture was reported to constitute a safety aspect that contributes to the improvement of the final product quality (Holzapfel, 1997; Ross *et al.*, 2002).

Amoa-Awua (1995), Amoa-Awua and Jakobsen (1997), Oyewole (1997), Holzapfel 1997, 2002, Kostinek *et al.* (2008), Sanni *et al.* (1998) reported that natural fermentation is an unpredicable and uncontrollable process characterised by variations that can affect the product quality in terms of safety and organoleptic attributes. These authors highlighted the approach of resorting to the use of a starter culture containing carefully selected microorganisms based on desirable biochemical properties, as a technological necessity and benefit to improve the process and make it more controllable. In this research, despite careful selection of mono-strain and multi-species cultures of softening bacteria based on strong capabilities of consistently causing cassava softening, there was not a clear guarantee that they would reproduce the same softening performance in mixed cultures with LAB (Tables 4.3.2.1, 4.3.2.3, 4.3.2.4, 4.3.2.7, 4.3.2.10 and 4.3.2.11). Similar technological issues of compatibility and loss of stability in mixed cultures were reported in other studies on selection of starter cultures appropriate for fermentation in developing countries, such as the fermentation of cassava for the production of *gari*; and probiotic starter cultures used for various food products (Holzapfel, 2002; Kostinek *et al.*, 2008).

Table 4.3.2.1: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 1A5

Starter culture combinations			Cassava pH		Softening	Visual attributes	
LAB	+	Soft. strain	Initial	Final	Occurrence	Cassava	Medium
K	+	1A5	7.89	3.19	-	white	clear
F	+	1A5		3.25	-	white	clear
N	+	1A5		3.26	-	white	clear
S	+	1A5		3.28	-	white +	clear +
T	+	1A5		3.49	-	white	clear
C1	+	1A5		3.50	-	white +	clear +
D	+	1A5		3.51	-	white	clear
Z	+	1A5		3.58	-	white +	clear +
R2	+	1A5		3.68	-	white +	clear +
C2	+	1A5		3.95	-	white +	clear +
F1	+	1A5		4.06	-	white	clear
F2	+	1A5		4.08	-	white	clear
R1	+	1A5		2.87	-	browning: top	clear

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.2: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 1A7

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
T	+	1A7	7.89	3.47	-	white	clear
D	+	1A7		3.51	-	white	clear
F1	+	1A7		4.31	+ / -	white	clear
R1	+	1A7		3.06	-	white +	clear +
K	+	1A7		3.27	-	white +	clear +
Z	+	1A7		3.62	-	white +	clear +
C1	+	1A7		3.65	+ / -	white +	clear +
F	+	1A7		3.94	-	brownish: top	yellowish
S	+	1A7		4.18	+ / -	brownish: top	yellowish
R2	+	1A7		4.72	+ / -	brownish: top	yellowish
N	+	1A7		3.85	+ / -	brown: top	clear
C2	+	1A7		4.33	+ / -	brown: top	clear
F2	+	1A7		3.88	+ / -	brown: top	clear

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.3: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 1A9

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
F	+	1A9	7.89	3.25	-	white	clear
S	+	1A9		3.29	-	white	clear
D	+	1A9		3.43	-	white	clear
T	+	1A9		3.51	-	white	clear
N	+	1A9		3.09	-	white +	clear +
K	+	1A9		3.12	-	white +	clear +
R1	+	1A9		3.15	-	white +	clear +
Z	+	1A9		3.64	-	white +	clear +
F1	+	1A9		3.87	-	white +	clear +
F2	+	1A9		4.02	-	white +	clear +
C1	+	1A9		3.69	-	brown: top	clear
R2	+	1A9		3.82	-	brown: top	clear
C2	+	1A9		4.01	-	browning: top	clear

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.4: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 12

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
T	+	12	7.89	3.24	-	white	clear
K	+	12		3.30	-	white	clear
N	+	12		3.19	-	white +	clear +
S	+	12		3.27	-	white +	clear +
R1	+	12		3.32	-	white +	clear +
C1	+	12		3.62	-	white +	clear +
Z	+	12		3.66	-	white +	clear +
F1	+	12		3.93	-	white +	clear +
F2	+	12		4.06	-	white +	clear +
R2	+	12		3.70	-	brown: top	clear
C2	+	12		3.99	-	brown: top	clear
F	+	12		3.20	-	white	yellowish
D	+	12		3.29	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.5: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 13

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
T	+	13	7.89	3.16	-	white	clear
R1	+	13		3.13	-	white +	clear +
S	+	13		3.26	-	white +	clear +
Z	+	13		3.61	-	white +	clear +
F1	+	13		3.70	-	white +	clear +
C2	+	13		3.93	-	white +	clear +
C1	+	13		3.47	-	brown: top	clear
R2	+	13		3.65	-	brown: top	clear
F2	+	13		4.04	+ /-	brown: top	clear
N	+	13		3.20	-	white	yellowish
K	+	13		3.25	+ / -	white	yellowish
F	+	13		3.31	-	white	yellowish
D	+	13		3.51	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.6: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 18

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
T	+	18	7.89	3.25	-	white	clear
R1	+	18		3.33	-	white +	clear +
S	+	18		3.35	-	white +	clear +
Z	+	18		3.44	-	white +	clear +
C1	+	18		3.60	-	white +	clear +
F1	+	18		3.98	+ / -	white +	clear +
F2	+	18		4.00	-	white +	clear +
R2	+	18		4.25	-	white +	clear +
C2	+	18		3.86	+ /-	brown: top	clear
N	+	18		3.19	-	white	yellowish
F	+	18		3.34	-	white	yellowish
K	+	18		3.34	+ / -	white	yellowish
D	+	18		3.50	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.7: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 7A1

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
S	+	7A1	7.89	3.28	-	White +	clear +
R1	+	7A1		3.31	-	white +	clear +
T	+	7A1		3.50	-	white +	clear +
Z	+	7A1		3.52	-	white +	clear +
C1	+	7A1		3.60	-	white +	clear +
F	+	7A1		3.84	-	white +	clear +
F2	+	7A1		3.97	-	white +	clear +
R2	+	7A1		4.25	-	white +	clear +
C2	+	7A1		3.99	-	White +	clear +
N	+	7A1		3.18	-	white	yellowish
K	+	7A1		3.34	-	white	yellowish
F	+	7A1		3.38	-	white	yellowish
D	+	7A1		3.56	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.8: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 7A7

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
S	+	7A7	7.89	3.27	-	White +	clear +
R1	+	7A7		3.27	-	white +	clear +
Z	+	7A7		3.59	-	white +	clear +
C1	+	7A7		3.59	-	white +	clear +
F2	+	7A7		3.93	-	white +	clear +
C2	+	7A7		3.96	-	white +	clear +
K	+	7A7		3.32	-	brown: top	clear
T	+	7A7		3.39	+ / -	brown: top	clear
R2	+	7A7		3.66	-	brown : top	clear
F1	+	7A7		4.02	+ / -	brown: top	clear
N	+	7A7		3.13	-	white	yellowish
F	+	7A7		3.18	-	white	yellowish
D	+	7A7		3.56	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.9: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 7A14

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
K	+	7A14	7.89	3.37	-	White	clear
T	+	7A14		3.35	-	white	clear
F1	+	7A14		3.90	+ / -	white	clear
C1	+	7A14		3.44	-	white +	clear +
Z	+	7A14		3.71	-	white +	clear +
F2	+	7A14		4.19	-	white +	clear +
N	+	7A14		3.24	+ / -	brown: top	clear
R1	+	7A14		3.37	+ / -	brown: top	clear
R2	+	7A14		3.68	-	brown: top	clear
C2	+	7A14		4.04	-	brown: top	clear
S	+	7A14		3.24	-	white	yellowish
F	+	7A14		3.25	-	white	yellowish
D	+	7A14		3.58	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.10: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 42

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
T	+	42	7.89	3.31	-	White	clear
K	+	42		3.34	-	white	clear
R1	+	42		3.18	-	white +	clear +
C1	+	42		3.63	-	white +	clear +
Z	+	42		3.75	-	white +	clear +
F1	+	42		3.88	-	white +	clear +
C2	+	42		4.00	-	white+	clear +
F2	+	42		4.08	-	white +	clear +
R2	+	42		3.73	-	brown: top	clear
N	+	42		3.21	-	white	yellowish
F	+	42		3.24	-	white	yellowish
S	+	42		3.49	-	white	yellowish
D	+	42		3.62	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.11: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 421

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
K	+	421	7.89	3.34	-	White	clear
T	+	421		3.34	-	white	clear
C2	+	421		3.99	-	white	clear
Z	+	421		3.67	-	white +	clear +
R1	+	421		3.42	-	brown: top	clear
C1	+	421		3.73	-	brown: top	clear
R2	+	421		4.31	-	brown: top	clear
F2	+	421		4.12	-	brown: top	clear
F1	+	421		4.13	-	brown: top	clear
F	+	421		3.26	-	white	yellowish
N	+	421		3.26	-	white	yellowish
S	+	421		3.56	-	white	yellowish
D	+	421		3.73	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Table 4.3.2.12: Starter culture combination 2: Evaluation of pH, degree of softening and colour induced by 13 individual strains of LAB with softening strain 411

Starter cultures combinations			Cassava pH		Softening	Visual attributes	
LAB		Soft strains	Initial	Final	Occurrence	Cassava	Medium
K	+	411	7.89	3.27	-	white	clear
T	+	411		3.36	-	white	clear
R2	+	411		3.65	-	white	clear
F1	+	411		3.68	+ / -	white	clear
C2	+	411		4.05	+ /-	white	clear
R1	+	411		2.99	-	white +	clear +
N	+	411		3.18	-	white +	clear +
T	+	411		3.36	-	white +	clear +
Z	+	411		3.71	-	white +	clear +
F2	+	411		4.02	-	white +	clear +
C2	+	411		4.05	-	white +	clear
F	+	411		3.25	-	white	yellowish
D	+	411		3.66	-	white	yellowish

Average results of three fermentations replicates
Softening: + no softening: - partial softening: + / - Extra white: white + extra clear: clear +

Using multispecies cultures of LAB and various combinations of multispecies cultures of softening bacteria induced low pH values (3.40 – 3.64), white and unsoftened cassava pieces. However, any combination of mono and mixed cultures of LAB and softening bacteria containing monostrain culture of the odour producer yielded the odour (Tables 4.3.4, 4.3.5). Tables 4.3.4 and 4.3.5 reveal that the use of multispecies cultures of LAB combined with multispecies of softening cultures each composed of arranged in groups of three softening strains and one odour-producing bacterium induced low pH of 3.2 – 3.64, i.e. rapid acidification. It was observed that multispecies cultures caused an accelerated acidification compared to mono cultures of LAB. The possible mechanism underlying the enhanced effects of the mixed cultures may be good compatibility and possible synergistic partnership, as reported by Mulder *et al.* (2004). Therefore, the use of this sort of starter culture clearly confers safety benefits, but also has an adverse impact on cassava softening.

There was no softening in all cases with any combination of organisms, however the characteristic odour was evident whenever strain 43 was incorporated with the starter cultures. The production of white cassava was accompanied by a final pH range: 3.40 – 3.64, and extra white cassava, by pH 3.32 – 3.36. Use of various inoculation concentrations and volume ratios of starter cultures containing monostrain or multispecies cultures of LAB showed no evident differences in reduction of pH values and organoleptic characteristics. However, Ogbo (2003) in his study to produce odourless fermented cassava, found that low numbers of LAB and subsequent low acid production played a part in absence of odour formation. Those results revealed a possible association between induced pH values and lack of softening of cassava, including its colour. The tendency in this research was that low or lower pH values were accompanied by the production of unsoftened white to extra white cassava. These observations were different from the characteristics obtained in natural fermentation of cassava.

Table 4.3.4: Starter culture combination 3: Effect of multi-species cultures on pH values, softening and odour of fermented cassava

LAB mixed cultures	Softening + odour producer strain	pH initial	pH average	Colour attribute	Softening and typical odour
Group 1	(1A5,1A7, 1A9)	7.85	3.40	white cassava, clear medium	absent
	(12, 13, 18)		3.46	white cassava, clear medium	absent
	(7A1, 7A7, 7A74)		3.50	white cassava, clear medium	absent
	(42, 421, 411)		3.64	white cassava, clear medium	absent
	(43)		3.52	white cassava, clear medium	no softening, odour positive
Group 2	(1A5, 1A7, 1A9)		3.35	extra white cassava extra clear medium	absent
	(12, 13, 18)		3.35	extra white cassava extra clear medium	absent
	(7A1, 7A7, 7A14)		3.36	extra white cassava extra clear medium	absent
	(42, 421, 411)		3.35	extra white cassava extra clear medium	absent
	(43)		3.32	extra white clear medium	no softening odour positive

Average results of three fermentations replicates
Group 1 LAB: D, F, K, N, S, T, Z Group 2 LAB: R1, R2, C1, C2, F1, F2

Table 4.3.5: Starter culture combination 4: Effect of mono-strain and co-cultures of LAB, softening strains and odour producer on production of characteristic odour.

Any combination of LAB cultures	Combinations of softening strains and odour producer cultures	Odour production
Mono and mixed cultures (2)	mono culture + odour producer	present and strong
Mono and mixed cultures (2)	mixed cultures + mono cultures odour producers	present and strong
Mono and mixed cultures (2)	mono culture of odour producer	present and strong
Mono and mixed cultures (2)	mono and mixed culture of softening strains only	absent

Average results of three fermentations replicates

Table 4.3.6 demonstrates that near neutral pH values, softening and brown colour of fermented cassava were produced in pH adjusted medium (6.0 – 10.0), and in medium with and without added glucose (2%) and Lab-Lemco (0.2%). Low concentrations (10^4 CFU/ml) in small inoculum volumes (0 – 100 µl) of LAB with high concentrations of softening bacteria (10^7 CFU/ml) in relatively large inoculation volumes (2 – 2.5 ml) induced a high degree of softening, pH values near neutral (5.75 - 7.15) and brown cassava. Larger inoculation

volumes (500 µl) of LAB and softening bacteria (2 – 2.5 ml) induced lower pH values (5.00 – 5.35), partial softening and white cassava. Microbial concentration and inoculation volume had an effect on physiological and organoleptic characteristics of fermented cassava.

The above results demonstrated that the inoculation concentration seemed to be important during the early stage of fermentation; high viable counts of softening bacteria appeared to have been a requirement to achieve the desirable attribute of softening. Similar observations were mentioned by Obilie and Amoa-Awua (2003) who used a high level inoculum of *Bacillus* species (10^9 CFU/ml) during studies on organisms responsible for cassava texture modification. Inoculation of high concentrations in large volumes of softening bacteria, and low concentrations in small volumes of LAB cultures tended to produce softened brown cassava. Highly concentrated inocula of LAB cultures with softening organisms led to unsoftened white cassava independent of inoculation concentration and volumes of softening bacteria cultures. The degree of softening was partly influenced by bacterial inoculum concentrations in controlled fermentation models. However, it was reported that the softening was highly dependent on the size of cassava pieces in natural fermentation and the correlation was that the smaller the cassava piece, the higher the degree of softening (Okafor *et al.*, 1984). In comparison with results shown in Table 4.3.6, use of additives and high alkaline conditions of the medium had no real effect on pH, softening and colour of cassava. The lower the LAB concentration, and the higher the relative concentration of softening bacteria, the higher the degree of softening and browning of cassava.

Okafor and Ejiofar (1990), Okafor *et al.* 1998) and Oyewole (2001) found that the texture, colour and odour of fermented cassava improved and intensified in advanced prolonged fermentation. By contrast, in this research, any combination of sole cultures of softening strains without LAB, produced consistent and rapid softening within 48 h, compared to irregular softening that occurred over three days in fermentations using co-cultures of LAB and mono-cultures of softening bacteria. The time of softening was comparable to that in natural fermentations. This duration of softening was comparable to those reported by Okafor *et al.*, (1984); Oyewole, (1991) and Amoa-Awua, (1995) in natural fermentations where *Bacillus* species were reported to have been detected in large numbers and high metabolic activities on the first day, and increased until the third day, and disappeared subsequently. Although it was reported that inclusion of a quickly-metabolised energy source and additional nutrients for growth enhance microbial stability (Salminen and Lee, 1995), in this study, there was inconclusive evidence to suggest that these additional factors had led to production of desirable microbiological, organoleptic and biochemical characteristics. It was concluded that these variables had no effect on softening, whether or not the fermenting medium was

supplemented, or the pH adjusted. However, it was evident that use of starter cultures containing low concentrations in small inoculation volumes of LAB, and high concentrations in large inoculation volumes of softening bacteria improved the softening results.

Table 4.3.6: Starter culture combination 5: Effect of supplementation and pH adjustment on softening, colour and final pH of fermented cassava

Lactic acid co cultures	Soft. mono culture	Additive	Cassava pH value final	Softening occurrence %	Visual attribute Cassava	medium	Inoculation volume LAB	soft strain
None	7A14	glucose	7.15	100% +	brownish	brown	0 µl	4 ml
K + R2	7A14	glucose	6.60	100% +	brownish	brownish	50 µl	4 ml
K + R2	7A14	glucose	7.09	100% +	brownish	brownish	100 µl	4 ml
K + R2	7A14	no glucose	6.93	100%+	brownish	brown	50 µl	4 ml
K + R2	7A14	lab-lemco	5.75	100% +	brownish	brown	50 µl	4 ml
None	7A14	lab-lemco	6.66	100% +	pinkish	brown	0 µl	4 ml
K + R2	7A14	no lab-lemco	6.91	100% +	brownish	brown	50 µl	4 ml
K + R2	7A14	pH: 6.0-10.0	5.00+0.35	10%	white	brownish	500 µl	4 ml
K + R2	7A14	pH: 6.0-10.0	5.00+1.52	100%	brownish	brownish	50 µl	4 ml
None	7A14	pH: 6.0-10.0	5.00+1.80	100% +	pinkish	brownish	0 µl	4 ml

Average results of three fermentations replicates
100%+: intensive softening

Table 4.3.7 indicates the effect of using co-cultures of selected *Lactobacillus plantarum* strains and other LAB on the pH values and softening. Co-cultures (9) of *Lactobacillus plantarum* strain K with another individual *Lactobacillus* spp. and mono-cultures of the softening strain 7A14 induced a high degree of cassava softening and pH values between 4.33 and 7.20. On the other hand only two co-cultures produced partial softening and lower pH value of 4.38 – 4.5. All co-cultures of *Lactobacillus plantarum* strain S with another individual no-LAB *plantarum* and mono-culture of the softening strain 7A14 produced partial softening and pH values between 4.22 and 7.26.

Lactobacillus plantarum had been reported to be dominant from the start to the end of natural fermentation of cassava and their high performance and its success was attributed to its dominance and synergistic relationships with other organisms (Oyewole, 2001).

The results in table 4.3.7 showed that starter cultures containing *Lactobacillus plantarum* and other individual LAB strains with softening strain 7A14 yielded complete softening of cassava pieces. In contrast, 87% of various combinations of starter cultures composed of LAB with softening bacteria, failed to produce softening, and 13% yielded unsatisfactory partial softening. *Lactobacillus plantarum* strains K and S were specifically selected to include with LAB co cultures in an attempt to obtain the desirable characteristics of low pH and softening of cassava at the end of fermentation. Table 4.3.7 clearly indicated that this type of a starter cultures induced cassava final pH values (4.33 -7.26), an intensive (100% +) as well a partial softening (<100%). Starter cultures containing strain K produced better softening results than those containing strain S. However, apart from displaying a potential good performance in terms of cassava softening, there was a cause for concern from a safety point of view, as fermented cassava pieces showed an undesirably high pH (4.33 – 7.26), i.e. greater than a value of pH below 4.0, recommended for satisfactory cassava fermentation (Holzapfel and Schillinger, 1995; Holzaphel, 1997; Coulin *et al.*, 2006). As consequence, the recommendation was that starter cultures containing co-cultures or multi-species cultures of less than six LAB with softening bacterial cultures in any combination should be approached with caution.

Table 4.3.7: Starter culture combination 6: Effect of *Lactobacillus plantarum* co-cultures of on the pH and softening of fermented cassava

***Lactobacillus plantarum* and LAB co-cultures and their pH values after growth in MRS broth for 48 h**		**Softening bacteria mono culture**	**Cassava pH value initial**	**final**	**Softening occurrence**
K + F	3.83	7A14	6.99	4.57	50%
K + N	3.86	7A14		4.38	50%
K + S	3.94	7A14		4.33	100% +
K + T	3.97	7A14		6.74	100% +
K + R1	4.02	7A14		6.64	100% +
K + R2	3.97	7A14		6.65	100% +
K + C1	3.90	7A14		6.65	100% +
K + C2	3.96	7A14		7.00	100% +
K + F1	3.91	7A14		7.20	100% +
K + F2	3.97	7A14		7.10	100% +
Control: N + R1	3.99	7A14		6.18	100%+
S + F	3.90	7A14	6.99	4.57	20%
S + N	3.89	7A14		4.55	20%
S + T	3.87	7A14		6.30	30%
S + R1	3.94	7A14		4.22	20%
S + R2	3.86	7A14		7.26	50%
S + C1	3.88	7A14		4.58	20%
S +C2	3.86	7A14		4.72	20%
S + F1	3.82	7A14		4.70	20%
S + F2	3.93	7A14		4.71	20%
Control: D + F1	3.62	7A14			0%

Average results of three fermentations replicates
Intensive softening accompanied by tissue disintegration

Table 4.3.8 shows the effect of backslopping the natural fermentation of cassava with starter cultures of LAB and softening strains, and of using glucose supplemented medium on pH and softening. The results indicated that the factors of adding glucose to the medium and backslopping failed to induce softening, but produced white cassava with low pH (3.74 – 3.84).

In this study the effect of backslopping, use of various inoculation volumes (0 - 500µl) of LAB cultures and medium with or without added glucose (2%) induced low pH 3.74 – 3.84 and a white cassava was usually observed in unmanipulated natural fermentation, as well as in controlled fermentation (Table 4.3.4). These variations had not influenced or initiated the natural fermentation to produce the softening; however, the outcome was inconclusive as softening was observed in backslopped fermentations (Okafor *et al.*, 1984; Oyewole, 1992; Amoa-Awua *et al.*, 1996; Holzapfel, 2002; Coulin *et al.*, 2006). Backslopping a natural fermentation is an old tradition still practiced at household level and by small scale food processing industries to produce cassava products, sauerkraut and sourdough (Holzapfel and Schillinger, 1995; Holzaphel, 1997; Harris, 1998; De Vuyst, 1999). This traditional technique is still in use with the intention of shortening the fermentation process, to encourage the expected changes and to minimise fermentation failures such as microbiological instability, which can lead to spoilage and survival of pathogens (Holzapfel and Schillinger, 1995; Wood, 1997; Harris, 1998; Sanni and Oguntoyinbo, 1998; Holzapfel, 2002; Kostinek *et al.*, 2008).

The hypothesis of initiating in advance the softening of cassava using mono-strain and multi-species cultures of softening strains for 24 h before adding the LAB cultures was not effective. The principle of the concept was that omitting LAB at this stage would allow softening bacteria, after an initial lag phase, to grow fast, dominate and cause softening before adding the LAB to initiate fermentation. Thus the softening bacteria would be well-established before the LAB were added (Holzapfel, 2002). The results of this investigation (not shown) revealed that this approach had induced low pH values but had not caused or enhanced softening of cassava.

Table 4.3.8: Starter culture combination 7: Effect of backslopping and glucose supplementation on pH and softening

Lactic acid co cultures	Soft. mono culture	Additive	pH values final	Softening occurrence %	Visual attribute cassava	medium	Inoculation volume LAB	soft strain
Natural fermentation of non sterile cassava with and without glucose supplementation								
None	none	glucose	3.80	0%	white	clear	0 µl	4 ml
None	7A14	glucose	3.84	0%	white	clear	0 µl	4 ml
None	none	none	3.74	0%	white	clear	0 µl	4 ml
None	7A14	no glucose	3.82	0%	white	clear	0 µl	4 ml
K + R2	7A14	glucose	3.74	0%	white	clear	100 µl	4 ml
K + R2	7A14	no glucose	3.79	0%	white	clear	50 µl	4 ml
K + R2	7A14	no glucose	3.74	0%	white	clear	100 µl	4 ml
K + R2	7A14	no glucose	3.76	0%	white	clear	500 µl	4 ml

Average results of three fermentations replicates

4.4 Conclusion

Using solely mono-strain, multi-strain and multi-species cultures of softening bacteria induced near neutral pH values between 6.0 & 6.96 in the fermented cassava and high pH values in the range of 8.47 to 11.55) in the fermenting medium, with a high degree of softening. The colour of cassava seemed to be strain dependent as white and slightly brownish cassava were produced by slimy strains, and brown by non-slimy strains. Starter cultures of mono-strain and multi-species LAB cultures and softening bacteria at equal inoculum concentrations tended to induce low pH between 2.87 & 4.31 white cassava but no softening, or a partial softening and brownish cassava. The colour was affected by the degree of cassava softening. There were no significant differences between the production of biochemical and organoleptic characteristics using mono-strain and multi-species cultures of LAB using various inoculation concentrations and volumes. The LAB displayed good stability traits compared with softening bacteria. The latter had a

tendency to lose their softening ability in mixed cultures. The characteristic odour was evident whenever strain 43 (*Clostridium beijerinckii*) was incorporated in the starter cultures.

The culture concentrations and volume sizes used for inoculation appeared to have significant impact on bacterial development during the early stage of fermentation. Therefore, high concentrations of softening bacteria and low viable counts of LAB were requirements to produce the desirable attribute of softening. A substantial degree of softening and an undesirably high pH of cassava were induced by mono-strain cultures of softening bacteria and small inoculation volumes of LAB co-cultures containing particularly one of the two selected *Lactobacillus plantarum* strains.

Medium supplementation with glucose or nitrogen including amino acids (Lab-Lemco), and pH adjustment of the fermenting medium to alkaline conditions led to neutral pH, softening and brown and pink cassava. These parameters affected the colour of the fermented cassava, but had no clear effect on softening. Initiating fermentation with softening cultures for 24 h before introducing LAB cultures had an effect on pH, but not on softening of cassava. The action of backslopping a natural fermentation of cassava with multi-strains cultures of LAB and a mono-strain of softening bacterial cultures produced desirable characteristics of low pH, white cassava but had not caused softening.

This research provided useful information indicating that starter cultures composed of *Lactobacillus plantarum* strains and any *Bacillus subtilis* could be further studied and technologically developed for the production of desirable characteristics in mixed cultures. However, it was recommended that this type of starter culture of LAB co-cultures and softening bacteria should be approached with caution and investigated further to ensure product safety. The finding also demonstrated potential opportunities to produce odourless, fermented, softened cassava, with or without the characteristic odour, using starter cultures of two *L. plantarum* strains, K and S, *Bacillus subtilis* strain 7A14 and *Clostridium beijerinckii* particularly for production of *foo-foo*. There are also opportunities to produce: fermented, moderately softened and brown cassava pieces using mono strains of any combination of LAB and softening bacteria, and fermented unsoftened white using mono-strain, multi-strains or multi-species cultures of LAB isolates.

CHAPTER V: GENERAL DISCUSSION

5.1 Effect of time, temperature and substrate composition on pH, microbial growth and softening

Natural fermentation of cassava is characterised by two major factors: first, development of a complex microflora that includes LAB, *Bacillus* and *Clostridium* species, *Enterobacteriaceae*, yeasts and moulds, and second, thus the process is reputed to be unpredicable, uncontrollable and highly prone to all sorts of variations (Oyewole, 1997; Giraud *et al.*, 1998; Holzapfel, 2002; Miambi and Ampe, 2003). The effects of time, temperature, substrate composition on pH, microbial growth and on softening were investigated in attempt to study the possible causes driving these variations. It was found that the time and temperature were the factors that influenced pH or acidification rate; microbial composition, growth and survival; and organoleptic attributes of softening. High acid production was metabolically linked to rapid microbial growth of reported acid producing bacteria such as LAB (Amoa-Awua and Jakobsen, 1995; Oyewole, 1997, 2000; Kostinek *et al.*, 2007). Lactic acid bacteria grew optimally at 37C° over 48 h and dominated throughout to the end of fermentation (Miambi and Ampe, 2003). In this temperature conditions (37°C), they display very good ability to decrease the pH to low levels compared with other fermenting conditions of temperatures below 37°C (Chapter I). Similar findings of LAB ability to lower pH were reported by Miambi and Ampe (2003), Holzapfel (2002), Kostinek *et al.* 2008) in their studies on cassava lactic acid fermentation. Occurrence of softening over 72 h tended to correspond with high rapid microbial growth and metabolic activity over 24 h – 72 h at 37°C of reported softening bacteria such as *Bacillus* species (Okafor *et al.*, 1998; Oyewole, 1990; Amoa-Awua *et al.*, 1996; Kimaryo *et al.*, 2000; Holzapfel, 2002). Cassava-softening organisms were characterised by a drastic decline of their populations to undetectable levels for 72 h onwards to the end of fermentation, due to acidic conditions and other inhibitory effects such as bacteriocin formation (Okafor *et al.*, 1984; Brauman *et al.*, 1996; Kostinek *et al.*, 2008).

The results discussed herein (Chapter I) showed the effects of time and temperature on production of desirable characteristics during a natural fermentation process of 5 days duration. It was found that extending fermentation to nine days led to no further increase of pH or microbial growth and this showed that extending fermentation to allow more time to develop or enhance the production of desirable characteristics was not effective. This outcome was probably due to limited metabolic activity observed during microbial

stationary phase at the end of fermentation, and was possibly caused by nutrient limitation or depletion, or by inhibitory effects of metabolite accumulation. A similar observation drew the attention of LeRoy and De Vuyst (2001) who confirmed that microbial growth may slow down or cease due to limited availability or depletion of essential molecules, which provide energy requirements, and building blocks such as amino acids required for cell synthesis.

Although it was found earlier that the softening was influenced by temperature, the occurrence of softening at all temperatures investigated: 25°C, 30°C, 37°C and 40°C and at different pH values seemed to be atypical, unregulated biochemical reaction. In this case, the overall softening results demonstrated that softening or tissue modification was an atypical hydrolytic reaction, and did not follow the usual regulations that modulate the enzymatic reactions such as optimum pH and temperature ranges and substrate specificity in natural fermentation (Stryer, 1995). Looking at the disorderly patterns of softening in natural fermentations, it seemed that softening was a random occurrence that was pH and temperature independent. On the contrary, however, softening was found to be consistently influenced by pH in controlled fermentations, where softening was associated with high pH values. Moreover, experiments reported by others involving cassava softening at 25°C and 30°C suggested that microorganisms such as yeasts and moulds, which require temperatures in this range for optimal growth, may also be responsible for softening at these lower temperatures (Amoa-Awua, 1995, Amoa-Awua *et al.*, 1997; Obilie and Amoa Awua, 2003). With regard to the effect of substrate composition, higher numbers of organisms were found at initially and at the end of fermentation of cassava containing the skin, than in peeled cassava. The increased numbers were probably attributable to a carryover of various microorganisms present on the skin. This demonstrated that the skin was contaminated before harvesting with microorganisms associated with the soil as reported by Adams and Moss (1995), thus increasing the overall microbial load during fermentation.

Increased pH conditions at initial fermentation of cassava with leaves included could have been a consequence of the fact that leaves contain a relatively high proportion of proteins, which are known to alter and increase the pH of a medium due to their buffering power (Stryer, 1995; Adams *et al.*, 1995; Ouoba *et al.*, 2004). Survival of high numbers of *Bacillus* species at the end of fermentation of cassava containing leaves was probably enhanced by increased availability of amino acids derived from leaf proteins. This was not surprising as cassava leaves were reported to contain more protein (6.8 g/100 g) than cassava tuber: 1.2 g/100 g (FAO/WHO, 1991; Bokanga, 1994b; El-Sharkawy, 2004).

The phenomenal high growth and recovery of *Bacillus* species at the end of fermentation was not unusual, as these organisms were reported to possess high proteolytic and peptidase activities, which enhance their dominance during alkaline fermentation for the production of *Bikalga*, a fermented *Hibiscus sabdariffa* (Ouoba *et al.*, 2003).

5.2 Selection of starter cultures of LAB and softening bacteria for fermentation of cassava

One of the aims of this study was to select potential starter cultures to optimise cassava fermentation. Therefore, after examining the factors influencing the natural fermentation, the next step was to isolate, identify and characterise the microorganisms responsible for acidification and softening (Chapter II and III). In these experiments, the phenotypic and genotypic characterisation of acidifying bacteria showed considerable species diversity among LAB (*Lactobacillus, Enterococcus, Pediococcus, Leuconostoc* and *Weissella*), and the overall dominance of *Lactobacillus plantarum* at different stages of fermentation. These identifications agreed with the results of other studies on cassava fermentation involving isolation and identification of LAB (Amoa-Awua *et al.*, 1996; Oyewole, 1997; Miambi and Ampe, 2003; Kostinek *et al.*, 2007, 2008).

The characterisation of LAB based on the ability to cause a rapid acidification, to grow at different pH, to possess antimicrobial and probiotic properties, linamarase activity and ability to degrade oligosaccharides, showed that all LAB isolated in these experiments demonstrated one or more of the above desirable properties and *Lactobacillus plantarum* displayed an outstanding performance. The natural fermentation is generally driven by a complex microflora, where microorganisms such as LAB are commonly found in the early stage, and due to their fast multiplication, they dominate throughout and survive to the end of fermentation due their ability to cause rapid acidification (Miambi and Ampe, 2003). The *Lactobacillus plantarum* strains showed exceptional growth, dominance and superior biochemical performance under most conditions. This was reported to be attributable to their ability to lower the pH of fermenting substrate, to resist highly acidic conditions, their antimicrobial and probiotic activities and ability to hydrolyse and degrade cyanogenic glucosides and oligosaccharides (Oyewole, 1997; Holzapfel, 1997, Holzaphel *et al.*, 2001; Kimaryo *et al.*, 2000; Ogunbariwo and Onilude, 2003; Kostinek *et al.*, 2007, 2008). A display of strongly effective antimicrobial activities against Gram positive and Gram negative bacteria and fungi by mixed cultures of LAB were observed in the fermentation of cassava reported herein. These activities were likely to be a consequence of a combination

of inhibitory effects of different biological factors such as lactic and acetic acids, hydrogen peroxide and bacteriocins produced by individual bacterial metabolic activities (Holzapfel, 2002). It was also reported that the mechanism underlying the enhanced effects appeared to have resulted from combined synergistic effects of differed LAB strains with specific properties, or from enhanced biological activity such as exchange of different metabolites caused by symbiosis of various strains (Drago *et al.*, 1997; Timmerman *et al.*, 2004).

One of the reasons cassava is fermented is to modify and organoleptically improve its texture through the breakdown of pectin during fermentation, and to obtain a desirable sour, tangy flavour (Oyewole, 1990; Amoa –Awua *et al.*, 1996; Kimaryo *et al.*, 2000). The results showed that 31isolates of two *Bacillus* species (*Bacillus subtilis, Bacillus cereus*) and one *Stapylococcus* species were found to cause consistent cassava softening in a relatively short time of 48 h compared to natural fermentation (72 h). *Bacillus subtilis* strains were found to be the dominant species and cause high degree of cassava softening and moreover, the process was readily reproducible. The present results agree with the findings of Essers (1995) and Amoa-Awua (1995) who reported that *Bacillus subtilis* was the dominant species identified as being responsible for the texture modification. Microorganisms such as *Bacillus* and *Clostridium* species, yeasts and moulds have been commonly reported to cause cassava tissue texture modification, and their involvement in the softening biotransformation is facilitated by their ability to produce a wide range of texture-modifying enzymes such as cellulase, polygalacturonase, pectinesterase and pectin lyase (Jakobsen and Olsen, 1995; Amoa-Awua and Jakobsen, 1995; Brauman *et al.*, 1996; Tamaru and Doi, 2001; Kimaryo *et al*, 2000). *Staphylococcus* species and *Bacillus cereus* were previously isolated from cassava fermentations (Holzaphel, 2002; Mante and Amoa-Awua, 2003) and in this study, these species were found to be able to cause the softening of cassava, and apart from this current finding, no previous studies have reported their involvement in the softening of cassava.

Previous research on the mechanism of cassava softening or tissue textural modification has been ambiguous, contradictory and lacking in uniformity. For example, some studies implicated endogenous pectinase, others involved pectinase enzymes produced by *Bacillus* and *Clostridium* species, yeast and moulds in the softening of cassava (Keleke *et al.*, 1996; Oyewole and Sobowale, 2002; Sanni *et al.*, 2003; Ogbo, 2003). Some *Clostridium* strains were reported to cause cassava softening by the above authors, but in this study, a different strain of *Clostridium* species (*Clostridium beijerinckii*) was found to cause only the

characteristic odour of fermented cassava; it was not implicated in the softening. Previous studies on the cause of odour production are vague and inconclusive, for examples: Okafor *et al.* (1984, 1998), Kimaryo *et al.* (2000), Oyewole and Sobowale (2008) reported the involvement of LAB in the development of the odour. This was also reconfirmed by Ogbo (2003) who demonstrated that treating cassava pieces with hydrogen peroxide and adding sodium nitrate before the fermentation starts, affects the LAB, therefore leading to poor growth, low acid production, and eventually, the absence of odour. The above proves that numerous efforts were continuously applied to find methods that improve the sensory quality of fermented foods through manipulation of starter cultures metabolism, and the example is the use of starter cultures of *Lactobacillus plantarum* strain to produce a fermented cassava product with a fruity aroma called *Kivunde*, a Tanzanian product (Kimaryo *et al.*, 2000). In focusing on the sensory characteristics of fermented softened cassava pieces, natural as well as controlled fermentations had tendencies to produce cassava pieces of different colour. Cassava fermentation using sole cultures of softening bacteria, particularly *Bacillus subtilis,* consistently induced a brown colouration of cassava more frequently than in mixed cultures containing LAB. Similar noticeable colour changes were observed in *Soumbala*, a fermented African locust bean produced in alkaline fermentation using *Bacillus subtilis* strains (Ouoba *et al.*, 2005). It is assumed that, due to lack of competition for nutrients, these organisms were able to grow profusely and it is suggested that the heavy growth caused visible accumulations of the bacterial cells on surface of cassava, hence the brown appearance could be related to that, or to metabolitic activities which induce pigmentation. Other influential factors on colour development, such as the duration of fermentation have been reported, and according to Oyewole (2001), a prolonged fermentation was found to intensify the (desirable) white colour of cassava pieces. These results show huge differencies between the cassava colour profiles produced by controlled fermentations using starter cultures and by natural fermentation. To highlight the significance of organoleptic attributes of fermented cassava, a consumer's preference survey demonstrated that fermented softened white cassava pieces are highly regarded in Sub-Sahara Africa (Oyewole, 2001).

5.3 Development of a starter culture by controlled fermentation of cassava

Lactic acid bacteria and softening bacteria were isolated from fermented cassava, and based on characteristics of inducing desirable changes in cassava , were characterised and 26 were selected for the starter culture development trials Chapter III. Therefore, their ability to adapt well to the cassava substrate suggests that their utilisation as potential starter cultures would produce a fermented cassava with defined and consistent characteristics and help to accelerate the process (Klaenhammer and Kullen, 1999; Kostinek *et al.* 2008). However, this was not always the case, as trials of starter cultures containing equal concentrations of LAB and softening bacteria cultures produced low pH values, white cassava, but only partial or no softening, compared to starter cultures containing a lower concentration of LAB and high concentration of softening bacteria. Similar high inoculation levels between 10^7 & 10^9 CFU/ml of *Bacillus* species were used to cause the softening (Obilie and Amoa-Awua, 2003) in the study on microorganisms responsible for texture modification in fermentation of cassava for the production of *akyeke.*

The reason why the softening failed to occur may be because of low viability of the softening bacteria, lack of competitiveness and failure to establish themselves as dominant during fermentation. It appeared that softening bacteria in this research failed to reach sufficient numbers to be able to cause the softening transformation. The lack of growth was probably caused by nutrient limitation due to microbial competition in mixed cultures, or by inhibitory effects as a consequence of highly acidic conditions, or other antimicrobial agents such as bacteriocins.

The softening bacteria seemed to be outnumbered, overpowered and dominated by substantial growth of LAB, with simultaneous production of acids known to be detrimental to *Bacillus* species (Okafor *et al.*, 1984; Oyewole, 2000). Coulin *et al.* (2006) confirmed a decrease of *Bacillus* species to below detection level to be a consequence of increased acid production during fermentation. In mixed cultures containing low concentrations of LAB and high concentrations of softening bacteria, the latter ones seemed to acquire stability, competitiveness and regain their ability to grow in high numbers, hence initiating metabolic activities that cause softening (Holzaphel, 2002). This clearly indicated that inoculation concentrations of microorganisms in mixed cultures is of great importance, and must be given careful consideration in order to capitalise on the characteristics of each culture to produce the desired quality of fermentation.

Ability to cause rapid acidification as well as softening are two of the desirable characteristics of starter cultures for optimisation of cassava fermentation: accelerated acid production was achieved when starter cultures with high concentrations of LAB (Kostinek *et al.*, 2008) and equally or less concentrated softening bacteria were used. In this research, softening of cassava was produced after using starter cultures of low concentrations of LAB combined with a high concentration of softening bacteria. A rapid softening occurred following use of starter cultures of mono- or multi-strain or species of softening bacteria at high and low concentrations.

In this study some results reveal that high concentrations of softening bacteria and low concentrations of LAB were likely to induce softening, albeit with both low and near neutral pH values. However, mono-strain cultures are likely to improve and help the process to be more controllable and predicable in terms of metabolic activity within the culture, but suffer the disadvantage of being prone to bacteriophage infection and mutation. On the other hand, multi-strains or species were reported to produce complex enhanced sensory characteristics due to multiple synergistic effects, to be less susceptible to deterioration and fluctuations and therefore are more suitable as starter cultures (Holzapfel, 2002; Kostinek *et al.*, 2008).

The offensive odour associated with fermented cassava has, from a commercial point of view, severely limited consumer acceptance of its products (Kimaryo *et al.*, 2000). In this study, a mixed starter culture that can produce odourless fermented softened cassava had been achieved, without resorting to chemical treatment as mentioned by Ogbo (2003). The characteristic odour was produced only by mono-strain cultures of odour-producing bacteria (*Clostridium beijerinckii*), and also by any selected combination of starter cultures of LAB and softening bacteria that also contained this organism. However, Okafor *et al.* (1984) and Oyewole and Sobowale (2008) showed that starter cultures of *Bacillus* strains and *Leuconostoc* or *Lactobacillus plantarum* strains were able to produce the characteristic odour.

5.4 Evaluation of use of starter cultures and their benefits

The effects of natural fermentation and the use of starter cultures for the rapid and high acidification rate, softening, colour and odour of fermented cassava were compared in Chapter I and IV.

Rapid acidification rate

According to Holzapfel (2002), Kostinek *et al.* 2008) and Kostinek (2008) the overall objective of applying starter cultures is to improve the fermentation process by making it more controllable and shorter, and to improve the product quality in terms of safety and sensory attributes. Therefore, an accelerated acidification over a 48 h target time has been achieved by fermentation using starter cultures of LAB combined with softening bacteria of well defined combinations and inoculation concentrations. Low acid production of different levels were observed in natural fermentation under similar conditions, while the use of starter cultures containing equal concentrations of LAB and softening bacteria were able to produce consistent low pH (rapid acid production) under similar conditions. Overall results show that the use of the above starter cultures is potentially valuable and seemed to generally improve reproducibility of rapid acidification that guarantees product safety and organoleptic attributes. This would be an important beneficial contribution to optimisation of cassava fermentation (Kostinek *et al.*, 2007, 2008).

Softening of cassava

The softening trends show minor differencies between natural fermentation and controlled fermentation using starter cultures. Natural fermentations require a longer period of 72 h for softening, show frequent softening failures and variations in the degree of softening production. This also occurred with certain combinations of LAB and softening bacteria in the starter cultures, so these combinations would not be beneficial. In contrast, a quick and consistent degree of softening of cassava was produced over 48 h by softening bacteria alone, without LAB. Therefore, these softening cultures were able to shorten the softening process, and would be considered a benefit only for people who are seeking a quick softening in disregard of induced high pH and lack of flavours known to be developed by LAB metabolic activities concomitant problems of toxicity and bacterial safety – could not be recommended. (Okafor *et al.*, 1984; Oyewole, 1997, 2000; Holzapfel, 1997, 2002; Van Kranenburg, 2002; Esayas *et al.*, 2008).

Colour of fermented cassava

There were significant differences between the colour of cassava produced naturally and by using starter cultures containing LAB and softening bacteria. Fermented softened white cassava pieces were produced only by natural fermentation and by co-cultures of low concentrations of *Lactobacillus plantarum* and a mono-strain culture of a high concentration of softening bacteria. Fermented softened and unsoftened brown cassava pieces were produced (respectively) by mono- and multi-cultures of softening bacteria and by natural fermentation. From an organoleptic point of view, and in consideration of other benefits such as safety, and reproducibility (Holzapfel, 2002), use of starter cultures containing co-cultures of *Lactobacillus plantarum* strains and mono-culture of softening bacteria is judged highly beneficial for the fermentation of cassava optimisation. Successful similar starter cultures were reported by Kostinek *et al.* (2008).

5.5 General conclusion

This study reconfirmed the unpredicability and variability that characterise natural fermentation of cassava and showed the time and temperature influence on pH; microbial growth, development and survival; and on cassava softening. Natural fermentation of cassava should not exceed five days, and cassava should be fermented at 37°C for at least for 72 h to achieve safety of the end product. In order to optimise the cassava fermentation, LAB, softening bacteria and odour producer strains were characterised. Thirteen LAB fulfilled the selection criteria of causing rapid acidification, having antimicrobial and probiotic properties, ability to degrade oligosaccharides and to possess linamarase. Twelve bacteria were able to cause a consistent and high degree of softening, and one organism had the ability to consistently produce the characteristic odour. These organisms were all chosen for development of starter cultures.

The starter culture development trials encountered technological issues particularly with softening bacteria in mixed cultures. Their interactions in mixed cultures with LAB at equal inoculation concentrations demonstrated the tendency to lose their ability to soften cassava, a previously satisfactory characteristic in single-strain controlled fermentations. In mixed cultures containing low concentrations of LAB (10^4 CFU/ml), and high concentration of softening bacteria (10^7 CFU/ml), softening bacteria re-established themselves and regained their softening capabilities. Therefore, this study highlighted the significance of inoculation concentration ratio between microorganisms in mixed starter cultures and their influence on desirable characteristics at the end of fermentation.

Use of starter cultures of LAB and softening bacteria has no effect on the duration and therefore did not shorten the fermentation process in term of softening. On the contrary, a quick cassava softening (excluding fermentation) was produced over 48 h compared to 72 h in natural fermentations. Desirable colour attributes of the fermented softened cassava were mostly produced in natural fermentation. The study demonstrated possibilities to develop potential starter cultures of LAB, softening bacteria and an odour producer strain, using various combinations to produce different desirable characteristics such as odour or odourless and white or brown / brownish fermented softened cassava. A comparison of the effects of natural fermentation and the use of starter cultures for the rapid acidification rate, softening and odour of fermented cassava, reveal that use of starter cultures particularly combinations containing *Lactobacillus plantarum* gave the most reproducible results. The study was useful to indicate that potential starter cultures comprise co-cultures of one *Lactobacillus plantarum* (strain K) with one of following strains: *Lactobacillus plantarum* T, *Weissella confusa* R1, *Leuconostoc mesenteroides* R2, *Enterococcus faecium* C1, *Pediococcus acidilactici* C2, *Lactobacillus rhamnosus* F1, *Lactobacillus hilgardii* F2, *Lactobacillus plantarum* S and mono-strain cultures of *Bacillus subtilis,* showed considerable potential for optimisation of fermentation of cassava. The research also showed that further refinements are required before introduction in small-scale fermentations.

5.6 Relevance

Currently, a substantial body of information on use of starter cultures in traditionally African fermented cassava products is available; however little is known about whether their technological properties are successfully expressed in mixed cultures, therefore, this study gave an insight of the mixed culture interactions and relative influence of the inoculum concentration ratio between the microorganisms in mixed starter cultures. At present, there are numerous ongoing researches on development of starter cultures of LAB, but starter cultures combining particularly LAB, softening bacteria and odour producer bacteria are lacking or very limited. Therefore, the study demonstrated possibilities to develop potential starter cultures comprising LAB, softening bacteria and odour producing strains in various combinations to produce different desirable characteristics of the fermented product, such as odour or odourless; white or brown / brownish fermented softened cassava that meets the diverse needs of the African population.

Findings from this study would be useful for further development of potential multifuctional starter cultures in terms of technological properties. The knowledge gained with starter cultures in controlled fermentations, may be also applied in traditional African fermentation.

5.7 Future work

Problems arose when developing starter cultures containing cultures of softening bacteria and LAB: the softening cultures showed deterioration in performance in that they failed to express their softening abilities in mixed cultures. Therefore, the challenge would be to study their stability and feasibility for use in a mixed culture. This would require a study of their interactions in the context of whether they would be able to dominate during controlled fermentation of cassava and cause softening of cassava.

Another possibility is to study whether they lose or gain plasmid-mediated key physiological properties important in fermentation. As multifunctional starter cultures, it is important to develop further work on the probiotic concept in relation to cell line adhesion models and determine their effects on cytokines and cholesterol levels using *in vitro* models of the human intestine.

Finally, it would be valuable to apply selected starter cultures to the undefined and uncontrolled environments of natural fermentations of cassava and study the technological properties and the feasibility of their introduction into small- scale fermentations.

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Appendix

Appendix 1:

Table 1: Operations involved in cassava submerged fermentation and solid state fermentation

Cassava tuber	
Submerged fermentation	**Solid state fermentation**
Peeled, washed, sliced	Peeled, washed, grated
Soaked in double volume water	fermentation without water
Wet sieved or	
Sedimentation	Dewatering
Dewatering / sun-drying	Sun-drying / roasting

Appendix 2:

Cassava products

Gari: Roots are peeled, washed and grated. The mash is placed in bags and squeezed for a minimum of 48 h to allow detoxification and fermentation. The dough is fried dry and stored in bags and sealed plastic packages, rehydrated and eaten with soups, sauces, and sometimes with sugar. Harvesting ⇒ peeling ⇒ washing ⇒ grating ⇒ load in sack ⇒ pressing ⇒ sifting ⇒ roasting ⇒ sieving ⇒ gari.

Fufu: Peeled or unpeeled tubers are soaked in water for 2 -3 days, sundried and millet into flour and cooked in hot water to make a stiff paste.

Chikwangua: Cassava pieces are soaked for 2 -3 days, and fermented pulp is sieved, steamed, kneaded and wrapped in leaves for boiling.

Agbéli mawé: Cassava tubers are washed, peeled and grated to pulp. The pulp is pressed to remove water to 50-55% moisture content, sieved to remove fibrous materials, and milled into fine flour. This is kneaded into dough that is kept during one to three days at ambient temperature allowing a natural lactic fermentation to take place to allow souring and texture modification. The ferment is cooked into a paste by mixing with hot water before consumption (Nout *et al.*, 1999; Amoa-Awua, 1997).

Akyeke or Atieke: Steamed, sour granulated cassava product where cassava tubers are peeled, washed, grated together with traditional inoculum. The mash is packed onto polythene sacks and allowed to ferment for 5-7 days. Water is added to the fermented meal and screened to decolourise the meal and also to remove some amount of starch, and the granules are sundried and afterward steamed before consumption (Obilie *et al.*, 2003). The fermentation is used to obtain the desired texture modification in the product and reduces the rubbery texture.

Appendix 3:

Table 2: Examples of African fermented foods

Name	Country	Substrate	Associated microorganism	Uses
Gari	West Africa	cassava	*Streptococcus* spp. *Lactobacillus* spp Yeast	Main meal
Foo-foo	West and Africa	cassava	Lactic acid bacteria	Main meal
kisra	Sudan	sorghum, millet	Lactic acid bacteria	bread
ogi	West Africa	Maize, sorghum, millet	Lactic acid bacteria, yeast	Beverage (light porridge
mahewu	South Africa	Maize	Lactic acid bacteria	Beverage (porridge)
Kenkey	Ghana	Maize, sorghum	Lactic acid bacteria and yeast	Beverage (porridge)
Kaffir	South Africa	Kaffir corn, Maize	*Lactobacillus* spp, Yeast	Beverage (alcoholic)
Pito	Nigeria, Ghana, Burkina Faso	Maize, Sorghum	*Candida* spp., *Penicillium* spp, *Lactobacillus* spp	Beverage (alcoholic)
Kishk	Egypt	Wheat and milk	Yeast, *Bacillus* spp *Lactobacillus* spp	Beverage (alcoholic)
Chang'aa (Nubian gin)	Kenya	Cane molasses	yeast	Beverage (alcoholic
Iru* (Soumbala, Dawadawa)	West and central Africa	Locust beans	*B. subtilis,* *B. licheniformis* *Staphylococcus* spp.	Condiment
Soy- Dawadawa*	West Africa	Soybeans	*Bacillus subtilis, B. lichenifromis, B.pumilus* *Staphylococus* spp, *Micrococcus* spp.	condiment
Ogiri*	South- eastern Nigeria	Melon seeds	*B. subtilis* *B. megaterium* *B. firmus*	Condiment
Ugba*	Sierra Leone, eastern Nigeria	Oilbean seeds	*B. subtilis* *Staphylococcus* spp. *Micrococcus* spp.	condiment
Kawal*	Sudan	*Cassia obtufalia*	*B. subtilis* *Propionibacterium* spp.	Condiment

Source: (Steinkraus *et al.*, 1986; Wang and Fung, 1996; Odunfa and Oyewole, 1998)

Appendix 4:

Table 3: Factors affecting the development of microbial association in food

Intrinsic factors	Environmental factors	Implicit factors
Nutrients	Relative humidity	Specific growth rate
pH & buffering capacity	Temperature	Synergism
Redox potential	Gaseous atmosphere	Antagonism
Water activity		Commensalism
Antimicrobial constituents		
Antimicrobial structure		

Source: Adams and Moss, 1995.

Appendix 5:

Table 4: Nutritional composition of cassava tuber and leaves

Nutrient	Unit	Cassava tuber 100g	Cassava leaves 100g
Water	g	62.5	80.5
Carbohydrate	g	34.7	9.6
Protein	g	1.20	6.8
Fat	g	0.30	1.3
Calcium	mg	33	206
Iron	mg	0.70	2.0
Vitamin A	I.U	trace	10.000
Thiamine B1	mg	0.06	0.16
Riboflavin B2	mg	0.03	0.30
Niacin	mg	0.06	1.80
Vitamin C	mg	36	265

Source: FAO Food composition Table (Nweke, 2002)

Appendix 6:

Table 5: Principal genera of LAB

Genus	Cell morphology	Fermentation	Lactate isomer	DNA (mole %GG)
Lactococcus	cocci in chains	homo	L	33-37
Leuconostoc	cocci	hetero	D	38-41
Pediococcus	cocci	homo	DL	34-42
Lactobacillus	rods	Homo / hetero	DL, D, L	32-53
Streptococcus	cocci in chains	homo	L	34-46
Enterococcus	cocci in chains	homo	L	38-40

Other LAB genera: *Carnobacterium, Vagococcus, Aerococcus, Tetragenococcus, Alloiococcus, Weissella.* Source: Adams and Moss, 1995.

Appendix 7:

Table 6: Factors contributing to microbial inhibition by LAB

Low pH
Organic acids
Bacteriocins
Hydrogen peroxide
Ethanol
Nutrient depletion
Low redox potential

Source: Adams and Moss, 1995.

Appendix 8:

Table 7: pH of potential pathogen cultures incubated with LAB cell free supernatants.

Incubation time	*Escherichia coli*	*Salmonella typhimurium*	*Bacillus cereus*	*Staphylococcus aureus*
Initial	4.26 ± 0.01	4.27± 0.03	4.62 ± 0.01	4.45 ± 0.01
24 hours	3.89 ± 0.03	3.83± 0.06	4.19± 0.19	4.06± 0.13
48 hours	3.99 ± 0.12	3.95± 0.15	4.32 ± 0.17	4.19 ± 0.11

pH values: mean values of three experiments

Appendix 9:

Table 8: pH measurement of potential pathogen cultures incubated without LAB cell free supernatant

Incubation time	*Escherichia coli*	*Salmonella typhimurium*	*Bacillus cereus*	*Staphylococcus aureus*
Initial	7.06 ± 0.15	7.05± 0.14	6.68 ± 0.06	6.51 ± 0.11
24 hours	7.05 ± 0.4	7.44± 0.65	7.11± 0.54	7.16± 0.59
48 hours	7.07 ± 0.43	7.41± 0.82	7.04 ± 0.47	6.77 ± 0.36

pH values: mean values of three experiments

Appendix 10:

Table 9: Organisms reported to cause cassava softening

Bacterial	Yeast and mould
Clostridium thermosulfurogenes	*Aspergillus alliaccus*
Clostridium species	*Aspergillus niger*
Bacillus subtilis	*Aspergillus oryzae*
Clostridium thermosaccharolyticum	*Fusarium coeruleum*
Clostridium thermocellun	*Mucor rodemsus*
Clostridium butyricum	*Neurospora sitophila*
Bacillus pumilus	*Rhizopus oryzae*
Bacillus licheniformis	*Saccharamyces cerevisiae*
Corynebacterium	
Erwinia carotovora	
Pseudomonas solanacearum	
Streptomyces scabies	

Appendix 11:

Table 10: Identity code of non LAB isolates

	Identity code		
11	1A1	7A1	411
12	1A2	7A2	412
13	1A2	7A3	413
14	1A3	7A4	42
15	1A4	7A5	421
16	1A5	7A6	43
17	1A6	7A7	
18	1A7	7A8	
2	1A8	7A9	
3	1A9	7A10	
4	2A	7A11	
5	3A	7A12	
6	4A	7A13	
7	5A	7A14	
8	5A	7A15	
9	6A	8A	
		9A	

Appendix 12:

Table 11: Organoleptic attributes of colour of cassava and its pH by softening cultures

pH	White cassava clear medium	Extra white cassava extra clear medium	White cassava yellowish medium	Brown cassava clear medium	Softening strain
Range	2.87 – 4.06	3.28 – 4.08			1A5
Average	3.38	3.68			
Range	3.47 – 7.31	3.06 – 3.62	3.85 – 6.88	3.94 – 6.72	1A7
Average	3.00	3.40	5.02	4.95	
Range	3.25 – 3.51	3.08 – 40.2		3.69 – 4.01	1A9
Average	3.37	3.45		3.84	
Range	3.24 – 3.30	3.19 – 4.06	3.20 – 3.29		11
Average	3.27	3.57	3.25		
Range	3.25 – 3.25	3.13 – 3.93	3.20 – 3.51	3.47 – 4.04	
Average	3.25	3.47	3.32	3.72	13
Range	3.25 – 3.25	3.33 – 4.25	3.19 – 3.50	3.86 – 3.98	18
Average	3.25	3.66	3.36	3.92	
Range		3.28 – 3.99	3.18 – 3.56	3.66 – 3.66	7A1
Average		3.63	3.37	3.66	
Range		3.27 – 3.96	3.18 – 3.56	3.32 – 4.02	7A7
Average		3.60	3.29	3.73	
Range	3.35 – 3.90	3.44 – 4.19	3.24 – 3.58	3.24 – 4.04	7A14
Average	3.54	3.78	3.37		
Range	3.31 – 3.34	3.18 – 4.08	3.21 – 3.62	3.73 – 3.73	42
Average	3.33	3.11	3.39	3.73	
Range	3.34 – 3.99	3.67 – 3.67	3.26 – 3.73	3.42 – 7.13	421
Average	3.56	3.67	3.45	4.74	
Range	3.27 – 4.05	3.18 – 4.02	3.18 – 3.66		43
Average	3.60	3.50	3.36		

Results (range) of three experiments replicates

Appendix 13:

Table 13: Softening isolates responsible for the fermented cassava coloration

Visual colour appearance	Softening strain			
Strong browning	1A5, 1A9	7A14	12, 13, 18	411
Slight browning	1A7	7A1		
White		7A7		412, 42, 421, 43

Results of nine controlled fermentations experiments

Table 14: pH profiles of cassava and liquid medium by softening strain mono culture

Cassava pH	**Softening strain inducing high pH**			
High pH values: 5.98 – 6.96	1A7, 1A7, 1A9	7A1, 7A7, 7A14	12, 13, 18	411, 42
Low pH values: 4.32 – 4.54	412, 421, 43			
Liquid medium pH	**Softening strain inducing high pH**			
High pH values: 10.31 – 11.5 3	1A5, 1A7	7A14	12, 13	411, 42
Medium pH values: 8.47 – 9.84	1A9	7A1, 7A7	18	
Lower pH values 3.61 – 4.95				412, 43

Results of nine controlled fermentations experiments

Appendix 15:

***Clostridium* species major end products from pyruvate**

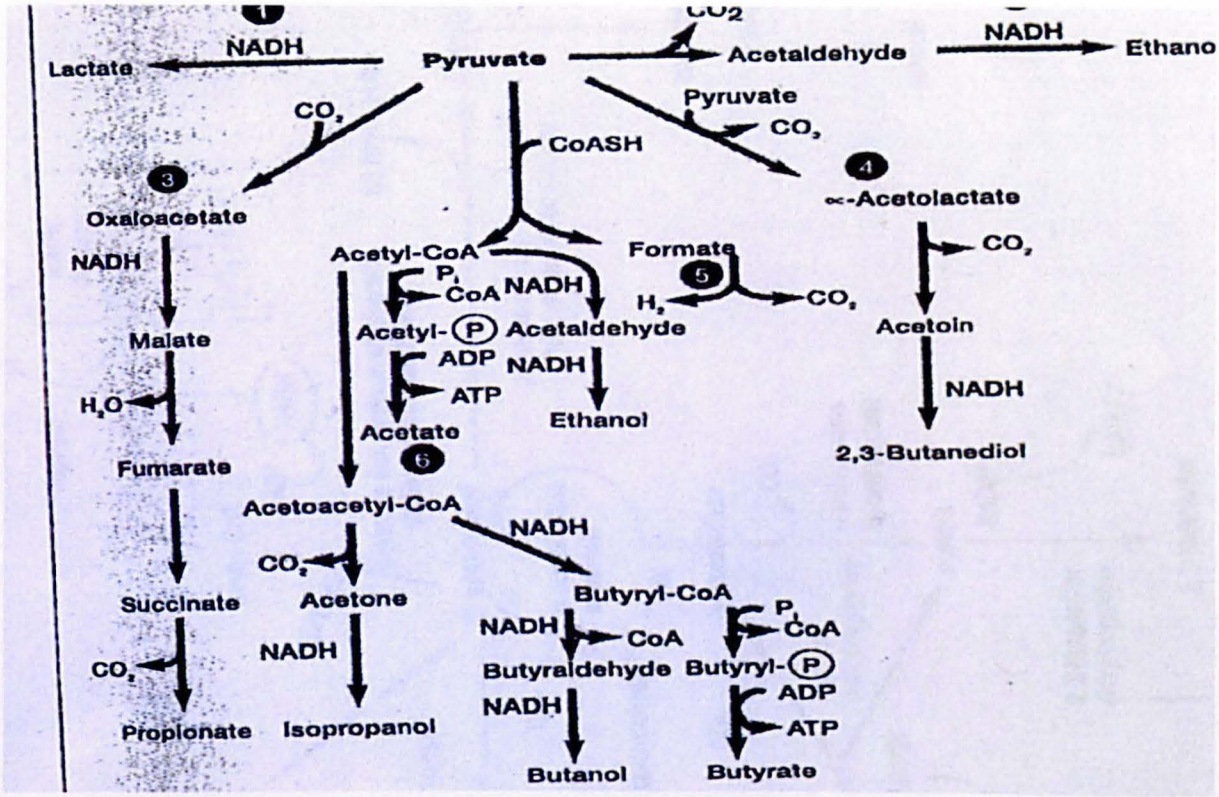

Figure 2: Some common microbial fermentation pathways (Madigan *et al.*, 2000)

1. **Lactic acid bacteria (*Streptococcus, lactobacillus* species), *Bacillus* species**
2. **...Yeast, *Zymomomonas* species**
3. **Propionic acid bacteria (*Propionibacterium* species)**
4. ***Enterobacter, Serratia, Bacillus* species**
5. **Enteric bacteria (*Escherichia coli, Enterobacter, Salmonella* species, *Proteus*)**
6. ***Clostridium* species**

Appendix 16:

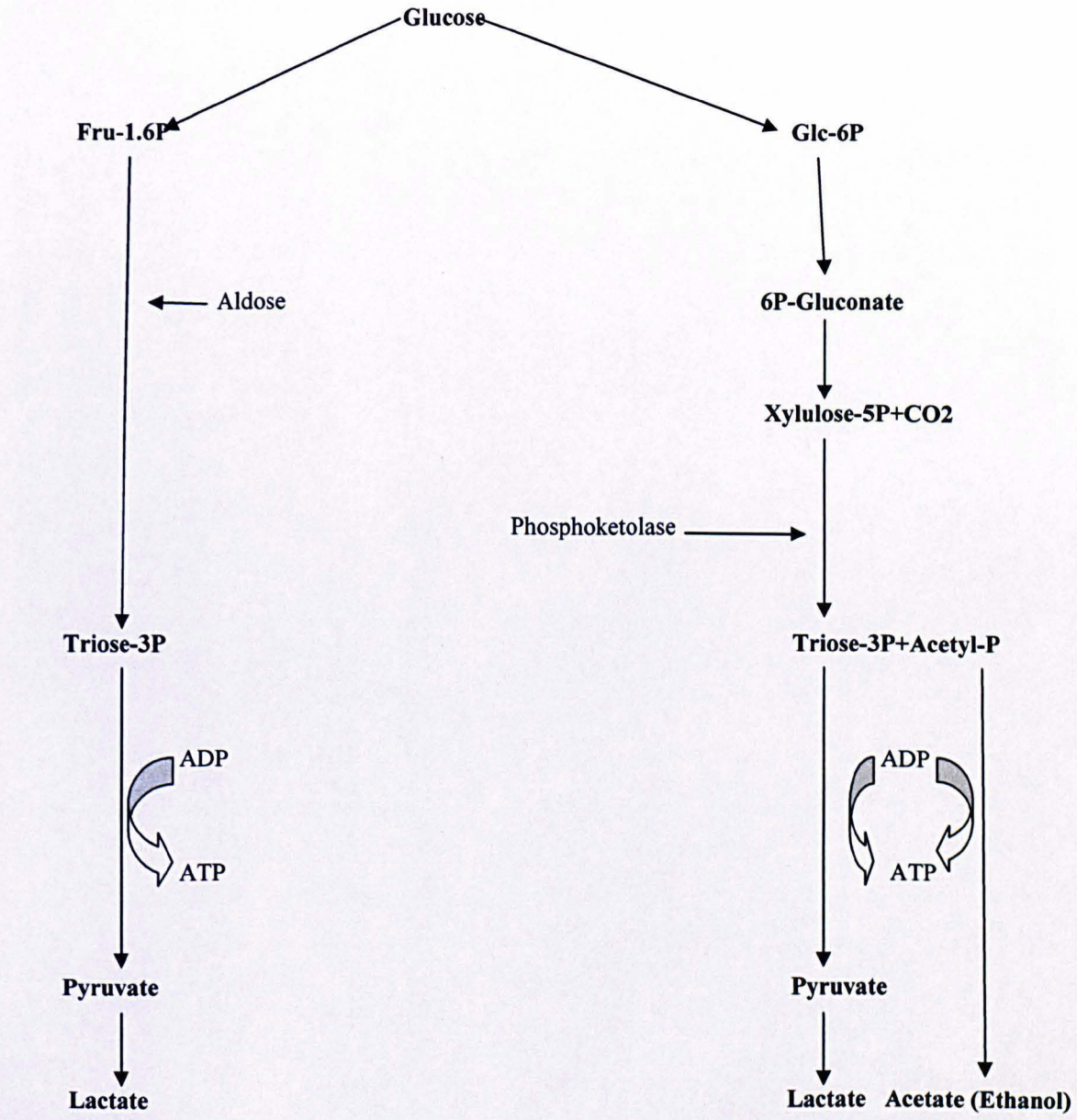

Figure 1: LAB homo- and heterofermentation pathways.

Abbreviation

- = negative

% = percentage

$(NH_4)^2SO_4$ = ammonium sulphate

+ = positive

< = less

> = more

≤ = less than

≥ = more than

& = and

µg = microgram

µl = microlitre

µmol = micromoler

A = initial absorbance

A_0 = final absorbance

Aspl = *Aspergillus fumigatus*

B. cereus = *Bacillus cereus*

B. subtilis = *Bacillus subtilis*

BCS = backslopped

BHT = brain heart infusion

bp = base pair

BP = Bird-Parker agar

C. beijerinckii = *Clostridium beijerinckii*

CaO_3 = calcium carbonate

CCY = Gram negative anaerobes agar

CFU = colony forming unit

cP = centipoise

Cy3 = cyanide dye

Cass= cassava

Clostri= Clostridium

DNA = deoxyribonucleic acid

DNS = dinitrosalicylic acid

dNTP = deoxyribonucleotide triphosphate

DRCM = double reinforced clostridial medium

E. casseliflavus = *Enterococcus casseliflavus*

E. faecium = *Enterococcus faecium*

ECC = *Escherichia coli* chromogenic

EDTA = ethylene diamine tetraacetic acid

EXT = extended

FAO = Food & Agriculture Organisation

Ferm = fermentation

FISH = fluorescent in situ hybridization

g = gram

GIT = gastrointestinal tract

GTBP =

h = hour

H_20 = water

H_2O_2 = hydrogen peroxide

HBA = horse blood agar

HCL = hydrochloric acid

HPLC = high performance liquid chromatography

ITS-PCR = intergenic transcribed spacer-polymerase chain reaction

K_2HPO_4 = potassium phosphate

L. higardii = *Lactobacillus higardii*

L. paracasei = *Lactobacillus paracasei*

L. plantarum = *Lactobacillus plantarum*

LAB = lactic acid bacteria

log = logarithm

M = molar

MEA = malt extracts agar

MEB = malt extracts broth

Mac = Macconkey agar

$MgSO_4$ = magnesium sulphate

min = minute

ml = millilitre

mM = micromoler

mm = millimetre

MPN = most probable number

MRD = maximum recovery diluent

MRS = De Man, Rogosa, Sharpe

MRU = Microbiology Research Unit

NA = nutrient agar

NaCL = sodium chloride

NaOH = sodium peroxide

NB = nutrient broth

nm = nanometre

NS = no softened

OD = optical density

P. acidilactici = *Pediococcus acidilactici*

PBS = phosphate buffer saline

PCR = polymerase chain reaction

PEMBA = Polymyxin B, egg yolk *Bacillus cereus* agar

Pen1 = *Penicillium expansum*

PGA = polygalacturonic acid

pH = intracellural pH

PNPG = para-nitrophenyl-beta-D-glucoside

RBC = Rose-Bengal chloramphenicol agar

RCA = reinforced clostridial agar

RCM = reinforced clostridial medium

rDNA = ribose deoxyribonucleic acid

Rep-PCR = repetitive-polymerase chain reaction

RNA = ribonucleic acid

RNA = ribonucleic acid

rpm = revolution per minute

rRNA = ribose ribonucleic acid

S = softened

S. typhimurium = *Salmonella typhimurium*

SAB = sabouraud dextrose agar

SDS = sodium lauryl sulphate

SFP = Shahid Ferguson perfringens

Soft. = softening

Spp = species

TBE = tris borate, ethylene diaminetetraacetic acid

TE = tris-hydrochloric acid ethylene diaminetetraacetic acid

TES = tris-hydrochloric acid ethylene diaminetetraacetic acid sodium chloride

UK = United Kingdom

UV = ultra violet

V = volt

v = volume

VRBGA = violet red bile glucose agar

w = weight

W. confusa = *Weissella confusa*

WHO = World Health Organisation

x = time

XLD = xylose-lysine-deoxycholate

α = alpha

β = beta

ρ = para

ρmol = picamoler