Selection of Lactic Acid Bacteria for use as Starter Cultures in Lafun Production and their Impact on Product Quality and Safety

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Declaration of Original Authorship

I confirm that the work presented in this thesis is my own and the use of materials from other sources was properly and fully acknowledged

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Abstract

Lafun is one of the fermented cassava products of West Africa. However, current production systems, which use spontaneous fermentation, result in variable product quality and reduced safety. Therefore, there is an interest in developing a manufacturing process to overcome these challenges. An important element that could resolve the above issues is the use of starter cultures. Therefore, this thesis focuses on the selection of lactic acid bacteria (LAB) from fermenting cassava that have potential use as starter cultures for lafun production and explores their impact on final product quality and safety. Lactic acid bacteria isolates (n=41) from spontaneous fermentation of cassava were characterised and screened for flavour compounds production. Four strains were identified as having the greatest potential use as starter cultures (n=4) and were genotypically identified as *Weissella koreensis* (2 strains), *Lactococcus lactis* and *Leuconostoc mesenteroides*. These four strains were further screened for antibiotic resistant genes and did not seem to contain any such genes. Following starter culture addition, subsequent processing operations may impact on lafun flavour. Therefore, fermentation time and drying of lafun were investigated to enhance the laboratory production of lafun. Acidification rate of the selected isolates was also assessed to also identify possible starter culture combinations where one strain is a strong acidifier while the other enhances flavour. The impact of the developed starters, singly and in combination, on lafun nutritional quality, rheological properties, volatile flavour profile, NMR metabolite profile, cyanide reduction and microbial load was evaluated. Three cassava varieties (Bitter: IBA30527; Fortified: IBA011371; and Sweet: TMEB117) were fermented, in duplicate, to produce samples for analyses. Fermentation for 48 h without pressing the fermented cassava roots favoured the yield

of desirable flavour compounds. The results support the adoption of fortified cassava for lafun production due to its positive flavour, nutritional and physical properties. *Leuconostoc mesenteroides* resulted in fermented product with the highest proximate values, *W. koreensis*-2 with the best physical properties, and combined cultures of *W. koreensis-1* and *L. lactis* the highest concentration of desirable flavour compounds (ketones). In addition, the concentrations of hydrogen cyanide found in LAB-produced lafun were below the recommended 10 mg kg^{-1}. These findings demonstrate the potential improvements in the safety and quality of lafun that could be achieved if raw materials, starter culture, and processing operations are optimised. In addition, it advances the development of these starter cultures and provide a basis for further research required for their development and adoption.

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Chapter One

1.0 Introduction

1.1 *Background*

Fermentation is a simple reaction that is cost-effective with high specificity and flexible application. In food fermentation, the metabolites generated by the fermenting organisms, especially lactic acid bacteria, inhibit the growth of spoilage and pathogenic organisms resulting in the extension of the shelf life of perishable produce. The metabolites also enhance flavour (Di Cagno et al., 2013). Modern techniques have since taken the technology beyond preservation such that fermentation is optimised towards generating desirable organoleptic properties and improving palatability in food. Food attributes such as flavour, aroma, texture, other physical properties and nutritional profile can be enhanced by fermentation (Terefe, 2016).

Cassava is a plant material that is mainly processed by fermentation. Initially tagged 'the food of the poor,' cassava has become a 21st-century multiuse crop that answers to climate change which is one of the main challenges of low-income countries (Howeler et al., 2013). Though mostly a tropical crop, it has some similarities with potato regarding its inedible starchy state when fresh, bland flavour when cooked, and similar methods of preparation, mashing, frying, boiling, and baking. It is a food security crop in the developing world (Sriwana & Erni, 2015). However, the main challenge facing the widespread usage of cassava roots by processors is because it is bulky and highly perishable, deteriorating within three to four days of harvest resulting in high post-harvest losses of cassava (Saravanan et al., 2016). Cassava, because of this, is often processed into dried forms, mostly after fermentation. When the moisture content and water activity is lowered, it becomes durable and is shelf-stable.

Production of fermented cassava products in Africa is still carried out in a traditional manner in a domestic setting (Huch et al., 2008) with only a few small and medium scale industrial operations. The techniques used are usually laborious and time consuming. Typically, such production utilise spontaneous fermentation with no implementation of good manufacturing practice (GMP) and hazard analysis and critical control point (HACCP) plans. Also, the problem with using spontaneous fermentation is that product quality is highly varied. This variation occurs due to initiation of fermentation by prominent microflora from the raw material, water and environment that vary according to time and place (Kimaryo et al., 2000). Initiation of fermentation may take longer during a spontaneous process therefore Huch et al. (2008) opined that, the risk of failure of the fermentation process is higher which results in the growth of spoilage microbes. The use of starter cultures in control fermentation of cassava is fundamental to achieving a safe product of consistent quality from a reproducible production procedure. Be that as it may, starter cultures should be developed from isolates of strains from previous spontaneous fermentation batch(es) selected for specific purposes or attributes (Edward et al., 2012).

Lafun is one such fermented product of cassava common in the Republic of Nigeria and the Benin Republic. It is traditionally produced by submerged and spontaneous fermentation of cassava. It is a fibrous powdered form of cassava. Two types are produced in Benin: ordinary lafun and Chigan lafun, and the latter differ in production based on the washing operations at critical control points, soaking is done in an enclosed container and soaked cassava pulp being dried on a raised area to prevent dust (Padonou et al., 2009). Nigerians in the southwest consume ordinary lafun, and it is a significant part of their diets, especially in rural areas.

1.2 Statement of Problem

Nigeria is the highest producer of cassava and three different fermented cassava products are produced there: garri, fufu and lafun. Consumption of lafun is dropping in Nigeria, with consumption predominently in rural areas. The drop in consumption of lafun is due to flavour quality defects that occur due to unstable fermentation. This instability is because its production has not benefitted from advances in process technology, especially concerning flavour improvement and quality with a concomitant improvement in safety. Therefore, production of a high quality consistent lafun with enhanced consumer acceptability, would extend the market for lafun, producing benefits to cassava producer and lafun manufacturer.

1.3 Justification for the Project

There is a need to optimise process conditions to enhance product quality regarding stabilising flavour production along with high sensory acceptability to permit full acceptance of lafun as a high-quality processed product. Despite various reports in the literature on lafun production from the bitter form of cassava, there is a scarcity of information on efforts to stabilise the flavour-enhancing process in lafun for maximum product quality and safety. There is also no information regarding the usage of sweet and fortified cassava in lafun production to investigate the impacts of these forms in the nutritional quality and safety of lafun. Therefore, there is an opportunity to standardise the method for production of lafun which is not currently available. In particular, the standardisation of the starter culture for desirable flavour components, rheological properties and safety characteristics, is the focus of this thesis.

1.4 Aims and objectives

1.4.1 General objective

The general objective of this thesis is to identify, screen and select LAB with potential as starter cultures for lafun production from three cassava varieties and determine their impact on product quality and safety.

1.4.2 Specific objectives:

- Isolation and identification of LAB during lafun production;
- Determination of best process conditions for the use of isolates selected based on acidification and flavour yield as the starter in lafun production;
- Production of lafun at laboratory scale using the selected starter cultures.
- Determination of the impact of the selected starter cultures and cassava variety (bitter, sweet and fortified forms) on the flavour, nutritional and physical properties of lafun.
- Assess the safety of lafun produced with the selected starter culture in terms of adequacy of cyanide reduction, potential to confer antibiotic resistance, and microbiological quality

1.5 Structure of the thesis

This thesis is divided into 5 Chapters:

Chapter 1 (Introduction) - Presents the background of the study and highlights both general and specific objectives. It also layout description of each chapter.

Chapter 2 (Literature Review) - Gives an overview of the relevant works on cassava, lafun and lactic acid bacteria regarding optimisation processes and quality assessments.

Chapter 3 (Screening of Lactic Acid Bacteria to Determine Their Potential as Starter Cultures for Lafun Production)- In this chapter, four identified isolates were selected for

their high potential in producing flavour compounds during lafun production. The safety of these selected organisms regarding antibiotics resistance was also determined at the gene level.

Chapter 4 (Optimising Processing of Cassava for the Production of lafun at Laboratory Scale)- This chapter presents the impact of varying processing conditions for lafun production, and the best methods were selected for use in the thesis

Chapter 5 (Impact of Selected Starters and Cassava Varieties on the Quality and Safety of lafun) - Effect of the selected LAB strains and cassava varieties on the physical, microbiological and nutritional properties of lafun was one of the objectives of this chapter. Flavour profile (volatile organic compounds content), metabolites fingerprint and safety (cyanide level reduction) of lafun produced with LAB starter cultures were also estimated.

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Chapter Two

2.0 Literature Review

2.1 Cassava, an indispensable root crop

Cassava, *Manihot esculenta*, has become an essential raw material for the production of many staples in Africa and Asia (Sanni et al., 2009). Although it is generally called cassava, it has different names in different countries (Table 2.1). It is a shrubby but slightly woody, tuberous plant of the *Euphorbiaceae* family (Figure 2.1) which is cultivated mainly for its starchy roots. Though mostly a tropical crop, it has some similarities with potato regarding its inedible starchy state when fresh, bland flavour when cooked, and similar methods of preparation, mashing, frying, boiling, and baking (Kimaryo et al., 2000). This drought-resistant crop has a similar carbohydrate content to plantain and yam. It is an important food crop in the developing world as millions of people consume fermented and unfermented cassava products as a carbohydrate source. Cassava is fermented to produce a range of different products (Figure 2.2). Among these are garri, fufu, lafun, kokonte, agbelima, akyeke, beer, cossettes, chikwangue, kanyanga, attieke, mapanga, kivunde, Kondugbala and makopa in Africa. In Asia the fermented products produced from cassava are peuyeum, pancakes, tapai/tapey singkong, rondho royal/monyos and cake tape (Akely et al., 2010; Kimaryo et al., 2000; Sanni et al., 2009; Echebiri and Edaba 2008).

2.2 Regional use of cassava in the food chain and beyond

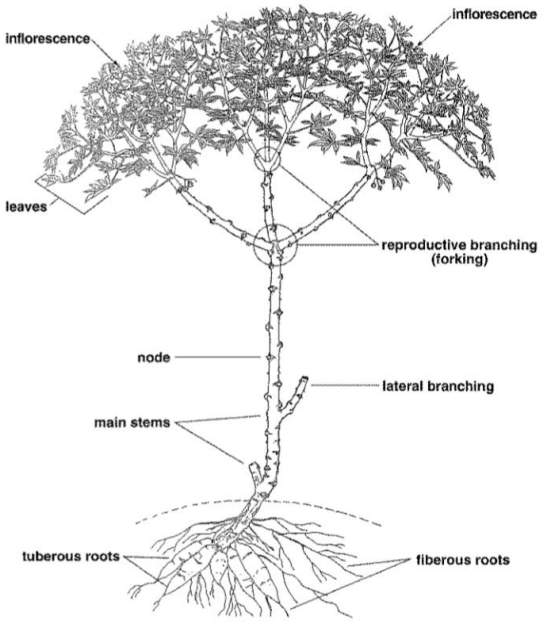
2.2.1 West Africa

Nigeria is the highest producer of cassava in the world (Table 2.2), accounting for about 20% of world production (Oyewole & Phillip, 2006; FAO, 2004). In Nigeria,

Table 2. 1: Names of cassava from different regions

Country	Region	Cassava given name(s)
Spanish America		yucca
Malaysia		tapioca
India		tapioca
	Hindi	sabudana, sago or jawwarisi
	Telugu	karrapendalam
	Malayalam	kappa
	Gujarati	mogho
	Kannada	mara genasu
	Tamil	kuchi kizhangu
Brazil		macacheria or mandioca
Tanzania & Kenya, Uganda & Rwanda, Burundi & Mozambique		muhogo muhogo muhogo
Congo		pondu
Nigeria		cassava
	Southwest Nigeria	ege or gbaguda
	Northern Nigeria	karaza or doyar kudu
	Southern Nigeria	igari
	South-south Nigeria	iwa unene or imidaka
	Southeastern Nigeria	akpu, jigbo or abacha

Sources, (Yadtec, 2013; Iwuagwu, 2012; Nassar, 2007)



A

B

C

Figure 2. 1: (A) Cassava plant diagram (Bokanga, 1999) (B) Cassava plants, and (C) Cassava roots (source: IITA, Ibadan, Nigeria)

apart from the fermented foods stated in section 2.3, cassava is also processed into flour of high quality that is used in pastries, confectionaries, baby foods, chips for animal feeds, and snacks. Cassava syrup concentrate can be used in beverage production while the hydrolytes can be used in pharmaceuticals (Echebiri and Edaba 2008) (Figure 2.3). Cassava has limited utilisation in Sierra Leone. However, its leaves serve as a vegetable source for fufu and boiled cassava. Its roots are also processed into flour cooked into a paste (Sanni et al., 2009). Ghanaians see cassava as most favoured among root crops. It has been used extensively in their brewery and textile industries (Kleih et al., 2013). It does have potential usage in plywood, pastry and pharmaceutical sectors (Adjekum, 2006). The greater use of cassava in central and southern Africa regions like other parts of the world is in the form of food. In Senegal, cassava is not being put into much use as a result of insufficient processing equipment and lack of processing units. Guédé et al. (2013) indicated that the Senegalese people consume cassava as a vegetable in their daily meals, although at a very low level.

2.2.2 Asia

About 74% of cassava had been estimated to be freshly consumed or processed for snacks in Asia. Dried cassava accounts for only 15% while 26% and 0.1% were noted to be used for tapioca cassava flour and cassava flour, respectively (Hermiati et al., 2012). It is well adapted to the continent and formed a secondary staple food in some of the countries. In India, the root is being processed to many dishes, some of which are foods and, in some cases, added to different kinds of gravies. It is sometimes boiled and taken with fresh fish curry or made into pearls that are used for sweet milk pudding, salted khichadi and vada deep-fried patties. On the industrial front, it is used

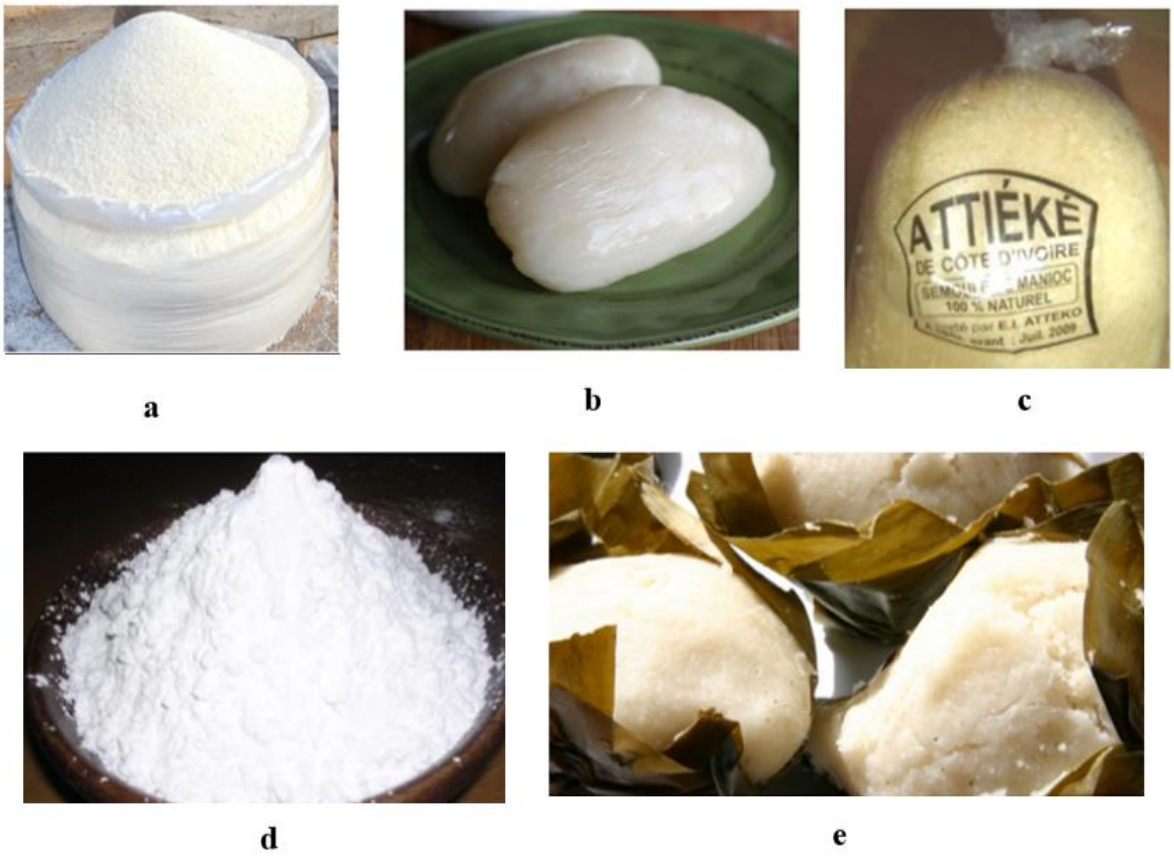


Figure 2. 2: Selected fermented cassava products: (a) gari (forfoeghgari 1.blog); (b) fufu (nigeriagalleria.com); (c) attieke (gastronomieafricaine.wordpress.com); (d) lafun (madamsabi.com) and (e) agbelima (bergamini.be)

Table 2. 2: Ranking of cassava producers' countries in the world by 2013

RANK	Country	Production Quantity (in Tonnes)	Production Value
1	Nigeria	52403455	$5,474,222,000
2	Brazil	25349088	$1,324,020,000
3	Indonesia	24009624	$2,457,975,000
4	Thailand	21912416	$2,151,145,000
5	Democratic Republic of the Congo	15569138	$1,610,134,000
6	Angola	14333509	$1,497,321,000
7	Ghana	14240867	$1,487,643,000
8	Mozambique	10093619	$1,054,409,000
9	Viet Nam	9897912	$1,033,965,000
10	India	8076000	$843,643,000
11	Uganda	4757800	$497,014,000
12	United Republic of Tanzania	4646523	$464,497,000
13	China, mainland	4500000	$423,075,000
14	Cambodia	4368159	$456,310,000
15	Malawi	4259301	$444,939,000
16	Cameroon	4082903	$383,861,000
17	Benin	3600000	$376,066,000
18	Madagascar	3490300	$364,607,000
19	Rwanda	2579399	$269,451,000
20	Paraguay	2453837	$131,141,000

Source: Food and Agriculture Organisation of the United Nations

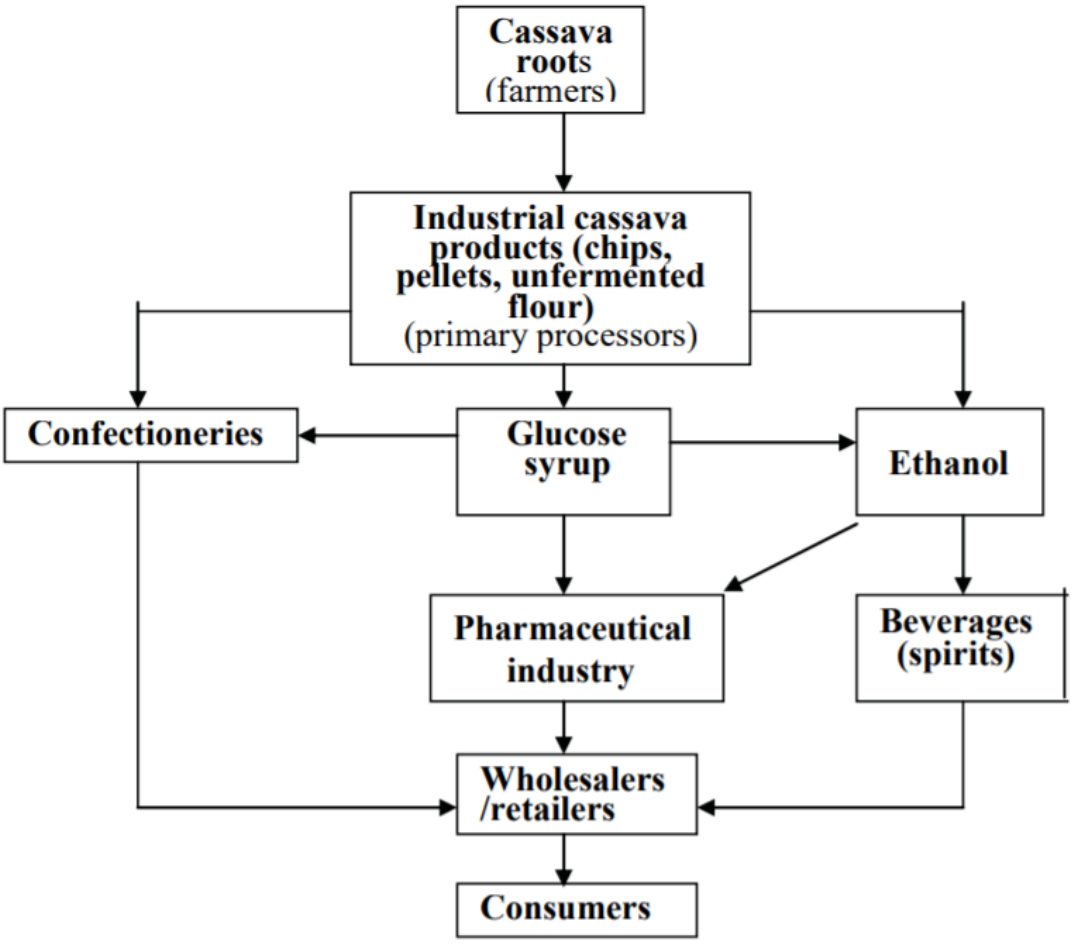


Figure 2. 3: Chains of industrial cassava products (Olusola Bandele Oyewole & Phillip, 2006)

as a raw material in animal feed, glucose syrup, chips, and flour. It is cultivated extensively for starch production used for the preparation of dextrin, ethanol, and liquid adhesive, among others (Edison & Srinivas, 2006). In Indonesia, apart from the fact that cassava is processed into snacks, foods, and flour, the potential to use its pulp to produce ethanol has been explored. The transformation of cassava from an inferior global food to a useful ingredient in the global supply chain was noted in Southeast Asia (Sriwana & Erni, 2015). This transformation is as a result of its useful application in the paper, glues, sweeteners, livestock feed, pharmaceuticals, alcohol, biofuel, and bioplastics industries.

2.2.3 South America and the Caribbean

Cassava featured extensively in Brazilian dishes as roasted flour (farofa), toasted flour (farinha de mandioca), cooked stew (vaca atolada), gravy-like gruel (pirão), and tapioca (beiju de tapioca) to mention a few. In addition to using it as food, cassava is also cultivated for starch production as well as animal feed. The aim of substituting cassava for imported sorghum and maize used in animal feeds necessitate an integrated cassava project in Colombia (Henry et al., 2002). The project eventually included the utilisation of fresh cassava in the production of flour and starch. The earlier-mentioned economic importance is in addition to its primary use in the preparation of soups (sancocho), bread (pandebono, bollo de yuca, and enyucados), meat pie (carimañola) and fish garnish.

2.3 Challenges in cassava utilisation

The presence of cyanogenic glycosides (phytotoxins) in cassava is a major setback in its usage as food raw material (Kwok, 2008). Cassava stores two forms of these toxins: linamarin and lotaustralin. Unless its β-glycosidic linkage is broken,

linamarin is relatively non-toxic within an intact cassava plant, just like other cyanogenic glycosides. But, hydrogen cyanide (HCN) gas is released as a result of cellular disruption which exposes the intracellular glucoside to the extracellular linamarase (Bolarinwa et al., 2016; Tewe, 1983). Disruption of cassava tissues could be by physical or chemical means during food preparation which is as a result of but not limited to maceration, application of high pressure or temperature, and release of mineral acids or enzymes (William, 2011; Kwok, 2008).

Based on the quantity of hydrogen cyanide (HCN) produced by cassava varieties, it had been possible to group them into two forms: sweet and bitter. While sweet cassava roots have less than 50 mg kg^{-1} HCN, there may be up to 400 mg kg^{-1} in the bitter form (both on a fresh weight basis) (Hahn et al., 1992). Therefore, there is a necessity to process this crop, to reduce the HCN content, before consumption. By thoroughly cooking after peeling, the HCN content of the sweet cassava roots are reduced to a safe level. However bitter cassava is traditionally fermented and cooked to reduce HCN to a level safe to consume. It is important to note that researchers had fortified both forms of cassava to give rise to yellow cassava roots (Figure 2.4).

2.4 The role of microorganisms in the spontaneous fermentation of cassava

A range of bacteria and fungi species had been identified with the fermentation of cassava appearing in succession. Among these are organisms belonging to the genera *Leuconostoc, Corynebacterium, Lactococcus, Weissella*, *Klebsiella* and *Saccharomyces* (Anike & Okafor, 2011; Padonou et al., 2009; Oyewole and Odunfa, 1988). Ihenetu et al. (2017) characterised *Escherichia coli*, *Lactobacillus sp.*, *Staphylococcus aureus*, *Aspergillus sp.*, *Candida sp.*, and *Bacillus sp*. in Abacha, a fermented cassava product consumed in Eastern Nigeria. A similar set of microorganisms were found in lafun samples from Ogun

Figure 2. 4: Cassava varieties collected from International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

and Oyo states, Western Nigeria, collected for a microbiological safety assessment study by Adebayo-Oyetoro et al. (2013). The presence of both spoilage and pathogenic microorganisms is notable in any spontaneous fermentation (Li et al., 2014), which is one of the reasons for starter culture development in food fermentation. Among the desirable organisms of wild fermentation of cassava, Lactic acid bacteria (LAB) species had a greater potential to be used as starters considering the significant roles they play.

2.5 Lactic acid bacteria and their role as starter cultures

The pure culture of lactic acid bacteria (LAB) ("*Bacterium lactis*") was first achieved by J. Lister in 1873 while the use of starter cultures in food (cheese and sour milk) production dated 1890. The first monograph of a typical LAB was by S. Orla-Jensen in 1919. LAB has DNA of a G + C content under 55 mol% (König and Fröhlich, 2009). Lactic acid bacteria are described as Gram-positive, catalase-negative, non-spore forming and non-motile bacteria that produce lactic acid as the end-product of metabolism either as a primary or secondary metabolite (Mokoena, 2017). They can be either coccus, coccobacillus or rod. They are ubiquitous normal flora of plants and animals; thus, are already present in the food we plan to ferment.

Lactic acid bacteria are widely employed in the fermentation of foods due to their roles (Leroy and De Vuyst, 2004). Among these roles are: 1. Producing antimicrobial substances that contribute to the safety of foods; 2. Enhancing organoleptic and nutritional properties; 3. Reducing anti-nutritional factors; 4. Ability to realise fast acidification of fermented products, thereby preserving them; 5. Surviving and dominating the indigenous population in the process showing antagonistic effect against spoilage organisms as well as pathogens; 6. Exerting probiotic effects in guts; 7. Contributing to the liberation of bioactive peptides that promote health (Proteolytic

LAB) or hydrolysing starch and thus increasing the energy density of starchy food (Amylolytic LAB) (Kostinek et al., 2007).

The potential of LAB to break down linamarin, a form of cyanide, is proven by different studies. Kimaryo et al. (2000) reported that four strains of *L. plantarum* have the capability of reducing the cyanide level of cassava roots fermented to produce kivunde, a Tanzanian food. They suggested a preference in submerged lactic fermentation to a spontaneous (either natural or back-slopping) fermentation regarding toxicological safety and improved quality of the final product. Their work agreed with that of Giraud et al. (1992) that LAB seem to display high linamarase activitiy in the fermentation of cassava, thereby producing standardised and non-toxic food products. Tefera et al., (2014) also demonstrated that *L. plantarum* and *L. mesenteroides* strains reduced free cyanide level up to 98% in fermented cassava roots.

2.5.1 Metabolism of lactic acid bacteria strains

The term 'Lactic Acid Bacteria' is conservatively used for genera in the order *Lactobacillales*, which includes *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Carnobacterium*, *and Weisella* (Makarova et al., 2006). Species used for food fermentations belong to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and the recently discovered *Carnobacterium* (Rattanachaikunsopon and Phumkhachorn, 2010) (Table 2.3). Lactic acid bacteria exist in homofermentative and heterofermentative metabolic groups, which are based on sugar fermentation patterns (Table 2.4). The former contains most species of *Lactococci*, *Enterococci*, *Pediococci*, *Streptococci*, and some *Lactobacilli* that ferment hexoses by the Embden-Meyerhof (E-M) pathway. The latter comprises *Leuconostocs*, some *Lactobacilli*, *Oenococci*, and *Weissella species* (Figure 2.5). The apparent dissimilarity on the enzyme

Table 2. 3: Fermented foods and beverages and their associated lactic acid bacteria (Rattanachaikunsopon and Phumkhachorn, 2010)

	Lactic acid bacteria
Dairy product	
- Hard cheeses without eyes	*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris*
- Cheeses with small eyes	*L. lactis* subsp. *lactis, L. lactis* subsp. *lactis var. diacetylactis, L. lactis* subsp. *cremoris,* *Leuc. menesteroides* subsp. *cremoris*
- Swiss-and Italian-type cheeses	*Lb. delbrueckii* subsp. *lactis, Lb. helveticus, Lb. casei, Lb. delbrueckii* subsp. *bulgaricus,* *S. thermophilus*
- Butter and buttermilk	*L. lactis* subsp. *lactis, L. lactis* subsp. *lactis var. diacetylactis, L. lactis* subsp. *cremoris,* *Leuc. menesteroides* subsp. *cremoris*
- Yoghurt - Fermented, probiotic milk	*Lb. delbrueckii* subsp. *bulgaricus, S. thermophilus Lb. casei, Lb. acidophilus, Lb. rhamnosus,* *Lb. johnsonii, B. lactis, B. bifidum, B. breve Lb. kefir, Lb.*
Fermented meats	
- Fermented sausage (Europe)	*Lb. sakei, Lb. curvatus*
- Fermented sausage (USA)	*P. acidilactici, P. pentosaceus*
Fermented vegetables	
- Sauerkraut	*Leuc. mesenteroides, Lb. plantarum, P. acidilactici Leuc. mesenteroides, P. cerevisiae, Lb. brevis,*
- Pickles	*Lb. plantarum, Leuc. mesenteroides, Lb. pentosus, Lb. plantarum* *P. acidilactici, P. pentosaceus, Lb. plantarum, Lb. fermentum*
- Fermented olives	
Fermented cereals	
- Sourdough	*Lb. sanfransiscensis, Lb. farciminis,* *Lb. fermentum, Lb. brevis, Lb. plantarum, Lb. amylovorus,* *Lb. reuteri, Lb. pontis, Lb. panis, Lb. alimentarius, W. cibaria*
Fermented fish products	*Lb. alimentarius, C. piscicola*

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

Table 2. 4: Differential characteristics of lactic acid bacteria based on morphology and physiology (Todar's Online Textbook of Bacteriology, accessed Oct. 21, 2016)

Genus	*Lacto-bacillus*	*Entero-coccus*	*Lacto-coccus*	*Leuconostoc*	*Pedio-coccus*	*Strepto-coccus*
Characteristic						
Morphology	rods	cocci	cocci	cocci	cocci in tetrads	cocci
CO_2 from glucose*	±	–	–	+	–	–
Growth						
at 10°C	±	+	+	+	±	–
at 45°C	±	+	–	–	±	±
in 6.5% NaCl	±	+	–	±	±	–
at pH 4.4	±	+	±	±	+	–
at pH 9.6	–	+	–	–	–	–
Lactic acid configuration	D, L, DL	L	L	D	L, DL	L

Key: + positive; - negative; ± varies between species; *test for heterofermentation of glucose

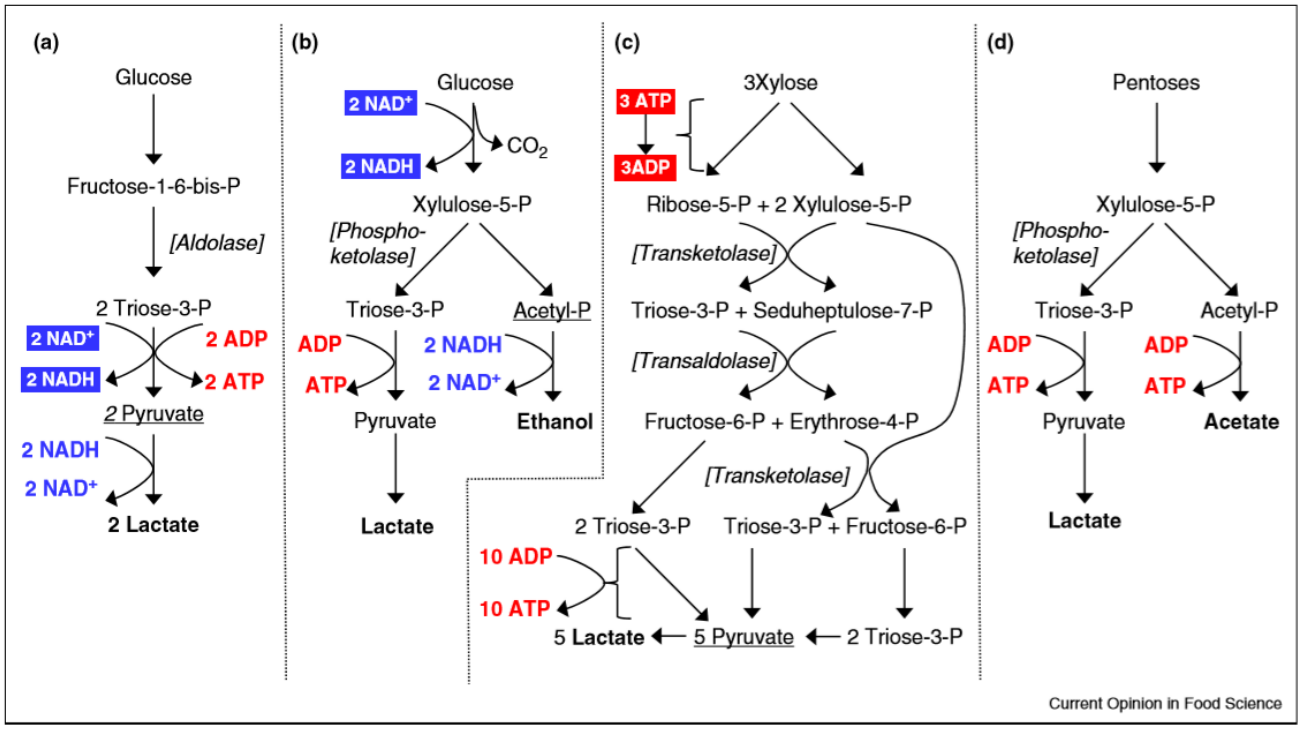


Figure 2. 5: lactic acid bacteria metabolism. (a) Homofermentative metabolism of hexoses via the Emden–Meyerhoff pathway. (b) Heterofermentative metabolism of hexoses via the phosphoketolase pathway. (c) Homofermentative metabolism of pentoses via the pentose phosphate pathway. (d) Heterofermentative metabolism of pentoses via the phosphoketolase pathway. Main end products are printed in bold; branching points of 'metabolic switches' are underlined. The formation of reduced/oxidized co-factors (printed in white on blue background or blue, respectively) is indicated if it occurs upstream/downstream of relevant metabolic branching points; ATP synthesis (printed in red) is shown to indicate the net ATP yield of the metabolic pathway. Image by (Gänzle, 2015)

level between both forms is the presence or absence of the main cleavage enzymes of the PK pathway (phosphoketolase) and the E-M pathway (fructose 1,6-diphosphate) (Gänzle, 2015). They are either microaerophiles or facultative anaerobes (non-respiratory), so they can grow through the bulk of the food except the surface. They possess superoxide dismutase and an alternative means of detoxifying peroxide radicals since they lack catalase.

2.5.2 Some lactic acid bacteria used in starter culture development

2.5.2.1 Lactobacilli spp.

Lactobacillus is a major group of LAB characterised by the formation of lactic acid as their sole end product (representing about 50-85%) of metabolism of carbohydrates. They are ubiquitous in habitat colonising plants, plant products, and some parts of the human body such as an oral cavity, gastrointestinal tracts (GIT) and even vagina. The microbiota of the GIT promotes the health of the host (Walter, 2008). They are Gram-positive, non-spore forming, non-motile, rod-shaped, facultative anaerobes or microaerophiles bacteria. Many are homofermentative while some species are heterofermentative. The genus has more than 180 species and is sub-divided into three groups: Obligately homofermentative (e.g., *L. acidophilus*); Facultatively heterofermentative (e.g., *L. plantarum);* Obligately heterofermentative (e.g., *L. brevis*).

The *lactobacilli* can grow at a pH as low as 4 and thus are more resistant to acidity than other LAB. Some of them are used as starter cultures for the production of fermented foods and drinks (Salvetti et al., 2012). Salvetti and colleagues discovered that *Lactobacillus brevis* comprised ten subspecies that are either facultative or obligate heterofermenters. They have GC content between 46 and 55%. *Lactobacillus collinoides*

comprises 5 subspecies that are all heterofermentative with GC content between 39.7 and 48.5%. *Lactobacillus plantarum* is very homogeneous regarding metabolic activities and comprises five subspecies that are facultative heterofermenters having GC content between 44 and 47%.

2.5.2.2 Lactococcus ssp.

As expected, *Lactococcus ssp* are gram-positive, catalase-positive, non-spore forming, non-motile bacteria occurring singly, in pairs or cocci chains. They are mainly homofermenters (Samaržija et al., 2001). They are very useful in the food industry as single starter cultures or mixed cultures with other LAB or yeasts. The type species of the genus used as a starter culture in commercial dairy fermentation is *L. lactis*(Song et al., 2017), which has received major interest in research and has two subspecies, *lactis* and *cremoris*. Members of this genus also play an important role in flavour compound production (Smit et al., 2005; Ayad, 2001).

2.5.2.3 Leuconostoc spp.

Leuconostoc was first isolated and identified by Cienkowski in 1878 (Hucker & Pederson, 1930) and have been known as part of starter cultures since the 1920s. The name is a mix of *leucus* (clear or colourless in Greek) and *nostoc* a generic Latin noun of an algal genus (Garvie, 1986). *Leuconostocs* are generally found in plant materials, fermented food, wines, milk and milk products occupying the same niche as *lactococci* and *lactobacilli* (Thunell, 2010) . Their importance in cheese production is extensively documented. Though they have an initial low growth rate, *leuconostocs* grow slowly and dominate the last stages of cheese fermentation (Frantzen et al., 2017). They are as a result of this usually employed in a combined culture with fast-acid-producing *lactococci.*

Leuconostoc spp. are heterofermentative LAB presently including, 13 species: *L. mesenteroides (*sub-species, *mesenteroides, cremoris, dextranicum,* and *suionicum), L. carnosum, L. palmae, L. citreum, L. fallax, L. lactis, L. gasicomitatum, L. gelidum, L. inhae, L. holzapfelii,L. kimchii, L. miyukkimchii,* and *L. pseudomesenteroides* (Alegría et al., 2013). Like other LAB species, *Leuconostoc* in fermenting medium participate in the formation of flavour and aroma compounds through the production of organic acids. These organic acids (lactate, malate and citrate) not only enhance the flavour profile of fermented products but also serve as preservative agents by their antimicrobial action alongside CO_2, diacetyl and hydrogen peroxide the organisms produce (Hemme & Foucaud-Scheunemann, 2004). *Leuconostoc* strains produce bacteriocins (pediocin-like ones), which also contribute to food safety by inactivating food spoilage organisms (Hitendra et al., 2015; Chang & Chang, 2010).

L. mesenteroides had been observed to be more active in sugar fermentation than the rest of the species (Erten, 2000) . Dimic (2007) observed that *L. mesenteroides* is one of the essential LAB participating in the fermentation of some foods. They have been isolated from the fermentation of cassava during the production of various indigenous food. They play a good role in acidification, cyanide reduction, taste improvement and bio-preservation (Tefera et al., 2014; Brauman et al., 1996).

2.5.2.4 Weissella spp.

Members of the genus *Weissella*, first proposed by Collins et al. (1993), are non-spore forming, Gram-positive, catalase-negative, heterofermentative except *W. ceti* (Vela et al., 2011), non-motile except *W. beninensis* (Padonou et al., 2010), facultative anaerobic, and alpha hemolytic bacteria that lack cytochrome. Their cells occur singly, in pairs or may be in short chains appearing as rods or coccobacilli. (Kamboj et al.,

2015). All species in this group produce lactic acid from glucose, except *W. paramesenteroides* and *W. hellenica* (Lee et al., 2002). They have similarities to *Lactobacillus* and *Leuconostoc spp.* regarding inherent resistance to vancomycin and certain morphological characteristics, thus, they have been mistakenly placed in the genus *Lactobacillus* or confused for *Leuconostoc spp.* (Safika et al., 2019; Fessard & Remize, 2017). After genotypic identification, they were placed in a unique genus, *Weissella*, named after the German microbiologist, Nobert Weiss, for his contribution to LAB taxonomy (Kamboj et al., 2015).

It is challenging to distinguish *Weissella* from *Leuconostoc* and heterofermentative *lactobacilli* using only morphological observations (phenotypic identification). However, identification of *Weissella* can be achieved by molecular methods among which are 16S rRNA gene sequencing, ribotyping, fluorescent-Amplified Fragment Length Polymorphism (fAFLP), Matrix-associated laser desorption ionisation Time-of-Flight (Maldi-ToF), and full genomic sequencing. Currently, the genome sequence of strains belonging to 21 species has been identified (Figure 2.6) among which are *W. koreensis, W. confusa, W. beninensis, W. hellenica, W. ceti, W cibaria, W. diestrammenae, W. paramesenteroides, W. fabalis, W. soli, W. fabaria, W. ghanensis, W. halotolerans, W. viridescens, W. kandleri, W. minor, W. oryzae, W. thailandensis, W. bombi, W. jogaejeotgali and W. uvarum* (Fusco et al., 2015). The majority of *Weissella spp.* occur in spontaneous fermented foods and participate to the final fermentation stage. They have been isolated from fermented vegetables (*W. koreensis*), fermented drink (*W.kandleri* and *W. koreensis*), and fermented cassava (*W. ghanensis, W. fabaria, W. fabalis*), among others (Fessard & Remize, 2017). Fessard and Remize (2017) investigated the use of *Weissella spp* as starters in sourdough fermentation and found

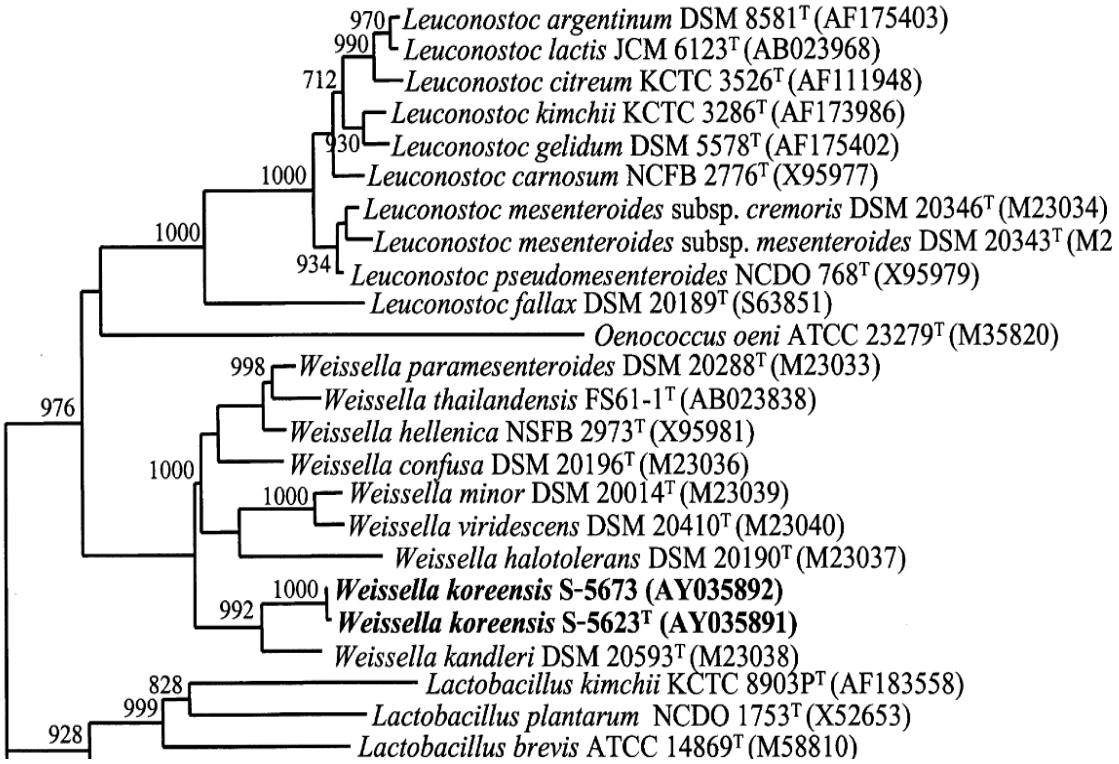


Figure 2. 6: Phylogenetic relationships between *W. koreensis sp*. nov. strains S-5623T and S-5673, other *W. species* and related bacteria, on 16S rDNA sequences basis, GenBank accession numbers given in parentheses. The branching pattern was produced by the neighbour-joining method. Numbers show bootstrap values "700. Bar, 0±1 nucleotide substitutions per nucleotide position (Lee et al., 2002).

that they compete well with other LAB checking their acidification and growth performance as well as their production of exopolysaccharides (EPS). Since they are frequently detected in a large variety of fermented foods, they could play a vital role in the fermentation process if attention is paid on their use as a starter in food fermentation. Pi et al. (2014) discovered that *W. koreensis* 521_CE exercised inhibitory effects on lipid accumulation and adipocyte differentiation in cultured and differentiating 3T3-L1 (adipocyte) cells. The authors thus suppose that the organism may have important anti-obesity effects that could be developed as a therapeutic substance. It was also reported recently that *W. koreensis* OK1-6 found in kimchi exhibited ornithine-producing capacity, L-ornithine is a non-protein and medicinal amino acid used as a food supplement. It was demonstrated that using *W. koreensis* OK1-6 as a starter for the production of kimchi enhanced anti-obesity and cardio-protective functionality (Moon et al., 2012; Park et al., 2012).

2.5.3 The role of lactic acid bacteria in cassava detoxification and nutrition improvement

Several LAB strains can degrade linamarin (which accounts for more than 80% of the cassava toxins) during submerged fermentation of cassava roots. The reaction has been noted to proceed in two stages (Nout & Sarkar, 1999). In the initial stage catalysed by β-glucosidase, linamarin is degraded to glucose and acetone-cyanohydrin and in the final stage by hydroxynitrile lyase; the latter is degraded to acetone and HCN. Although Westby et al. (1997) argued that it was pointless to develop starter cultures with potential for linamarase activity to ferment grated cassava roots, there are many studies in favour of achieving up to 98% cyanide reduction in fermented cassava flour using mixed-starters. The β-D-Glucosidase activity, which is common in LAB releases an extensive array of secondary metabolites in crops, some of which improve flavour in

fermented foods (Michlmayr and Kneifel, 2014). An example of such plant metabolite is deglycosylated that does not only improve fragrance in fermented products but also increases the bioavailability of health-promoting plant metabolites. Several flavour compounds, including organic acids (both volatile and non-volatile), occur in fermented cassava products as a result of LAB metabolic activities, some of which are discussed in the next section.

2.5.4 Flavour compounds in lactic acid bacteria fermentation

Flavour is typically a sensation that results when taste buds in the tongue convey information about many volatile and non-volatile components having different chemical and physicochemical properties. While the volatile ones influence both aroma and taste, the non-volatile compounds contribute mainly to the taste. An extensive range of chemicals are regarded as flavour compounds. Some as alcohols (e.g., geraniol), phenolic compounds (e.g., eugenol), aldehydes (e.g., benzaldehyde), esters (e.g., isoamyl acetate), dicarbonyls (e.g., diacetyl), short to medium-chain free fatty acids (e.g., butanoic acid), lactones (e.g., γ-lactones and δ-lactones), and methyl ketones (e.g., 2-pentanone) (Longo and Sanromán, 2006).

2.5.4.1 Non-volatile flavour compounds

In the literature, the non-volatile organic compounds produced by LAB and that characterise the flavour profile of fermented foods are mainly lactate, malate, succinate, and citrate (Tefera et al., 2014; Hemme & Foucaud-Scheunemann, 2004; Brauman et al., 1996).

2.5.4.1.1 Lactic Acid

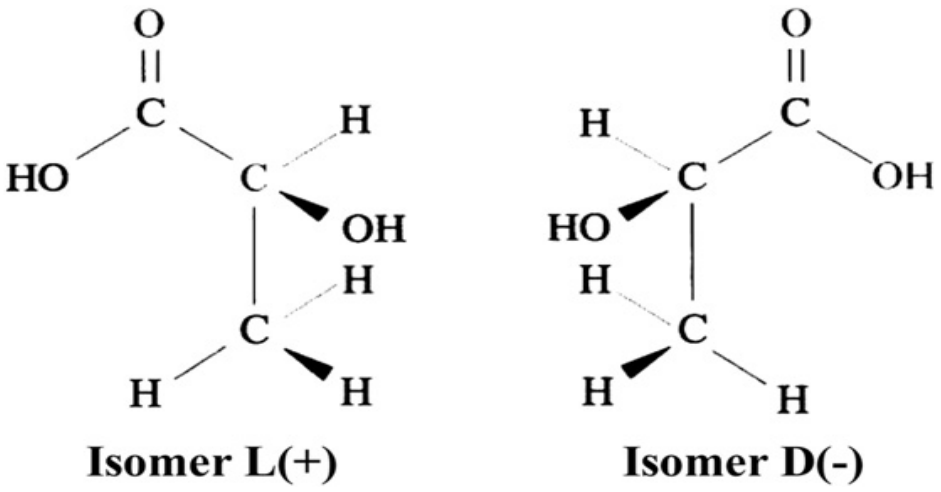
Lactic acid (IUPAC ID: 2-Hydroxypropanoic acid; $C_3H_6O_3$) is an organic compound which in its pure and anhydrous state is a white crystalline solid that is

water-soluble with a low melting point (about 16.8 °C) and clear in its liquid state. It has 122 °C boiling point and a molar mass of 90.08 g mol^{-1}. It can be produced naturally and synthetically. It has got two optical forms: L(+) and D(-). The L(+)-lactic acid is the biological isomer, naturally formed in living cells (Purac, 2008). Lactic acid is indeed present in many food products such as fufu, lafun, gari, cheese, soy sauce, bakery products, yoghurt, confectionery, beef, salads, pickled vegetables, dressings, sourdough, ready meals and alcoholic beverages (Savadogo et al., 2007).

2.5.4.1.2 Citric acid

Citric acid ($C_6H_8O_7$) can exist in two forms viz: an anhydrous (water-free) which crystallises from hot water or a monohydrate from cold water. It is possible at 78 °C to convert the monohydrate to the anhydrous form (Frank, 2005). The citric acid (IUPAC ID: 2-Hydroxypropane-1,2,3-tricarboxylic acid), an intermediate in the citric acid cycle, is a weak organic tricarboxylic acid. It has a molar mass of 192.12 g mol^{-1}, melting point at 156 °C and a boiling point at 310 °C. Citric acid exists in higher amounts in citrus fruits, constituting about 8% of the dry weight of their juices (i.e., 47 g L^{-1}) and small amounts in a variety of vegetables. It is used mainly as a flavour enhancer and an acidifier, in food and beverages, giving them a longer shelf life (FDA, 2010). Saha et al. (2013) reported that a fixed ratio of citric acid to malic acid gave a better inhibitory effect than using one acid as a preservative.

Fermentation is the most economical way of producing citric acid up to more than 90% because operations are smooth and stable with less technical skills (Swain et al., 2011). Fermentation process involves preparation and inoculation of the sample or raw material that will be left for some days to ferment. Citric acid is then recovered



(a) (b)

Figure 2. 7: (a) L-Lactic acid structural formula (b) D-Lactic acid structural formula (Martinez et al., 2013)

afterwards. Fermentation can be in three types: solid-state fermentation submerged fermentation and surface fermentation. The most favoured technique, however, is the submerged fermentation that is employed in the fermentation of cassava to produce lafun (Soccol and Vandenberghe, 2003).

2.5.4.1.3 Malic acid

Malic acid ($C_4H_6O_5$, IUPAC ID 2-Hydroxybutanedioic acid) is an organic compound and an active ingredient in many sharp sour (tart taste) foods, especially fruits and vegetables. It is a dicarboxylic acid produced by all living organisms (Figure 2.8). It exists in 2 forms (L- and D-enantiomers), although L-isomer is the only natural form. The pleasant taste experienced when a juicy cherry or apple is being eaten is somewhat caused by malic acid. It is also found in grapes and wines in a very high concentration of about 5 g L^{-1} although apple is the fruit mostly known to contain malic acid. It is a primary flavour of rhubarb (Jensen, 2007). It is commonly used as an additive and a flavour enhancer in a variety of foods (Saha et al., 2013).

2.5.4.1.4 Succinic acid

Succinic acid readily reacts with other molecules to form esters. It is the flavour of the esters formed that scientists used to describe its features. Succinic acid (IUPAC ID: Butanedioic acid; $C_4H_6O_4$) also is a dicarboxylic acid, which is white, odourless and solid at room temperature. It is formed through microbial fermentation (Song and Lee, 2006). Succinic acid, a by-product of sugar fermentation, is used in beverage and food industries as an additive, an acidity regulator, and a dietary supplement. Its great potential to convert too many valuable products and its ease of production made it lucrative as bio-based chemical according to a 2004 report from the United State Department of Energy (Vaswani, 2010).

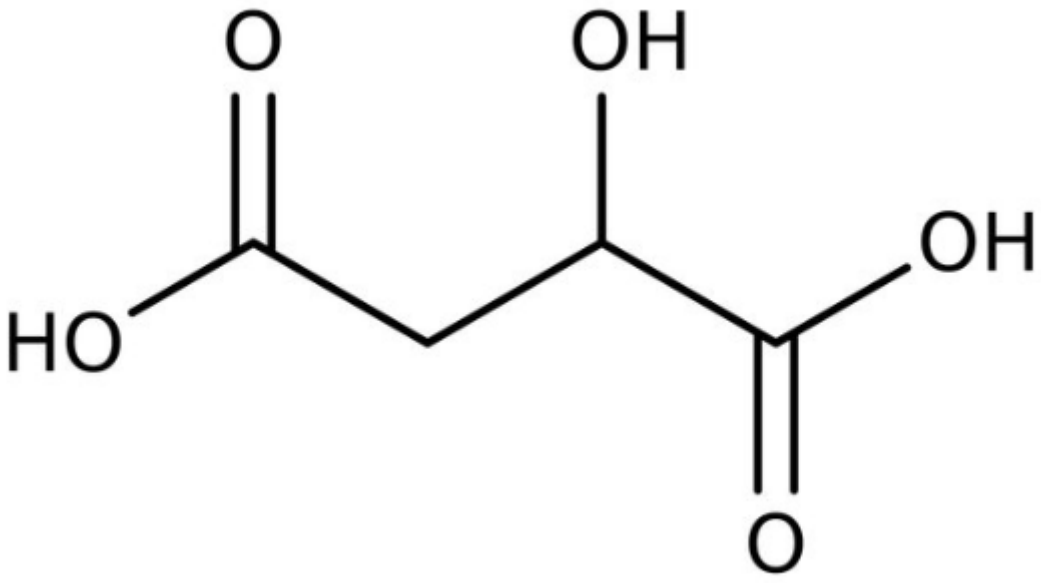


Figure 2. 8: Malic acid structural formula (fishersci.co.uk)

2.5.4.2 Volatile flavour compounds

The volatile organic compounds found in fermented cassava products are mainly in classes: aldehyde, ketone, organic acid, alcohol, furan, and, enones and driving the aroma characteristics of the products. The structures of some of the odour-active compounds containing carbon, hydrogen and oxygen are presented in Figure 2.9.

2.5.4.2.1 Aldehydes

Aldehydes (R-CH=O) are organic compounds that have one alkyl (or aryl) group and one hydrogen-bonded to the carbonyl carbon with a single covalent bond. They are named using the same system as other organic compounds, with the suffix -al to designate the presence of the molecule of a carbonyl group (McNaught & Wilkinson, 1997). They are formed by partial oxidation of primary alcohols. The volatile aldehyde has a strong odour, and many have been employed as ingredients in flavour and several studied in food flavour chemistry. Aldehydes differ in the smell with most of the lower molecular weight smelling bad (rotten fruits) or greeny, but some of the higher molecular weight aldehydes smell quite pleasant so also are some aromatic ones (Parker, 2014). With the chemistry process of autoxidation, aldehyde will degrade in air. Thus an aldehyde like hexanal is used to determine the oxidation level of some food materials and products (Fuentes et al., 2014; García_Llatas et al., 2007; Jeremiah, 2001).

2.5.4.2.2 Ketones

Ketones like aldehydes are carbonyl compounds but with two hydrocarbon groups attached (RC(=O)R') (Moss et al., 1995). The hydrogen atom that is attached to the carbonyl group in aldehydes makes it very easy to oxidise. Ketones, on the other hand, are resistant to oxidation since they do not have that hydrogen atom but are only oxidised by powerful oxidising agents that can break carbon-carbon bonds. Though

volatile profile of many foods contains hundreds of odour-active compounds but just a few of the number give character to the food. Ketones generally smell pleasant, and some of the structurally complex ones have a crucial role in food flavour and aroma by actually giving flavour character to the food (Parker, 2014).

2.5.4.2.3 Volatile organic acids (VOA)

Organic acids exist in fermented products as a result of microbial metabolism. They also occur in the raw material by hydrolysis and biochemical changes during storage (Shukla et al., 2010). While non-volatile acids are products of the metabolism of carbohydrates, protein and lipids, volatile acids are derived from CO_2 produced during the metabolism of same nutritive substances. The common VOA in cassava fermentation is acetic acid (also known as ethanoic acid). It is a simple carboxylic acid having the methyl group (CH_3) linked to the carboxylic acid group (COOH) to form a chemical formula CH_3COOH (Jones & Templeton, 1958). In 1845 German chemist Hermann Kolbe synthesised acetic acid from inorganic compounds for the first time (Goldwhite, 2003). It has a characteristic pungent odour. It is the acid of vinegar, which is why it was named after the Latin word for vinegar, 'acetum' and is formed, together with lactic acid, in the fermentation of cassava like other fermented foods (Coulin et al., 2006). Acetic acid is used as a condiment or an acidity regulator in some food industries (Silva & Lidon, 2016).

2.5.5 Application of lactic acid bacteria as starter cultures for cassava fermentation in selected food products

2.5.5.1 Gari

Gari, consumed by around 200 million people (Kostinek et al., 2005), is a granular product of fermented cassava made by fermenting and roasting mash

produced from grated cassava roots. In the previous study by Collard, (1963), *Corynebacterium sp* were was shown to be present in the fermenting cassava to produce gari and producing both acid and flavour. However, more recent studies reported that this bacterium is only present in low numbers and that LAB are the predominant microorganisms in of the production of gari (Okafor, 1977; Kimaryo et al., 2000; Kostinek et al., 2005). Therefore attention has concentrated on developing LAB starter cultures for gari production as well as freeze-drying these cultures (Yao et al., 2009; Huch et al., 2008).

The inoculation of starters is usually after grating the cassava roots into the mash. Kostinek et al., 2005 reported that *L. fermentum* and *L. fallax* strains produce high concentration of antagonistic substances like bacteriocins and hydrogen peroxide. Also, in their study, they found that *L. plantarum* strains were producing acid faster than all the LAB strains tested and having the highest linamarase activity. Thus, they recommended the inclusion of *L. plantarum, L. fallax* and *L. fermentum species* in a mixed culture as starters for gari production. Oguntoyinbo (2007) agreed with the recommendation as well as their observation that the strains of these three species had a high sugar fermentation profile for indigestible stachyose and raffinose sugars.

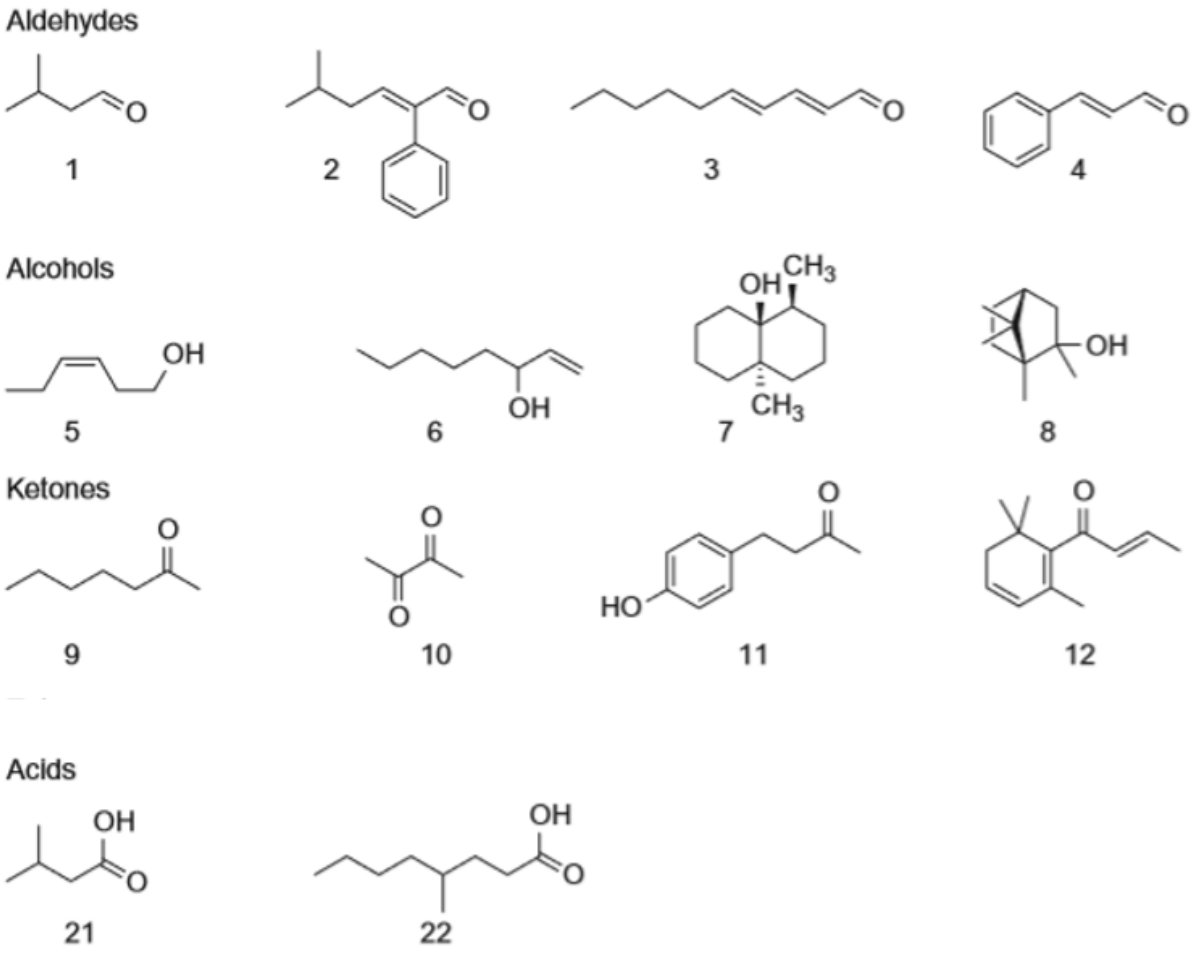


Figure 2. 9: Examples of odour-active compounds containing carbon, hydrogen and oxygen (Parker, 2014)

2.5.5.2 Fufu

Fufu or Foo-Foo as it is called in Central Africa, is a regular meal in both West and Central Africa. It is produced traditionally by solid-state fermentation where the cassava is not steeped in water but allowed to ferment in a solid-state under a hydraulic press. The mesh is subsequently sieved after fermentation and cooked in boiling water. However, in control fermentation, it must be steeped in water (Figure 2.10). Many studies describe the role of LAB in the spontaneous fermentation of retted cassava roots. LAB succession was found to start with *L. lactis* followed by *L. mesenteroides*. The former was said to displace epiphytic micro-floral, and the later regulates the fermentation process. The fermenting medium is lastly dominated by *L. plantarum* (Brauman et al., 1996). Sobowale et al. (2007) evaluated the effect of *L. plantarum* strains in fufu production. They determined that fufu, produced using *L. plantarum* as the starter culture, had more desirable pasting quality and flavour in comparison to the traditionally produced samples.

2.5.5.3 Kivunde

Kivunde (bada in Tanzanian, and inyange in Burundi), is a fermented cassava product made by heaping roots together for fermentation. It is traditionally produced by spontaneous or 'back-slopping' fermentation (Abass et al., 2018). In the latter fermentation option, a percentage of liquor from a previous kivunde spontaneous fermentation is added to initiate the fermentation process. Moulds of the genera *Penicillium, Cladosporium, Rhizopus, Mucor, Aspergillus and Fusarium* were found to dominate the fermentation of cassava into kivunde (Kimaryo et al., 2000). Abass et al. (2018) showed that the traditional methods of processing cassava roots into kivunde among other products have safety issues concerning high levels of some mycotoxins.

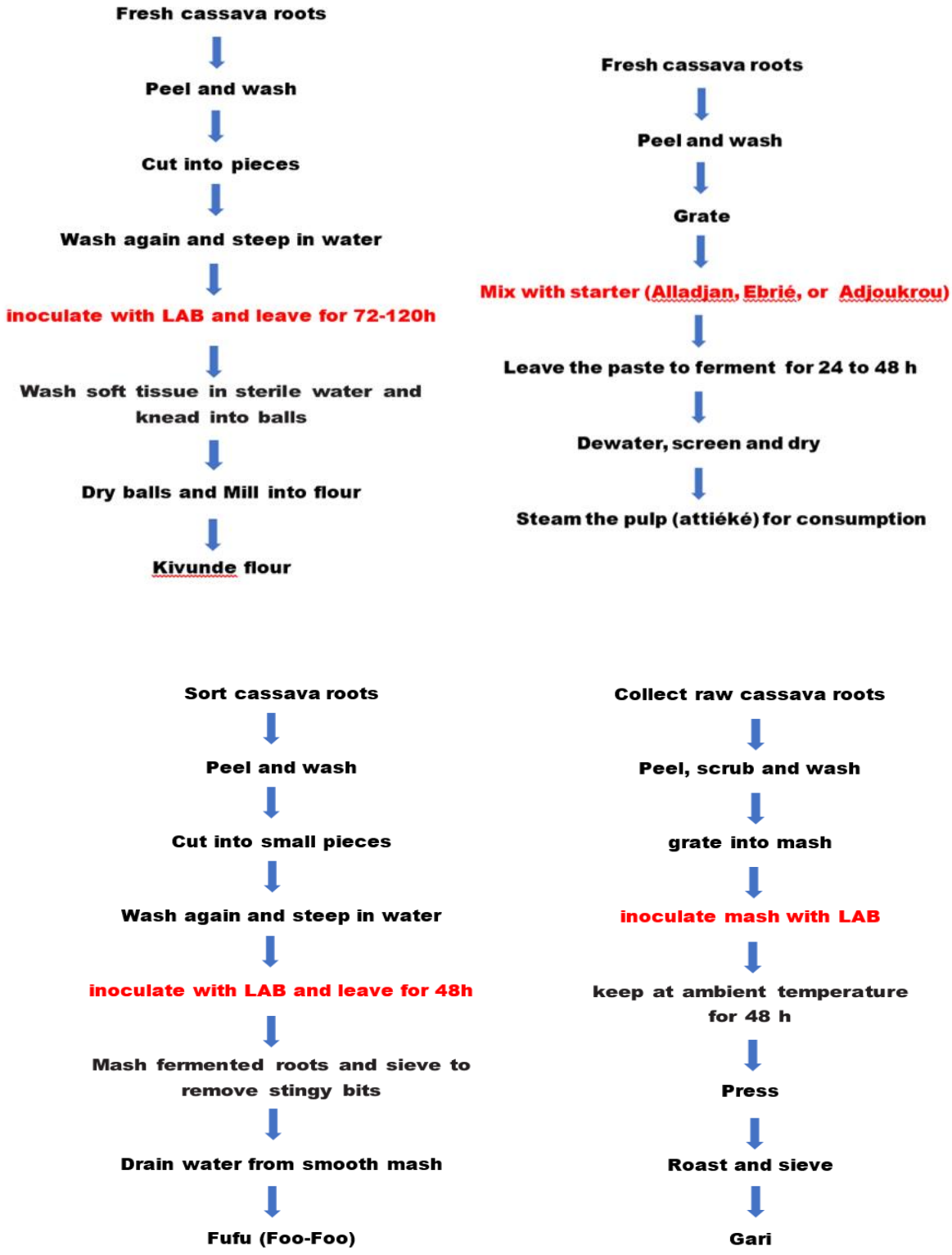


Figure 2.10: Flow diagrams of the production of some fermented cassava products at ambient temperature showing stages of lactic acid bacteria inoculation

The potential of *L. plantarum* to improve the quality and safety of this product was, however, demonstrated by Kimaryo et al. (2000).

2.5.5.4 Attiéké and Agbelima

Attiéké and Agbelima are indigenous cassava fermented doughs in Côte d'Ivoire and Ghana respectively. Attiéké is the main fermented food product in Côte d'Ivoire and forms a significant part of their diet (Djeni et al., 2011). It can be produced using three different traditional inocula: Alladjan, Ebrié, and Adjoukrou, made from boiled cassava roots (Bouatenin et al., 2012). As expected of any sourdough (Gobbetti, 1998; De Vuyst & Neysens, 2005), LAB were the dominant microflora in the production of attiéké. The activity of LAB in a sourdough had been attributed to their fermentation of certain carbohydrates such as fructose and maltose that are present in this type of food. Secondly, LAB also cause rapid acidification that inhibits a-amylase of the flour but improves water-binding of the dough (De Vuyst & Vancanneyt, 2007; Bouatenin et al., 2012). To confirm the report of their previous study, Bouatenin et al. (2017) used LAB strains (*L. plantarum* and *Leuconostoc mesenteroides*) among other microbes as starters, singly and in combination for attiéké. They reported that acid production responsible for sourness and detoxification of the product were the job of LAB strains.

Agbelima like attiéké is a sourdough cassava meal which souring is achieved by LAB such as *Leuconostoc mesenteroides, Lactobacillus brevis* and *L. plantarum* (Amoa-Awua et al.,1996; Mante et al., 2003). It is produced traditionally by using kudeme; an inoculum derived also from cassava roots (Ellis et al., 1997). Cyanide detoxification was expectedly achieved by LAB as reported in the work of Awua et al. (1996) that revealed the role of LAB in the production of agbelima.

2.6 Lafun, a fermented cassava flour

One of the ways in which cassava is prevented from rapid spoilage after harvest is by processing the roots into lafun, a fermented cassava flour. Cassava is cut into small pieces, washed, steeped in water for 3 to 4 days, drained, sun-dried and milled into powder (flour) (Figure 2.11). Many studies favour a minimum of 48 h fermentation of cassava to achieve a reasonable level of cyanogenic glucoside hydrolysis. Dietary cyanogen has been noted to result in tropical ataxic neuropathy (TAN) and diabetes mellitus, while it can also aggravate iodine deficiency disorders (IDDs) (Ojo and Deane, 2002). Oyewole (1992) found that the characteristics of the finished product depend, to a large extent, on whether cassava roots are peeled or not; the size to which the roots are cut and whether the fermentation water is changed at intervals during fermentation. During fermentation, roots become softened, the flavour is developed, the endogenous cyanides are degraded, and vitamins, essential amino acids, proteins are biosynthesised (Okoro, 2016; Olapade et al., 2014; Brauman et al., 1996). The deficiency relating to low protein content in lafun is made up for by accompanying other foods with high protein content as it is never consumed alone. Lafun is cooked in boiling water into a stiff porridge (oka) and consumed with various stews, preferably vegetables and source of protein which is mostly fish or beef (Padonou et al., 2010).

There is no formal standard or quality control on lafun processing methods regarding code of hygiene, food safety and nutritional value in the traditional production (Figure 2.12) (Falade & Akingbala, 2010). The primary processors are mainly farmers. So, cassava roots typically are harvested and processed close to the point of harvest and sold to consumers directly or to wholesalers or retailers, or exported abroad (Olusola Bandele Oyewole & Phillip, 2006). This lack of control indicates that lafun production has challenges associated with quality and safety. Unfortunately,

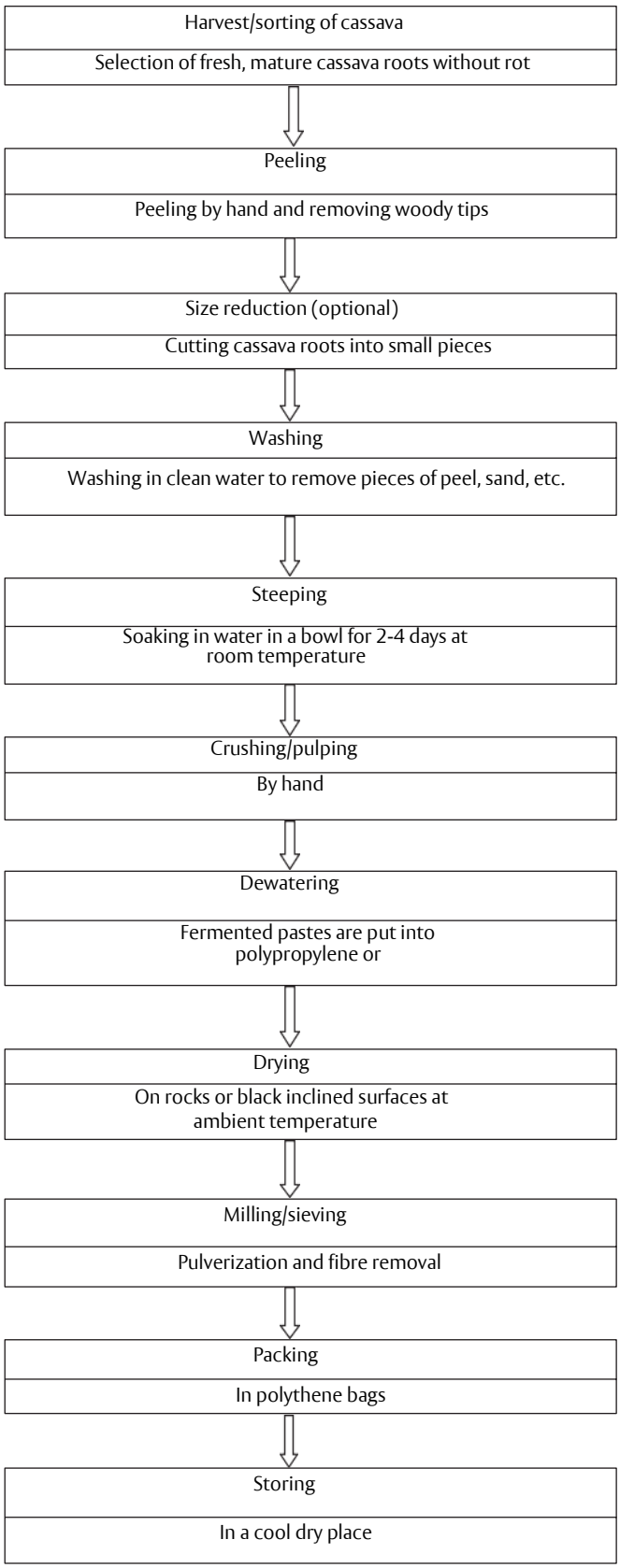


Figure 2. 11: Flow diagram of traditionally produced lafun adapted from Adebayo-Oyetoro et al., (2013)

there are difficulties getting reliable data on the scale of what the problem of the lack of regulatory might be.

Lateef & Ojo (2016) in their work reported the occurrence of *Staphylococcus aureus, Salmonella Typhimurium, Escherichia coli, Bacillus cereus, Aspergillus fumigatus, Klebsiella oxytoca, Aspergillus flavus, Rhizopus oryzae, Aspergillus niger,* and *Absidia corymbifera* in lafun samples. Their results showed that consumption of lafun produced with poor hygiene is a potential hazard of public health significance relating to food poisoning from multi-drug resistant bacteria. They, however, identified critical control points in steeping, drying and storage stages. Figure 2.13 shows how the stages of steeping and drying during lafun production are typically carried out traditionally. Sun-drying the fermented cassava mesh could take up to 2 weeks, depending on the ambient temperature and atmospheric humidity, making the control of drying conditions very difficult (Tunde-Akintunde et al., 2007). Lafun is the focus of this work to upgrade processing methods that result in the near absence of HCN in products and extreme reduction of the repulsive lafun odour. The production process would be done under modern techniques that include quality checks through standard biological analyses, to improve the quality of lafun.

Figure 2. 12: Lafun processing environment

a

b

c

d

e

Figure 2. 13: Lafun steeping and drying stages; a and c show steeping conditions; b, d and e are different drying sites

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Chapter Three

3.0 Screening of Lactic Acid Bacteria to Determine Their Potential as Starter Cultures for Lafun Production

Abstract

There is growing interest in improving the quality and consistency of lafun produced in Nigeria. This could be achieved by developing starter cultures for its production, as opposed to relying on spontaneous fermentation. However, there is a paucity of information available regarding the suitability of lactic acid bacteria in this regard. Therefore, the objective of this study was to assess the potential of naturally-occurring lactic acid bacteria (LAB) in fermenting cassava to be used as starter cultures for lafun production. Lactic acid bacteria isolated from spontaneous submerged fermentation of cassava were screened for non-volatile organic acid production, antibiotic susceptibility, and the presence of antibiotic resistance genes. A total of 60 representative colonies were isolated from fermenting cassava roots. Only 41 isolates were gram positive and catalase negative. API kit tentatively identified 37 out of the 41. The unidentified four isolates were later identified genotypically along with the four of the identified strains that were selected for further research. All isolates (n=41) were screened for the production of citrate, malate, succinate, lactate and acetate. They produced between 0.1 and 1.7 mg g^{-1} of citrate, 0.3 and 1.3 mg g^{-1} of malate, 1.9 and 7.9 mg g^{-1} of succinate, 10.0 and 34.3 mg g^{-1} of lactate, and 0.7 and 2.5 mg g^{-1} of acetate. Strains with highest lactate and succinate with low acetate production were favoured in the final selection to enhance lafun flavour. Four strains were selected and genotypically identified as *Weissella koreensis* (2 strains), *Lactococcus lactis* and *Leuconostoc mesenteroides*. The selected strains and the unidentified isolates were

found to be resistant to metronidazole while *Lactococcus lactis* was the only strain susceptible to vancomycin. None of the selected LAB strains carried a functional resistance gene. The results of this study suggest that the four selected strains may have potential as starter cultures for lafun production and should be further studied to determine their full potential.

***Keywords*:** Cassava, fermentation, non-volatile organic compounds, antibiotic susceptibility

3.1 Introduction

Cassava is an important crop and serves as a food security crop in the developing world (Kolawole et al., 2010). However, the main challenge inhibiting the widespread usage of cassava roots by processors is its high perishability (Adejumo et al., 2015) and the presence of more than one form of cyanogenic glycosides toxin (Hahn *et al*., 1992). As a result, cassava is often processed into dried forms which is more durable, shelf stable with lower volume and easily transported (Westby, 2002). Fermentation is one of the traditional ways to reduce cassava toxicity and with thorough cooking; the volatile hydrogen cyanide gas is released, to render the product safe.

Lafun is a fermented cassava flour, which is produced traditionally by spontaneous and submerged fermentation. The use of spontaneous fermentation in food generally has the disadvantage of generating variable product quality. Also, undesirable organisms participating in the fermentation process could pose a health risk to the consumers. However, using food-grade microorganisms as starter cultures can resolve these challenges. LAB participate in cassava fermentation playing an essential role in taste, texture, safety and quality. They prevent spoilage by secreting growth-inhibiting substances, such as organic acids (Leroy and De Vuyst, 2004).

Selection of appropriate LAB starter cultures for production of lafun requires the isolation and identification of LAB strains from spontaneous fermentation of cassava (Reginensi et al., 2013). Identification of LAB could either be phenotypic or genotypic. Phenotypic classification is based on their morphology, physiology, metabolism, growth requirements, among others. Genotypic identification techniques include the 16S rRNA gene and the full genomic sequencing. Different metabolic capabilities are revealed by phenotypic tests, which provide basis for identification. However, the ambiguities that arise from their very similar nutritional requirements and growth

conditions can be resolved by molecular techniques (Belhadj et al., 2014; Van Hoorde et al., 2008). In addition, molecular techniques can be used to investigate the antibiotic resistance genes in potential starter strains. This is because a starter culture could be an agent that introduces antimicrobial resistance genes into the food chain (Flórez et al., 2007).

Antibiotic resistance can either be intrinsic or acquired (Mathur & Singh, 2005). The innate ability of bacterial species to withstand the activity of an antimicrobial agent, without the effect of a specific antibiotic resistance mechanism associated with a resistance gene or a mutation is called intrinsic resistance (Fraqueza, 2015). However, bacteria can acquire resistance either from spontaneous gene mutations or horizontal gene transfer (Pariza et al., 2015). In theory, the emergence of antimicrobial resistance is an adaptation process that involves a complex series of events in humans, animals and the environments over a prolonged period. The food chain creates a convenient pathway by which humans can easily contact antibiotic resistance bacteria (Verraes et al., 2013). The transfer of antimicrobial resistance genes among bacteria has facilitated the spread of antimicrobial resistance in the food chain. Therefore, screening LAB that is a potential starter culture for antibiotic resistance is a vital part of the selection criteria. Apart from that, acidification and organic acid production rate are properties that should also be considered during selection (García-Hernández et al., 2016). Organic acids are essential components of taste and aroma in lafun. Oyewole (1992) stated that the organic acids produced during the fermentation of cassava contribute to the characteristic flavour of the product.

To date, production of lafun is still limited to spontaneous fermentation at cottage industry level, resulting in product instability regarding its flavour, quality and

safety. There has been limited research focusing on the development of starter culture for the production of lafun, particularly regarding their role in determining the flavour of the final product. Therefore, the objective of this study was to characterise LAB isolated from spontaneous fermentation of cassava in order to select strains with potential for further study on identification and description of volatile flavour components, lafun rheological properties and lafun metabolite fingerprinting. The isolates were subjected to a combination of phenotypic and genotypic (a taxonomic polyphasic system) identification. Strains were selected according to their potential for production of desired non-volatile organic compounds in lafun. In addition, the selected strains were screened for the presence of antibiotic genes. The information gathered in this work would be useful in both small-scale and commercial production of lafun with consistent quality.

3.2 Materials and methods

3.2.1 Experimental design

The LAB that participated in the submerged and spontaneous fermentation of cassava were first isolated and identified phenotypically. These LAB strains (n=41) were then assessed on their capacity to produce non-volatile organic compounds using HPLC. Selection criteria for the strains to be used as starter cultures were set based on the highest ability to produce desirable flavour compounds. Genotypic identification was carried out for the selected strains (n = 4) and four isolates that were not identified phenotypically. Lastly, antibiotic susceptibility profile of the selected LAB and the four isolates that were not identified phenotypically were determined.

3.2.2 Cassava roots collection and initial preparation

Raw cassava roots were purchased from a local shop in Reading, United Kingdom and used on the day of purchase. In the initial spontaneous fermentation experiment to isolate LAB, the cassava roots were washed thoroughly, peeled, cut into small sizes of about 3 cm in diameter and washed again. In the later controlled fermentation experiment, samples were washed, peeled, cut into smaller sizes of about 1 cm in diameter to be able to go through Erlenmeyer flask and then were washed again (Figure 3.1).

3.2.3 Isolation and identification of lactic acid bacteria

During the spontaneous fermentation, 1000 g of prepared cassava roots were steeped into 1000 ml of water. They were placed in temperature-controlled cabinets at 37 ± 2 ^{o}C for 48 h. Samples were taken at 6 h, 24 h and 48 h, and plated after serial dilutions on De Man, Rogosa and Sharpe (MRS) agar and M17 agar (Al-kotami et al., 2015). Plates were incubated anaerobically at 37 ± 1 ^{o}C for 48 h. Colonies were grouped

Cassava roots

↓

Peeled, washed and rinsed thoroughly

↓

Cut into small pieces

↓

Fermented (spontaneous or control fermentation)

↓

Dried

↓

Milled

Figure 3. 1: Flow diagram of lafun processing

into categories based on colony morphology: size- small, medium or large; opacity- transparent or opaque; surface- smooth, glistening, rough or dull. Representative colonies from the incubated plates were purified by repeated streaking (Kunduhoglu et al., 2012). To determine the genera of the different isolates, the first step was to characterise them by Gram staining and catalase test. Strains were maintained in an appropriate cultured broth (1860 µl) in 140 µl of Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, UK) and stored in the freezer at -80 ^{o}C.

3.2.3.1 Phenotypic characterisation of isolated lactic acid bacteria

The LAB isolates were identified using 49 biochemical tests on the API fermentation strips (API 50 CHL Kit-bioMérieux, UK). Frozen strains were sub-cultured on MRS and M-17 broths before sub-culturing on MRS and M-17 agar for 48 h at 37 ± 1 ^{o}C. The purity of the colonies on the agar plates was verified by microscopic examination. API 50 CH strips were made ready by first preparing the incubation box. Approximately 10 ml of deionised water was distributed into the honeycombed wells of the tray to create a humid atmosphere. Strips were removed from their packaging and then placed appropriately in the incubation tray. An ampule of API Suspension Medium (2 ml) was used to make a heavy suspension (S) of the bacteria from the culture using a sterile swab. Then, a suspension with a turbidity equivalent to 2 McFarland was prepared by transferring the required number of drops of suspension 'S' into the ampule of API Suspension Medium (5ml) and this number of drops was recorded as 'n'. An ampule of API 50 CHL Medium was inoculated by transferring twice the number of drops of suspension 'S' (i.e. 2n) into the ampule and was used immediately. The tubes of the strip were filled with the inoculated API 50 CHL Medium and covered with mineral oil. The strips were incubated aerobically at 37 ± 1 ^{o}C for 48

h and results were recorded on the result sheets at both 24 h and 48 h. The biochemical profile obtained for each strain was identified using the apiwebTM identification software with database (V5.1).

3.2.3.2 Genotypic characterisation of some selected lactic acid bacteria

Wizard® Genomic DNA purification kit (Promega, Uk) was used and the procedure followed was that of Abed (2013) with some modification. Cells were grown overnight in 1.5% glycine containing media for adequate cell lysis. De Man, Rogosa and Sharpe (MRS) and M17 broths were used with respect to the type of medium used for LAB isolation. The cultured broth (5 ml) was centrifuged at 8,765 g for 3 min at 25 °C to pellet the cells and the supernatant removed. The cells were re-suspended in 900 µl of 50 mM EDTA (Sigma-Aldrich, UK). A total volume of 120 µl of lysozyme solution (40 mg ml^{-1}) (Sigma-Aldrich, UK] was added to the cell suspension and gently pipetted to achieve efficient cell lysis. The sample was incubated in a water bath at 37 °C for 60 min, with occasional mixing. The suspension was centrifuged at 8,765 g and 25 °C for 3 min, the supernatant was removed, and the pellet was gently re-suspended in 900 µl Nuclei lysis solution. At this stage, the cells were incubated at 80 °C for 5 min to lyse the cells and cooled at room temperature. Four microliters of RNase solution were added to the cell lysate.

The tubes were inverted ten times to mix, incubated again at 37 ºC for 60 min with occasional mixing by inversion, and cooled to room temperature. Then 300 µl protein precipitation solution was added to the RNase-treated cell lysate and vortexed vigorously for 30 s. The sample was incubated on ice for 7 min and centrifuged for 15 min at 8,765 g. The supernatant containing the DNA was cautiously transferred to a clean 1.5 ml Eppendorf tube containing 600 µl of room temperature isopropanol

(Fisher Scientific, UK). The mixture was gently mixed by inversion until the thread-like strands of DNA formed a visible mass. This was followed by centrifugation for 10 min at 8,765 g, the supernatant carefully poured off and the tube was drained on clean absorbent paper. The pellet was washed with 70% ethanol (600 µl) with gentle inversion to remove residual contaminants and the ethanol was discarded. The pellet (DNA) was rehydrated by suspending in 60 µl DNA rehydration solution and incubated overnight at room temperature. The DNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, USA) and subsequently it was stored at -20 ^{o}C until sent on dry-ice to MicrobesNG, IMI – School of Biosciences, University of Birmingham for full genomic sequencing.

3.2.4 Production of lafun by control fermentation

Lafun was produced using each isolated strain (n=41) in triplicate. The prepared cassava roots (100 g) were weighed, sterilised using 75% ethanol for 10 min and steeped in 200 ml sterile deionised water in a sterile 250 ml Erlenmeyer flask under aseptic conditions. The samples were inoculated singly with a 1000 µl cultured broth of each isolate (8.0 log CFU ml^{-1}) and were left in an anaerobic chamber (Don Whitley Scientific, UK) at 37 ± 1 ^{o}C for 48 h**.** The pH of the fermentation water was checked before (0 h) and after (48 h) fermentation. The samples were freeze-dried over four days (Sentry 2.0 freeze drier, VirTis SP Scientific, UK) and subsequently milled with a coffee blender (Cuisinart, UK). The flour obtained was packed into 60 ml sterile containers and stored at 2 ^{o}C before subsequent analyses.

3.2.5 Quantitation of non-volatile organic compounds in lafun

3.2.5.1 Non-organic acids extraction protocol

The extraction of organic acids from lafun samples was carried out using the method described by Bouzas et al. (1991). Lafun (1 g) and 10 ml of 0.013 N H_2SO_4 (mobile phase) were added in a disposable sterile 50 ml centrifuge tube. This was thoroughly mixed using an electric mixer (Heidolph Multi Reax, UK) for 15 min at 9.4 $x10^{-5}$ g and centrifuged for 10 min at 4 ºC and 1834 g. The supernatant was filtered into glass bottles using 0.22 µm Minisart® syringe filter (Sartorius, Germany). The filtered supernatant (1 ml) was pipetted into an HPLC vial and the remaining was kept in the freezer for subsequent analysis.

3.2.5.2 HPLC quantification of lafun samples

The concentrations of selected organic acids from lafun samples were analysed by high-performance liquid chromatography (HPLC) on an Agilent HP 1100 series with Vacuum degasser, Isocratic pump and 100 vial capacity Autosampler, HP 35900 interface, Agilent HP 1050 Diode array detector, ERC-7515A RI detector and Chemstation A10.01 software. The analysis was performed with an Aminex HPX-87H column (Bio-rad, Hercules, CA) of size 300 x 7.8 mm and particle size 9 µm. The column oven was set at 50 ºC and the UV absorbance wavelength was 210 nm. The mobile phase was 0.013 N H_2SO_4 prepared with HPLC water (Fisher Scientific, UK) running an isocratic gradient set at a flow rate of 0.6 ml min^{-1} and run time of 30 min. The sample injection volume was 20 µl (Eyéghé-Bickong et al., 2012). Analytical grade reagents were used as standards and were run to make a standard curve. Calibration curves for each acid were prepared (0.01 – 1.0 g l^{-1}).

3.2.6 Antibiotic susceptibility profile of and gene detection in the selected lactic acid bacteria and the four unidentified by API kit

Antibiotic susceptibility profile of selected LAB (n=8) was carried out using the agar diffusion test method. Seven antibiotics of standardised concentration were used namely: Chloramphenicol (C; 30 µg), metronidazole (MTZ; 30 µg), vancomycin (VA; 30 µg), gentamicin (CN; 120 µg); tetracycline (TE; 30 µg), penicillin G. (P; 10 µg) and erythromycin (E; 15 µg). The method used in this study was a modified Kirby-Bauer disc diffusion test that uses a standardised turbidity approach for inoculum preparation (Hudzicki, 2012). Inoculums of the isolates were prepared by suspending colonies from a 48-h cultured plate in 5 ml of maximum recovery diluent (MRD) to achieve a 1.0 McFarland Standard which was equivalent of bacterial suspension containing 3.0×10^8 cfu ml^{-1}.

After that, a 200 µl of inoculum suspension was pipetted and smeared evenly onto the surface of either MRS or M-17 agar using the spreading technique. All inoculum suspensions were used within 15 min. Once the inoculated plates were prepared, multiple antimicrobial susceptibility discs (Oxoid, Fisher Scientific, UK) were firmly applied to the plate surface with proper spacing using a sterile forceps. The prepared plates were then incubated at 37 ^oC for 48 h inside an anaerobic workstation (Don Whitley Scientific, UK). Susceptibility was assessed by the measurement of the diameter of the inhibition zone around the disc. The zone diameters (mm) were measured and used to categorise the strains as susceptible, intermediate or resistant using the British Society for Antimicrobial Chemotherapy (BSAC) (2015) breakpoint tables. This test was repeated in triplicate. The results from the full genomic sequencing were equally used to detect resistance genes in the strains using Comprehensive Antibiotics Resistance Database (CARDB).

3.2.7 Data analysis

The data for this study were analysed using basic descriptive statistics aided with the use of Microsoft Excel and XLSTAT statistical package (XLSTAT 2018.1).

3.3 Results and discussion

3.3.1 Enumeration and phenotypic identification of lactic acid bacteria isolates

LAB is a dominant part of the microbiota during fermentation of lafun (Padonou et al., 2009), and they showed an increase in their colony count from 8.30 x 10^3 CFU ml^{-1} at 6 h to 1.74 x 10^8 CFU ml^{-1} at 24 h (Table 3.1). This dominance agrees with the findings of Brauman (1996) who found that in spontaneous cassava fermentation LAB were largely predominant, accounting for over 99% of the total microflora. Isolations performed at 48 h were all too numerous to count. Based on colony morphology which are size, opacity and surface, 60 representative colonies were isolated, of which 41 were gram-positive and catalase negative. API kits identified 2% of the strains as *Lactobacillus brevis* (the least abundant strain); 10% as *Lactobacillus plantarum*; 10% as *Leuconostoc mesenteroides*; 19% as *Lactobacillus collinoides*; and 49% as *Lactococcus lactis* which was the most dominant strain. Furthermore, four isolates (10%) remained unidentified by the API kit.

The isolates (n=41) were tested against 49 biochemical strips. None of the isolates showed a metabolic reaction in 19 tests. The results of these tests that were negative (glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-αD-mannopyranoside, inulin, D-melezitose, xylitol, D-lyxose, D-fucose, L-fucose, L-arabitol) are omitted from Table 3.2. The tests that showed reaction after incubation were categorised as positive, weak positive and very weak positive if the colour observed was yellow, green or light green respectively.

The API profiles of the LAB isolates indicated that they metabolise the main plant carbohydrates effectively. Fifty-four percent of the isolates tested positive for L-

arabinose (l-Ara) which is a plant monosaccharide (Kotake et al., 2016). Other monosaccharides such as D-galactose, D-glucose, and D-fructose were generally fermented by the isolates (Table 3.2). Hermiati et al. (2011) reported a very high level of glucose in cassava pulp and found both arabinose and mannose too. Like glucose, all the isolates metabolised mannose. Ketiku and Oyenuga (1972) reported that only sucrose, maltose, glucose and fructose were found in cassava root-tuber with sucrose accounting for the highest proportion of the sugars. The report of the latter authors regarding sucrose was also supported by Sharma et al. (2016) who stated that sucrose is 69% of the total sugars in cassava. The highest level of glucose in cassava pulp recorded by Hermiati et al. (2011) must have resulted from sucrose fermentation that proceeds through extracellular hydrolysis of the sugar, producing glucose and fructose (Batista et al., 2004). Only 41% of the isolates fermented sucrose.

All the isolates tentatively identified as *L. lactis* do not metabolise sucrose. This is in agreement with the findings of Bonestroo et al. (1992) that obligatory homofermentative species possess very low fermenting capacities on sucrose. There may not be a problem using these organisms in the fermentation of cassava as they all metabolised glucose and fructose. Maltose was also fermented by 100% of the isolates. Thus, it could be deduced that any isolates selected from the groups of LAB tested would be able to metabolise the sugars in cassava roots effectively. All the genera identified were previously isolated from cassava fermentation (Padonou et al., 2009).

Table 3. 1: Enumeration of microbial growth at 6 h, 24 h and 48 h based on colony size determined by its diameter (mm)

Agar type	Time (h)	Number of colonies			cfu ml^{-1}
		Small	Medium	Large	
MRS	6	28	7	-	3.50×10^4
	24	57	48	-	1.05×10^8
	48	-	-	-	Too numerous
M17	6	50	22	11	8.30×10^3
	24	103	55	16	1.74×10^8
	48	-	-	-	Too numerous

The diameter of colonies up to 1mm was categorised as small; 1mm > 3mm was medium; 3mm ≥ 4mm was large

Table 3. 2: Biochemical profile of 41 lactic acid bacteria strains on the ***apiweb***^{TM} identification software

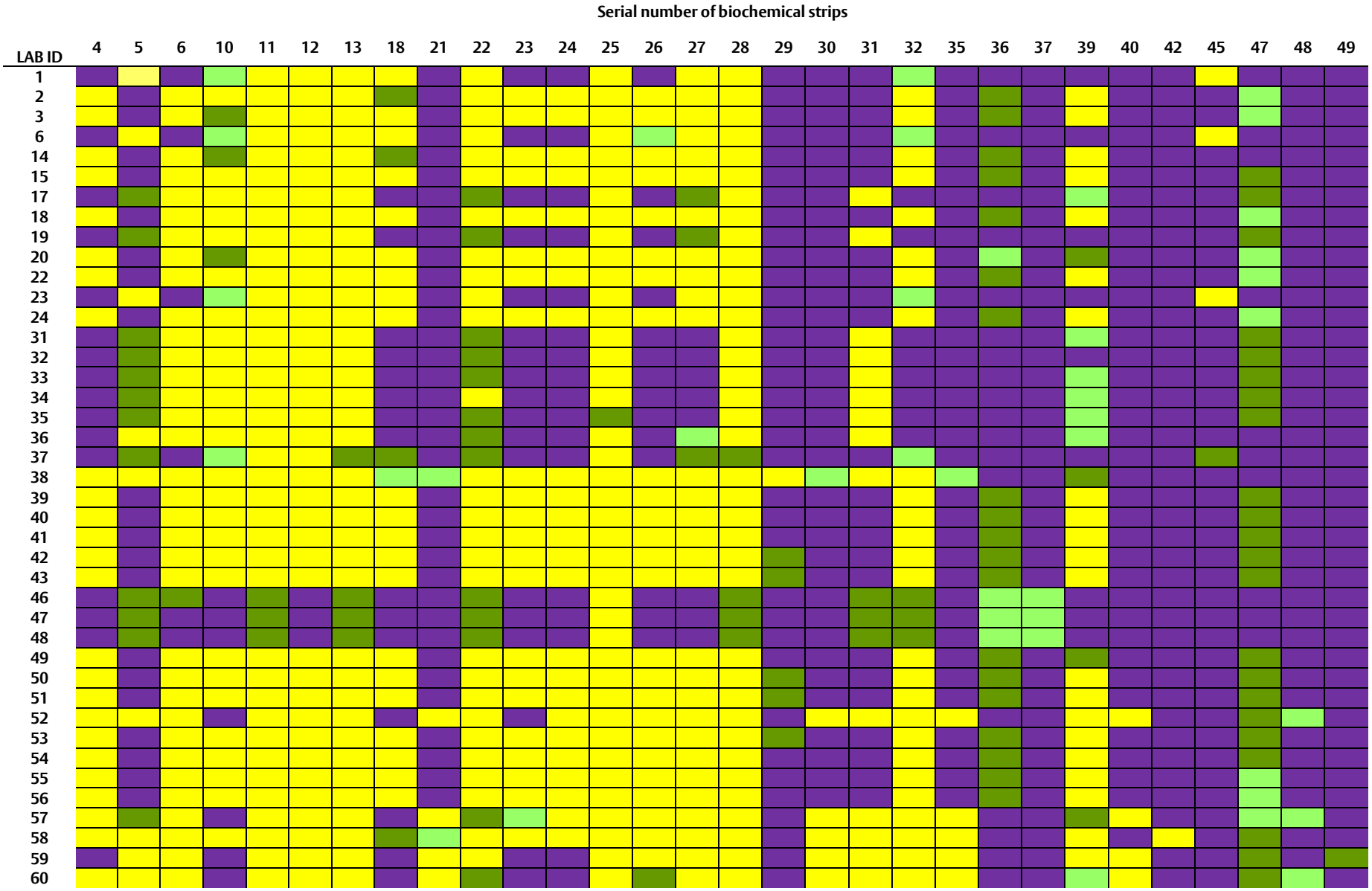

Table 3.2 continued

Key: Biochemical strips (there were no reactions in the strips with *)

*1 GLYcerol	*16 DULcitol	31 D-SACcharose	*46 L-ARabitoL
*2 ERYthritol	*17 INOsitol	32 D-TREhalose	47 potassium GlucoNaTe
*3 D-ARAbinose	18 D-MANnitol	*33 INUlin	48 potassium 2-keto Gluconate
4 L-ARAbinose	*19 D-SORbitol	*34 D-MeLeZitose	49 potassium 5-keto Gluconate
5 D-RIBose	*20 Methyl-αD-Mannopyranoside	35 D-RAFfinose	
6 D-XYLose	21 Methyl-αD-Glucosamine	36 AmiDON (starch)	Negative
*7 L-XYLose	22 N-AcetylGlucosamine	37 GLYcoGen	
*8 D-ADOnitol	23 AMYgdain	*38 XyLiTol	Positive
*9 Methyl-βD-Xylopyranoside	24 ARButin	39 GENtiobiose	
10 D-GALactose	25 ESCulin ferric citrate	40 D-TURanose	Weak positive
11 D-GLUcose	26 SALicin	*41 D-LYXose	
12 D-FRUctose	27 D-CELiobiose	42 D-TAGatose	Very weak positive
13 MaNnosE	28 D-MALtose	*43 D-FUCose	
*14 L-SorBosE	29 D-LACtose	*44 L-FUCose	
*15 L-RHAmnose	30 D-MELibiose	45 D-ARabitoL	

Key: LAB ID

1- *L. plantarum*	22- *L. lactis*	38- Unidentified	50- *L. lactis*
2- *L. lactis*	23- *L. plantarum*	39- *L. lactis*	51- *L. lactis*
3- *L. lactis*	24- *L. lactis*	40- *L. lactis*	52- *L. mesenteroides*
6- *L. plantarum*	31- *L. collinoides*	41- *L. lactis*	53- *L. lactis*
14- *L. lactis*	32- *L. collinoides*	42- *L. lactis*	54- *L. lactis*
15- *L. lactis*	33- *L. collinoides*	43- *L. lactis*	55- *L. lactis*
17- *L. collinoides*	34- *L. collinoides*	46- Unidentified	56- *L. lactis*
18- *L. lactis*	35- *L. collinoides*	47- Unidentified	57- *L. mesenteroides*
19- *L. collinoides*	36- *L. collinoides*	48- Unidentified	58- *L. brevis*
20- *L. lactis*	37- *L. plantarum*	49- *L. lactis*	59- *L. mesenteroides*
			60- *L. mesenteroides*

3.3.2 Evaluation of non-volatile organic compounds in lafun

To determine the level of select non-volatile organic compounds produced by the 41 LAB strains, each was used in control fermentation of cassava to produce lafun, and the concentration of organic compounds was subsequently determined using HPLC. The compounds quantified were citric acid, malic acid, succinic acid, lactic acid and acetic acid (Table 3.3). All strains produced high amounts of lactic acid leading to a rapid drop in pH to an average pH of 4.2 at 36 ± 1 °C temperature after 48 h. This is in agreement with the findings of Brauman et al. (1996) that LAB produce high amount of lactic acid during cassava fermentation that reduced the pH drastically. *L. lactis* (Lab ID 43) was the most efficient strain for lactic acid production, as expected for a homofermenter.

Since LAB ferment glucose primarily to lactic acid, this ability was used to screen the strains. The average lactic acid production across all 41 strains was calculated (17.9 mg g^{-1}) and this was used as a threshold value. Therefore 21 strains which produced lactic acid in the range of 16.5 mg g^{-1} to 34.3 mg g^{-1} were selected. These 21 strains were taken further to produce lafun singly to assess their consistency of flavour compounds production (Table 3.4). The concentrations of both citric acid and malic acid among all LAB strains were negligible. The strains produced quite a high concentration of lactic acid with exceptions of *L. lactis* (Lab ID 22), *L. plantarum* (Lab ID 23) *and L. lactis* (Lab ID 24) that produced 3.3, 5.4 and 5.7 mg g^{-1} respectively. Eleven strains produced an average of 10 mg g^{-1} lactic acid. *L. lactis* (Lab ID 56) showed the highest level of succinic acid, a by-product of sugar fermentation.

The selection of starters was based on high lactic acid and succinic acid with low acetic acid production (Figure 3.2). Lactic acid plays a preservative and flavouring role; succinic acid is an additive, acidity regulator and a dietary supplement (menopause

supplement that safely relieves up to 12 menopause symptoms); while acetic acid is considered an off flavour due to its pungent aroma (Meilgaard, 1982). Four organisms selected were: *L. collinoides* (Lab ID 36; lactic: 10.1 ± 0.3 mg g-1, succinic: 2.3 ± 0.2 mg g-1, acetic: 1.2 ± 0.1 mg g-1); *L. lactis* (Lab ID 43; lactic: 14.6 ± 0.5 mg g^{-1}, succinic: 2.6 ± 0.2 mg g^{-1}, acetic: 0.9 ± 0.1 mg g^{-1}); *L. lactis* (Lab ID 56; lactic: 13.0 ± 0.2 mg g^{-1}, succinic: 3.4 ± 0.0 mg g^{-1}, acetic: 1.4 ± 0.1 mg g^{-1}) and *L. brevis* (Lab ID 58; lactic: 12.5 ± 0.3 mg g^{-1}, succinic: 2.8 ± 0.2 mg g^{-1}, acetic: 1.4 ± 0.0 mg g^{-1}).

3.3.3 Genotypic identification of the selected lactic acid bacteria and the four unidentified by API kit

The accurate identification of the LAB strains that are going to be used in the control fermentation of cassava to produce lafun is of fundamental importance in this study that aims to enhance lafun flavour profile and improve its production. Accurate identification requires genotypic characterisation of these potential starter cultures. Thus, full genomic sequencing of the four selected strains as starters for the control fermentation of cassava and the four isolates that were not identified phenotypically using API kit was carried out to determine their genotypic identity and check if they are carrying antibiotic resistance genes.

The unidentified organisms with lab codes 38, 46, 47 and 48 were genotypically identified as *W. koreensis*, *L. lactis*, *L. lactis* and *Paenibacillus sp*. respectively. Three of the selected strains with lab codes 36, 56 and 58 that were identified phenotypically as *L. collinoides, L. lactis, and L. brevis respectively* were genetically characterised as *W. koreensis* (lab code 36 and 56) and *Leuconostoc mesenteroides* (lab code 58; Table 3.5). Only isolate with lab code 43 was identified as *L. lactis* at both phenotypic and genotypic levels. It is a typical recommendation in the literature (Belhadj et al., 2014) that the

Table 3. 3: Concentration of selected non-volatile organic acids produced by lactic acid bacteria isolates (n = 41) during initial screening experiment

		pH		Non-volatile organic acids (mg g^{-1})				
LAB ID	**Strain**	**0 h**	**48 h**	**Citrate**	**Malate**	**Succinate**	**Lactate**	**Acetate**
1	*L. plantarum*	6.5	4.4	2.6	0.4	5.3	12.8	0.0
2	*L. lactis*	6.3	4.5	1.3	0.3	3.7	10.0	1.9
3	*L. lactis*	6.5	4.5	1.3	0.4	4.3	10.0	2.3
6	*L. plantarum*	6.5	4.4	1.6	0.4	4.8	11.6	0.0
14	*L. lactis*	6.4	4.3	2.4	0.8	3.5	11.6	1.7
15	*L. lactis*	6.9	4.3	0.3	0.3	2.2	14.5	0.0
17	*L. collinoides*	6.5	4.1	0.1	0.3	2.5	12.2	0.0
18	*L. lactis*	6.4	4.2	0.3	0.4	4.5	24.9	0.0
19	*L. collinoides*	6.7	4.2	0.8	0.8	2.1	13.5	0.0
20	*L. lactis*	6.2	4.4	1.4	0.7	4.2	10.0	0.0
22	*L. lactis*	6.6	4.2	1.2	1.0	2.6	19.6	0.0
23	*L. plantarum*	6.4	4.2	0.2	0.8	4.5	21.0	0.0
24	*L. lactis*	6.5	4.3	0.2	1.0	6.5	23.5	0.0
31	*L. collinoides*	6.4	4.3	0.9	0.6	3.2	13.6	0.0
32	*L. collinoides*	6.2	4.3	0.2	0.9	5.2	20.1	0.0
33	*L. collinoides*	6.2	4.3	0.1	0.3	3.3	16.5	0.0
34	*L. collinoides*	6.3	4.1	0.2	0.4	3.4	10.6	0.0
35	*L. collinoides*	6.3	4.3	0.1	0.3	4.0	14.5	0.0
36	*L. collinoides*	6,2	4.2	0.1	1.0	3.7	16.5	0.0
37	*L. plantarum*	6.4	4.4	0.2	0.2	3.0	11.6	0.0
38	Unidentified	6.3	4.0	0.0	0.6	2.3	25.0	1.7
39	*L. lactis*	6.6	4.0	0.6	1.2	1.9	25.0	0.0
40	*L. lactis*	6.8	4.2	0.2	1.0	4.1	13.5	0.0
41	*L. lactis*	6.7	4.3	0.2	0.7	4.5	15.5	0.0
42	*L. lactis*	6.4	4.1	0.1	0.4	1.9	16.5	0.0
43	*L. lactis*	6.4	3.9	0.7	1.3	3.5	34.3	1.5
46	Unidentified	6.5	3.9	0.2	0.9	3.1	30.8	1.4
47	Unidentified	6.6	3.9	0.1	0.6	2.6	22.1	1.4
48	Unidentified	6.6	4.0	0.1	0.6	2.0	19.1	0.0
49	*L. lactis*	6.4	4.2	0.1	0.8	2.2	23.3	1.5
50	*L. lactis*	6.4	3.9	0.0	0.8	2.7	25.7	1.3
51	*L. lactis*	6.8	4.3	1.1	1.1	4.0	13.0	0.0
52	*L. mesenteroides*	6.7	3.9	0.8	0.2	6.2	12.9	0.0
53	*L. lactis*	6.4	3.9	1.7	0.7	3.1	27.8	0.7
54	*L. lactis*	6.8	4.0	0.1	1.0	4.7	12.9	0.0
55	*L. lactis*	6.4	3.9	1.4	0.7	2.8	27.4	1.0
56	*L. lactis*	6.5	3.9	1.1	0.4	3.3	26.7	1.2
57	*L. mesenteroides*	6.5	4.0	1.0	0.0	7.9	13.5	0.0
58	*L. brevis*	6.4	4.1	0.0	0.7	2.1	19.3	0.0
59	*L. mesenteroides*	6.8	3.8	0.3	0.4	7.9	16.5	0.0
60	*L. mesenteroides*	6.8	4.0	0.9	0.0	4.0	14.5	2.5

Table 3. 4: Concentration of selected non-volatile organic acids produced by lactic acid bacteria isolates (n = 21) during final screening experiment

		Non-volatile organic acids (mg g^{-1})				
LAB ID	**Strain**	**Citrate**	**Malate**	**Succinate**	**Lactate**	**Acetate**
18	*L. lactis*	1.0 ±0.1	0.0	0.1±0.0	9.2±0.2	0.0
22	*L. lactis*	1.8±0.1	0.8±0.1	2.5±0.2	3.2±0.3	2.9±0.2
23	*L. plantarum*	2.2±0.2	0.3±0.0	2.8±0.2	5.4±0.3	2.2±0.2
24	*L. lactis*	1.4±0.1	0.3±0.1	0.8±0.1	5.7±0.3	2.1±0.2
32	*L. collinoides*	0.6±0.0	0.3±0.0	1.6±0.1	11.2±0.2	1.8±0.1
33	*L. collinoides*	0.2±0.0	0.0	2.0±0.1	11.2±0.2	1.4±0.0
36	*L. collinoides*	0.3±0.0	0.0	2.3±0.2	10.1±0.3	1.2±0.1
38	*Unidentified*	1.5±0.1	0.3±0.0	2.2±0.1	10.6±0.3	1.0±0.0
39	*L. lactis*	0.4±0.0	0.3±0.0	2.3±0.2	12.9±0.0	1.7±0.1
42	*L. lactis*	1.7±0.1	0.7±0.1	1.4±0.2	6.5±0.5	1.0±0.1
43	*L. lactis*	1.4±0.1	0.0	2.6±0.2	14.6±0.5	0.9±0.1
46	*Unidentified*	0.9±0.0	0.0	1.8±0.1	9.6±0.4	0.8±0.0
47	*Unidentified*	1.0±0.1	2.5±0.2	0.2±0.0	8.7±0.2	0.2±0.0
48	*Unidentified*	1.0±0.1	3.2±0.3	0.2±0.0	8.9±0.2	0.2±0.0
49	*L. lactis*	1.5±0.1	0.0	0.2±0.0	9.8±0.2	0.3±0.1
50	*L. lactis*	1.5±0.1	0.0	0.3±0.1	9.6±0.4	0.2±0.0
53	*L. lactis*	0.8±0.0	0.3±0.0	2.9±0.2	12.5±0.4	2.2±0.2
55	*L. lactis*	0.7±0.0	0.4±0.0	2.5±0.1	13.1±0.2	1.6±0.1
56	*L. lactis*	0.8±0.0	0.3±0.0	3.4±0.0	13.0±0.2	1.4±0.1
58	*L. brevis*	1.8±0.1	0.7±0.1	2.8±0.2	12.5±0.3	1.4±0.0
59	*L. mesenteroides*	1.5±0.1	1.8±0.1	2.2±0.1	12.2±0.9	3.0±0.3

All results are presented as means of three replicates ±SD

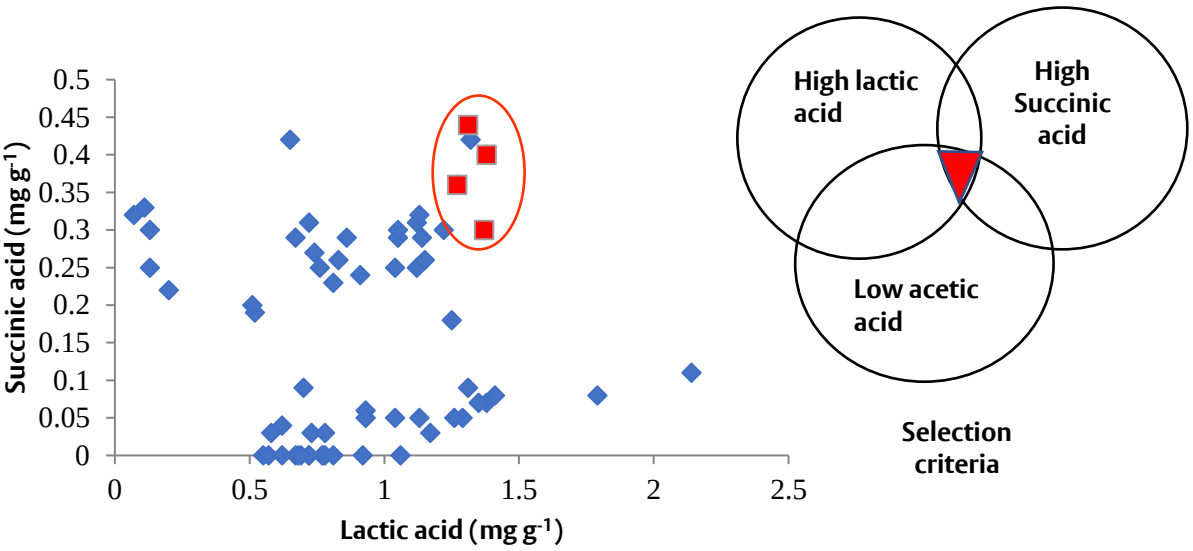

Figure 3. 2: Plot of lactic acid against succinic acid, red squares points to the lactic acid bacteria that met the set criteria

phenotypic identification method should always be supported with genotypic identification since the former method often fails to separate between phylogenetically related LAB species and subspecies (Adiguzel & Atasever, 2015; Conter et al., 2005; Gevers et al., 2001).

3.3.4 Antibiotic susceptibility profile (in-vitro) of the selected lactic acid bacteria strains and the four unidentified by API kit

The absence of transferable antimicrobial resistance genes is an important prerequisite for the selection of safe starter cultures. Fermented food can contain bacterial with the potential to convey antimicrobial resistance if such organisms are used as starter cultures (Verraes et al., 2013). Therefore, it is critical to determine the antibiotic susceptibility profile of the selected starter cultures used for cassava fermentation in this study. The antimicrobial susceptibility test using disc diffusion method was performed for the selected strains (Table 3.6). The antibiotics used were: Chloramphenicol (C; 30 µg), metronidazole (MTZ; 30 µg), vancomycin (VA; 30 µg), gentamicin (CN; 120 µg); tetracycline (TE; 30 µg), penicillin G. (P; 10 µg) and erythromycin (E; 15 µg). In general, all tested isolates were resistant to metronidazole and sensitive to chloramphenicol, gentamicin, tetracycline, erythromycin and penicillin G.

The resistance of *W. koreensis*, *L. lactis,* and *L. mesenteroides* to metronidazole is in agreement with other studies (Fraqueza, 2015; Mathur & Singh, 2005). The resistance to metronidazole is due to the absence of hydrogenase activity in LAB (Delgado et al., 2005). Metronidazole require a sufficiently low redox potential environment to be effective because it has a very low reduction potential (E17 – 486

Table 3. 5: The top families and genera that the reads map to as calculated using Kraken software

Sample code	**Unclassified**	**Unclassi-fied (%)**	**Most frequent Family**	**Most frequent Family (%)**	**2nd Most frequent Family**	**2nd Most frequent Family (%)**	**Most frequent genus**	**Most frequent genus (%)**	**2nd Most frequent genus**	**2nd Most frequent genus (%)**	**Most frequent species**	**Most frequent species (%)**	***Escherichia coli***	***Escherichia coli* (%)**
36	Unclassified	85.06	*Leuconostocaceae*	7.11	*Lactobacillaceae*	3.41	*Leuconostoc*	3.83	*Lactobacillus*	3.28	*Weissella Koreensis*	2.95	*Escherichia coli*	0.01
43	Unclassified	63.21	*Streptococcaceae*	34.09	*Enterococcaceae*	0.44	*Lactococcus*	31.99	*Streptococcus*	1.99	*Lactococcus lactis*	31.35	*Escherichia coli*	0.00
56	Unclassified	85.42	*Leuconostocaceae*	6.68	*Lactobacillaceae*	2.87	*Leuconostoc*	3.49	*Weissella*	2.91	*Weissella Koreensis*	2.91	*Escherichia coli*	0.00
58	Unclassified	79.13	*Leuconostocaceae*	17.94	*Lactobacillaceae*	0.66	*Leuconostoc*	17.74	*Lactobacillus*	0.57	*Leuconostoc mesenteroides*	7.21	*Escherichia coli*	0.01
38	Unclassified	85.85	*Leuconostocaceae*	6.39	*Lactobacillaceae*	3.11	*Leuconostoc*	3.54	*Lactobacillus*	2.98	*Weissella Koreensis*	2.53	*Escherichia coli*	0.02
46	Unclassified	63.84	*Streptococcaceae*	33.41	*Enterococcaceae*	0.43	*Lactococcus*	31.39	*Streptococcus*	1.90	*Lactococcus lactis*	30.79	*Escherichia coli*	0.01
47	Unclassified	62.93	*Streptococcaceae*	34.23	*Siphoviridae*	0.48	*Lactococcus*	32.15	*Streptococcus*	1.96	*Lactococcus*	31.54	*Escherichia*	0.00

mV) (Dhand & Snydman, 2014). This can only be provided by hydrogen producing organisms. However, LAB only exhibit a lactic acid fermentation and therefore will prevent the activation of the drug. Thus, the resistance of LAB against metronidazole was agreed to be innate by researchers (Rozos et al., 2018). Although most food-associated LAB have a 'Generally Regarded As Safe' status, the proof of absence of transferable antibiotic resistance in starter strains is still required before food production. This was the particular case for *Weissella spp*. that was selected as the starter culture used in this study, though it had yet to acquire GRAS status, they have already been widely used for fermentation in the food industry (Fessard & Remize, 2017).

Among these tested antibiotics, *L. lactis* strains were particularly sensitive to penicillin. The level of sensitivity can be assessed by comparing the size of inhibitory zones of the same antibiotics between isolates. Gentamicin was found to be the least effective for inhibiting the growth of bacteria where the zones ranged from an average of 22 to 26 mm, whereas for penicillin, the zones of inhibition reached 32 mm up to 42 mm. *L. lactis* had a higher sensitivity to all the antibiotics tested compared to other isolates as it always exhibited the largest inhibitory zones. *L. lactis* was the only strain sensitive to vancomycin. Elliott & Facklam (1996) could not find resistance to vancomycin in both *L. lactis* and *L. garvieae* that they investigated even though previous researchers reported vancomycin resistance in *lactococci*.

In general, *Weissella spp*. are highly resistant to a wide range of antibiotics, including metronidazole and vancomycin reported by some researchers (Kamboj et al., 2015). The mechanism of action of vancomycin is through its interaction with the peptidoglycan precursors in the cytoplasmic membrane (cell wall), followed by binding with the d-alanine/d-alanine terminus of the pentapeptide, inhibiting polymerisation

of peptidoglycan precursors of the microorganism. The resistance of vancomycin found in both *Weissella* and *Leuconostoc* strains in this study is due to the replacement of the terminal d-alanine residue into d-lactate or d-serine in the muramyl pentapeptide. The replacement prevents vancomycin from binding and therefore results in resistance to the antibiotic. These findings agree with other studies whereby both strains exhibited resistance to vancomycin due to their intrinsic resistance ability (Abriouel et al., 2015; Nelson, 1999). Concerning strains comparison, *Weissella* has relatively low sensitivity to antibiotics tested across the table, where they generally exhibited smaller inhibitory zones than the others.

3.3.5 Antibiotic resistance genes in the selected lactic acid bacteria strains and the four unidentified by API kit

The selected LAB strains (n=4) and the phenotypically unidentified isolates (n=4) were screened for the presence of antibiotic resistance genes. The results presented in Table 3.7 indicated that all strains identified as *L. lactis* at the molecular level and *W. koreensis-2* would be resistant to the lincosamide class of antibiotics. This class are known to be derivatives of sulfur-containing galactoside and an amino acid isolated in the 1960s containing lincomycin, clindamycin (a semi-synthetic derivative of lincomycin) and pirlimycin (an analog of clindamycin). Their mechanism of action is through protein synthesis inhibition by binding to the 50S subunits of bacterial ribosomes. They could be bactericidal or bacteriostatic in their spectrum of action. They are effective against most Gram-positive organisms, but specific resistance to this class of antibiotics results from the enzymatic inactivation of lincosamides drugs. There could be a cross-resistance among macrolides, lincosamides, and streptogramin group B antibiotics (MLSB resistance). The MLSB resistance happens when binding of

the drug to the target 50S ribosomal subunit is prevented (Morar et al., 2009) as these three classes share similar molecular target and have related chemical structures.

The disc diffusion antibiotic test showed however that *L. lactis* among the selected LAB was highly susceptible to erythromycin a macrolide. Good susceptibility was also observed in *W. koreensis-2*, confirming that there cannot be a cross-resistance from these organisms. Verraes et al. (2013) discussed that cross-resistance is mostly common among Gram-negative bacteria because of their outermost cell layers that serve as barriers against many antibiotics. Since lincosamides share similarities with macrolides (e.g. erythromycin) concerning the mode of action, the resistant gene observed cannot be a functional one. The selected LAB carrying a resistant gene to lincosamides had previously shown an antibiotic susceptibility phenotype to erythromycin.

There were no antibiotic-resistant genes in *W. koreensis-1*, *W. koreensis-3* and *L. mesenteroides* (Table 3.7). Both *W. koreensis-1* and *L. mesenteroides* are part of the selected strains as starters for lafun production. *Paenibacillus sp.* detected at the molecular level is not a LAB, but it is equally a Gram-positive but spore-forming bacterium that was initially put in the genus *Bacillus*. It was first isolated from the mycorrhizosphere of sorghum plants (Rieg et al., 2010). The antibiotic-resistant genes screening indicated that this species isolated from lafun has ten different antibiotic resistant genes. The species was also found in humans as opportunistic infections, which is hard to treat for its antibiotic resistance among other numerous reasons. In recent time, some *Paenibacillus* sp. have been discovered to be the cause of bacteremic infections in humans. The species implicated are *P. thiaminolyticus*, *P. konsidensis*, *P. alvei*, and *P. polymyxa* (Rieg et al., 2010). The isolation of *Paenibacillus sp.* in the spontaneous fermentation of cassava in this study strengthens the objective of using LAB that has the

Table 3. 6: Antibiotic resistance patterns of the selected lactic acid bacteria strains and the four unidentified by API kit interpreted as zone diameters obtained by agar diffusion method

	Types of antibiotics (Diameter of zone of inhibitions (mm)						
Strain	C(30)	MTZ (50)	VA (30)	CN (120)	TE (30)	P (10)	E (15)
W. Koreensis-1	28.00 ±1.73	R	R	23.00 ±1.00	26.33 ±0.58	33.00 ±1.00	23.00 ±1.73
L. lactis	32.67 ±1.15	R	24.00 ±0.00	24.33 ±0.58	35.67 ±0.58	38.00 ±3.46	33.33 ±1.15
W. Koreensis-2	27.67 ±2.89	R	R	24.00 ±1.73	25.67 ±0.58	33.00 ±1.00	23.33 ±1.15
L. mesenteroides	30.67 ±5.03	R	R	20.33 ±2.31	32.33 ±2.08	33.00±1.00	28.00 ±2.65

Key: R means resistance

Table 3. 7: Antibiotic resistant gene(s) in the selected lactic acid bacteria strains and isolates that were not identified by API kit

Strain	Best_Hit_ARO	Best_Identities	Drug Class	Resistance Mechanism	AMR Gene Family
W. koreensis-1	ND	ND	ND	ND	ND
L. lactis	lmrD	96.53	lincosamide antibiotic	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
W. koreensis-2	lmrD	97.67	lincosamide antibiotic	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
L. mesenteroides	ND	ND	ND	ND	ND
W. koreensis-3	ND	ND	ND	ND	ND
L. lactis-2	lmrD	96.53	lincosamide antibiotic	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
L. lactis-3	lmrD	96.53	lincosamide antibiotic	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
Paenibacillus sp.	vgbC	91.55	streptogramin antibiotic	antibiotic inactivation	streptogramin vgb lyase
	tetB(48)	98.89	tetracycline antibiotic	antibiotic efflux	major facilitator superfamily (MFS) antibiotic efflux pump
	mphI	94.48	macrolide antibiotic	antibiotic inactivation	macrolide phosphotransferase (MPH)
	CatU	92.63	phenicol antibiotic	antibiotic inactivation	chloramphenicol acetyltransferase (CAT)
	cipA	94.51	phenicol antibiotic; pleuromutilin antibiotic; streptogramin antibiotic; lincosamide antibiotic; macrolide antibiotic; oxazolidinone antibiotic	Antibiotic target alteration	Cfr 23S ribosomal RNA methyltransferase
	LlmA 23S ribosomal RNA methyltransferase	99.65	lincosamide antibiotic	antibiotic target alteration	Llm 23S ribosomal RNA methyltransferase
	TaeA	96.91	pleuromutilin antibiotic	antibiotic efflux	ATP-binding cassette (ABC) antibiotic efflux pump
	AAC(6')-34	95.05	aminoglycoside antibiotic	antibiotic inactivation	AAC(6')
	vanI	48.44	glycopeptide antibiotic	antibiotic target alteration	glycopeptide resistance gene cluster; van ligase
	tetA(48)	95.04	tetracycline antibiotic	antibiotic efflux	major facilitator superfamily (MFS) antibiotic efflux pump

history of safe use in control fermentation of food for human consumption. The results here indicated that the four selected LAB strains are suitable to incorporate in fermentation regarding safety concerns on antibiotic resistant gene transfer from starters to consumers.

3.4 Conclusion

The development of LAB starter cultures that will enhance lafun flavour was explored in this study. It was possible to isolate and characterised LAB from spontaneous fermentation of cassava using both phenotypic and genotypic methods. The strategy of using both identification methods for LAB strains provided accuracy and helped to overcome difficulties with inherent variation in wild isolates. All isolates showed high capabilities for fermenting the sugars in cassava. The identified LAB produced non-volatile organic acids such as lactic acid that have been used as flavouring agents in food industry. The rate of production of flavour compounds was used in the selection of four LAB strains as starters for lafun production. The selected LAB strains are suitable to incorporate in lafun processing regarding safety concerns on antibiotic resistant genes. Overall, the findings of this study would greatly facilitate flavour enhancement in lafun from a developed and improved starter system. The four selected strains would be further studied to determine their impact on volatile flavour compound production in lafun. In addition, to establish consumers' safety by ensuring the strains can reduce the level of cyanide content of cassava during lafun production.

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Chapter Four

4.0 Optimising Processing of Cassava for the Production of Lafun at Laboratory Scale

Abstract

Lafun is one of several fermented cassava-based products. The objective is to determine the impact of mechanical pressing on flavour retention, the impact of refrigerated storage on cassava quality and the acidification rate of potential starter monocultures. The findings provide a basis for optimising lafun production using selected starter cultures at laboratory scale. The experimental design was in four parts: assessment of freezing as a preservation method for imported cassava roots (bitter-IBA30572; fortified-IBA011371; sweet-TMEB117 from IITA, Nigeria) by microbial enumeration over a period of 3 months; determination of the pressing effect on drying time of fermented cassava, and flavour quality of lafun using a pneumatic press; estimation of time (24 h or 48 h) needed for fermentation to obtain higher levels of desirable flavour compounds; and determination of rate of acidification during cassava fermentation by LAB strains. Four LAB strains that were selected as optimal from Chapter 3: *Weissella koreensis-1*; *Lactococcus lactis*; *Weissella koreensis-2* and *Leuconostoc mesenteroides* were used in control fermentation of cassava. Bacterial counts assessed monthly showed a decrease from 3.0×10^4 to 1.8×10^3 CFU g^{-1} and from 6.0×10^2 to 3.0×10^2 CFU g^{-1} in bitter (IBA30572) and fortified (IBA011371) cassava respectively but increased slightly in sweet cassava (TMEB117). Yeast counts declined, and no mould found in all the cassava varieties. The results suggest that pressing affects drying time as it was shortened from 96 to 24 h. The concentrations of compounds such as lactate, citrate, malate and succinate were, however, reduced substantially by 22%, 76%, 53%

and 45% (v/v) respectively, when pressing was applied. Fermentation up to 48 h enhance non-volatile flavour compounds production. Finally, *Weissella koreensis-1* had the highest rate of acidification within 12 h (from pH 7.68 to 5.1) and maintained the pH with no significant difference up to 48 h. Combining *Weissella koreensis-1* with *Lactococcus lactis* that is capable of lowering pH down to 4.43 within 48 h in control fermentation of frozen cassava roots without pressing before drying could maximise flavour retention in lafun.

***Keywords*:** Cassava, microbial enumeration, fermentation, flavour, acidification, pressing

4.1 Introduction

Cassava, (*Manihot esculenta*), had been cultivated in South America for nearly eight millennia before becoming an import to all other continents (Bull et al., 2011). It is a shrubby tuberous plant of the *Euphorbiaceae* family, cultivated mainly for its starchy roots. As an essential staple in some parts of the world, it has different names in different regions: manioc in Francophone countries; yucca in Spanish America; tapioca in Malaysia and India. In Nigeria, it is known as ege or gbaguda by the people in the southwest; karaza or doyar kudu in the north; igari in the south, iwa unene or imidaka in the south-south; akpu, jigbo or abacha in the southeast (Iwuagwu, 2012).

It is an important raw material used to produce many staples in Africa and Asia (Ele, 2014). However, farmers and processors face a significant challenge with its rapid deterioration that can occur within few days from harvest which reduces both its storage life and market value (Uchechukwu-agua *et al*., 2015). To overcome this challenge cassava can be fermented to increase its shelf life. Different fermented and unfermented products have been derived from cassava roots across the globe. Unfermented products include tapioca, starch, snacks and cassava flour used in gluten-free bread, pastries and other gluten-free products. These unfermented products are produced from sweet cassava with reduced cyanide content that is reduced by simple food preparation. The bitter variety, however, must be processed by fermentation to attain a cyanide level that is not toxic for consumption (Ifeabunike et al., 2017).

Spontaneous fermentation and back-slopping are conventional production methods employed traditionally by indigenous processors. The classic problem with these methods is that the product quality and sensory quality are inconsistent due, in part, to the microflora of the production environment, hygiene of the production plant,

the variety of cassava used, processing equipment availability and the drying method employed (Kimaryo *et al*., 2000). To increase cassava root utilisation, food scientists and technologists have given increased attention to upgrading traditional processes, thereby optimising products.

There are various fermented cassava flour products among which is lafun. It is a staple in the Republic of Nigeria and the Republic of Benin. It is produced traditionally by submerged and spontaneous fermentation of bitter cassava roots to a fibrous powder that is sieved before cooking. Two types are produced in Benin: ordinary lafun and Chigan lafun, and the latter differ in the production method and more specifically in the washing operations at critical control points. Soaking is done in an enclosed container and soaked cassava pulp is being dried on a raised area to prevent dust (Padonou et al., 2009). Nigerians in the southwest consume ordinary lafun, and it is a significant part of their diets, especially in rural areas.

Most lafun handlers are indigenous producers with little or no knowledge about food quality and safety or good manufacturing practices (GMP). Indigenous processors are limited in their understanding of the fermentation process. They do not consider toxin hazards neither do they monitor water quality, fermentation temperature, and raw material quality. However, monitoring these aspects of fermentation process is very important to achieve a good quality product.

With the issues of product consistency, quality and safety, lafun processing needs to be transformed into a standardised modern process. In chapter 3, isolates that could have a positive impact on the flavour profile of lafun were identified by a control fermentation method using selected lactic acid bacteria (LAB). The work presented in this chapter aims to identify the processing conditions to be used at laboratory scale to

enhance the flavour profile of the lafun produced by the isolates selected in chapter 3. Three varieties of typed cassava were imported for use in this study and subsequent research. Therefore, it was necessary to determine the impact of frozen storage on microbial quality of the cassava roots. To improve the efficiency of water removal from the fermented cassava, mechanical pressing and its impact on organic acid content of the lafun was also assessed. Finally, the acidification rate of the LAB strains was checked to identify which isolates could be used in combination in the next stage of this research.

4.2 Materials and Methods

4.2.1 Raw cassava root samples

Three optimised cassava varieties: bitter-IBA30572; fortified-IBA011371; sweet-TMEB117 were sourced from International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. These cassava roots were harvested and waxed on the day of export and shipped by air freight to the University of Reading arriving on the third day after harvest. The samples were prepared according to good manufacturing practices in the food processing pilot plant at the University. These roots were used to determine the impact of frozen storage on the microbial quality of the cassava. In order to determine the impact of pressing on the concentration of organic acids in lafun, raw cassava roots (untyped) were obtained from an African shop in Reading, United Kingdom. These roots were used on the day of purchase.

Regardless of the source, all roots were washed thoroughly to remove sand and stones. They were peeled and rewashed after cutting into pieces of about 2 cm. The pieces were frozen and held at -18 ^{o}C for further processing. The frozen roots were always blanched for 2 min at 100 ^{o}C to inactivate a significant part of the background microflora and cooled under a UV light with a fan before the addition of the starter culture.

4.2.2 Frozen storage and microbial counts

Cassava roots were washed thoroughly, peeled, cut into pieces of about 2 cm and washed again. The pieces were packed in Ziploc polyethylene bags, and blast frozen at -20 ^{o}C and subsequently held at -18 ^{o}C. A sample of the prepared roots were not frozen but held at 2 ^{o}C as a control. Total plate counts were carried out monthly for 3 months to enumerate both bacteria and fungi. Ten grams of cassava roots were put in

a stomacher bag with 90 ml of Maximum Recovery Diluent (MRD) added, and the bag placed in a Colworth Stomacher for 1 min (10^{-1} dilution). Solid particles were allowed to settle before transferring 10 ml of liquid to a sterile glass. A dilution series of the supernatant was prepared up to 10^{-7} using sterile MRD. Sterile plates of Plate Count Agar (PCA) and Potato Dextrose Agar (PDA) were inoculated in duplicate with 0.1 ml of each dilution and incubated at 30 ^{o}C for 48 h and 25 ^{o}C for 120 h respectively. Plates were counted on a Gallenkamp Colony Counter and expressed as colony forming units (CFU g^{-1}) of the sample (Eq. 4.1).

$$No.\ of\ CFU/g = \frac{\text{No.of colonies counted X Dilution factor}}{\text{volume of the sample taken}} \qquad \text{Eq. 4.1}$$

4.2.3 Mechanical pressing of fermented cassava roots

The assessment of the pressing effect on the drying time of fermented cassava and flavour quality of lafun was carried out and the untyped cassava roots from the local African Shop were used. Prepared roots (750 g) were fermented spontaneously in 1,500 ml of potable water for 48 h in a temperature-controlled cabinet at 35 ^{o}C. Fermented roots were divided in half. One portion was pressed in a 9.5 cm (internal diameter) metal mould using a horizontal pneumatic press (TAYBAR, England) at 1.8 bar until excess water was removed. The pressed roots were then freeze-dried for 24 h (CHRiST Gamma 2-16 LSC, UK). The second half of the fermented cassava roots were freeze-dried without prior pressing for 96 h. The moisture content of each was determined after drying. Both samples were quantified for non-volatile flavour compounds using high-performance liquid chromatography (HPLC).

4.2.4 Quantification of some non-volatile flavour compounds throughout fermentation

In Chapter 3, 41 LAB strains were isolated and identified from lafun produced through spontaneous fermentation. Each isolate was assessed for acid and flavour production in lafun. Strains were selected based on lactic acid and succinic acid production for enhanced safety and flavour. Therefore, four strains were selected: *Weissella koreensis-1*; *Lactococcus lactis*; *Weissella koreensis-2* and *Leuconostoc mesenteroides* based on these criteria. They were used in control fermentation of prepared bitter-IBA30572 cassava roots. Cassava roots (100 g) were steeped in 200 ml of sterile water and inoculated with a 1000 µl monoculture broth of each strain (8.0 log cfu ml^{-1}). These were placed in an anaerobic chamber at 37 ^{o}C for 48 h to ferment**.** The samples were taken out at 24 h and 48 h and freeze-dried in a Sentry 2.0 freeze drier (VirTis SP Scientific, UK) for 96 h. Dried samples were milled with a coffee blender (Cuisinart, UK). The flour obtained was packed into 60 ml plastic bottles and stored at -18 ^{o}C prior to subsequent analysis.

4.2.5 Quantification of non-volatile organic compounds in lafun samples

4.2.5.1 Non-volatile organic acids extraction protocol

The extraction of organic acids from lafun samples was carried out using the method described by Bouzas et al. (1991). Lafun (1 g) and 10 ml of 0.013 N H_2SO_4 (mobile phase) were added in a disposable sterile 50 ml conical centrifuge tube. The mixture was thoroughly homogenised using an electric mixer (Heidolph Multi Reax, UK) for 15 min at 9.4 x 10^{-5} and centrifuged for 10 min at 1834 g and 4 ^{o}C. The supernatant was filtered into glass bottles using 0.22 µm Minisart® syringe filter (Sartorius, Germany) and 1 ml was pipetted into HPLC vial while the remaining was kept in the freezer for subsequent analysis.

4.2.5.2 HPLC quantification of lafun samples

The concentrations of select organic acids from lafun samples were analysed by high-performance liquid chromatography (HPLC) on an Agilent HP 1100 series with vacuum degasser, binary pump, 100-vial capacity autosampler, column oven, a variable wavelength detector, and Chemstation A10.01 software. The analysis was performed with an Aminex HPX-87H column (Bio-rad, Hercules, CA) of size 300 x 7.8 mm and particle size 9 µm. The column oven was set at 50 °C and the UV absorbance wavelength was 210 nm. The mobile phase was 0.013 N H_2SO_4 prepared with HPLC water (Fisher Scientific, UK) running an isocratic gradient set at a flow rate of 0.6 ml min^{-1} and run time of 30 min. The sample injection volume was 20 µl (Eyéghé-Bickong et al., 2012). Analytical grade reagents were used as standards and were run to make a standard curve. Calibration curves for each acid were prepared (0.01 – 1.0 g l^{-1}).

4.2.6 Monitoring of lactic acid bacteria strains acidification rate

Prepared bitter-IBA30572 cassava roots were fermented with the monoculture of selected LAB as per section 4.2.4. The initial pH (Seven CompactTM pH/Ion S220, METTLER TOLEDO) of the fermenting medium was determined at 0 h and readings subsequently taken at 12-hour intervals for 48 h. The experiment was repeated in triplicate.

4.2.7 Statistical Analysis

The data collected were analysed using basic descriptive and inferential statistics aided with the use of Microsoft Excel and XLSTAT statistical package (XLSTAT 2018.1). One-way analysis of variance (ANOVA) was carried out with $p<0.05$ indicating a significant difference.

4.3 Results and Discussion

4.3.1 Frozen storage and microbial counts

The purpose of determining the impact of blast freezing and frozen storage on imported cassava roots was to inhibit the metabolic activity of the roots microbes and prevent microbial infestation. This way, its microbiological quality is kept for three months until used. The treatment was necessary because the subsequent research work requires the availability of raw cassava roots as there is no access to freshly harvested roots. The yeast counts at 30 days were 5.0×10^2 and 9.0×10^2 for fortified and sweet cassava varieties respectively. However, no yeast was detected at 90 days. The log counts of bacteria for bitter and fortified cassava varieties were significantly reduced ($p < 0.05$) from 30 to 90 days and slightly higher ($p < 0.05$) in sweet cassava. The reduction observed in the log counts of yeast for bitter cassava from 30 to 90 days was significant ($p < 0.05$) (Table 4.1). The 1.2 log reduction observed in the bacteria count of bitter cassava indicated that the freezing method successfully reduced the number of viable bacteria tenfold. The presence of mould in the roots refrigerated at 2 ^oC was already visible within one month of storage (Figure 4.1a). This shows that freezing prevented deterioration of the cassava varieties and the growth of mould (Figure 4.1b) (Oranusi et al., 2014).

In food processing, lowering the temperature of raw materials or food products inhibits the growth of microbes as molecular mobility is depressed and consequently chemical reactions and biological processes are slowed down at low temperature. Rapid freezing using a blast freezer is an essential part of the freezing process that helped achieve an acceptable quality root after months of storage.

Table 4. 1: Microbial counts of frozen cassava stored over three months

Cassava variety	Microbe	30 days		60 days		90 days	
		CFU g^{-1}	Log CFU g^{-1}	CFU g^{-1}	Log CFU g^{-1}	CFU g^{-1}	Log CFU g^{-1}
Bitter	Bacteria	$3.0x10^4$	4.5	$1.0x10^4$	4.0	$1.8x10^3$	3.3
	Yeast	$6.9x10^3$	3.8	$3.0x10^3$	3.5	$1.0x10^3$	3.0
Fortified	Bacteria	$6.0x10^2$	2.8	$8.0x10^2$	2.9	$3.0x10^2$	2.4
	Yeast	$5.0x10^2$	2.7	$1.0x10^3$	3.0	ND	-
Sweet	Bacteria	$3.0x10^3$	3.5	$3.0x10^3$	3.5	$4.0x10^3$	3.6
	Yeast	$9.0x10^2$	3.0	$1.0x10^3$	3.0	ND	-

Results are the means of duplicates. ND, not detected

A B

Figure 4. 1: Prepared cassava roots stored at (A) 2 ^{o}C, and (B) -18 ^{o}C

Rahman and Velez-Ruiz (2010) reasoned that the rate of freezing a plant material determines its final quality. This is because the damage of the cell wall and general dehydration of the plant cell, and ice crystal formation depend on how rapid freezing is achieved. Blast freezing prevents disruptive bulky ice crystals formation that damage or alter the cell structure and give the undesirable product while thawing (USDA, 2013). Proper storage of the fresh roots is one of the verified measures for keeping quality and extending cassava shelf life. Rapid freezing of a well-prepared cassava roots using a blast freezer and holding in -18 ^{O}C had proven to be an excellent control measure for preventing deterioration of cassava roots.

4.3.2 Mechanical pressing of fermented cassava roots

A vital part of any food production is the regular supply of the products to meet consumers demand. It is crucial that in food optimisation and standardisation, demand challenges are taken into consideration while also maintaining food quality (Matopoulus et al., 2012). In the production of lafun (both traditionally and technologically improved procedures), significant time is required for drying. Therefore, the reduction of drying period was considered in this work. Pressing of fermented roots is not a stage in the production of lafun as it is in the processing of some cassava products like gari and attiéké. Akely et al. (2010) noted that the pressing step in attiéké production plays a vital role in determining product's paste properties and loading. Hence, pressing step was introduced to lafun processing to determine its impact on the concentration of lafun flavour components.

The effect of mechanical pressing on the time of drying and concentration of non-volatile flavour compounds in the final product, lafun, was assessed. A relationship was established between pressing fermented roots before drying and the time of

drying. Water (19.9% v/w) was mechanically pressed from the fermented root and thus required freeze-drying for just 24 h (Moisture content = 1.01%), whereas, the batch that was not pressed took 96 h to freeze-dry (Moisture content = 1.42%). Though 'pressing' shortened drying time from 96 h to 24 h, it would cause a significant reduction in the concentration of desired flavour components in the pressed batch (Table 4.2). The application of mechanical pressing is therefore not acceptable in the processing of lafun where lactic acid is required to play a major role in flavour enhancement.

4.3.3 Quantification of some non-volatile flavour compounds for the period of fermentation

Part of improving the processing of lafun was to investigate the effect of reduction in fermentation time on non-volatile organic compounds concentration. Lafun, like most fermented cassava flour is processed for about 8 days with a fermentation period between 48 h and 72 h (Nwabueze & Odunsi, 2007; Oboh & Elusiyan, 2007). In this experiment, the concentration of flavour compounds in lafun produced at 24 h and 48 h were compared to check if it is possible to shorten fermentation time without reducing the level of desired organic acids. The quantity of lactic acid (flavour compound of significant interest) could significantly increase from 24 h to 48 h in lafun produced with LAB strains (Figure 4.2). Some scientists had studied the effect of fermentation time on cyanide reduction and cassava lafun yields and found that between 36h to 48 h fermentation time is favourable (Tefera et al., 2014; Nwabueze & Odunsi, 2007). However, this is the first time the effect of fermentation time on the concentration of flavour compounds in the lactic fermentation of cassava has been investigated.

Table 4.2: Effect of pressing on the concentration of non-volatile organic compounds content (mg g^{-1}) of lafun on dry basis

	Treatment		
Non-volatile organic acids (mg g-1)	**Pressed**	**Not pressed**	**P[A]**
Citrate	0.3	2.2	***
Malate	1.0	3.3	***
Succinate	1.3	3.4	***
Lactate	6.8	10.7	***

[A]Probability as obtained from ANOVA that there is a difference between means: ns, no significant difference ($p > 0.05$); * significant at 5% level, ** significant at 1% level and *** significant at 0.1% level

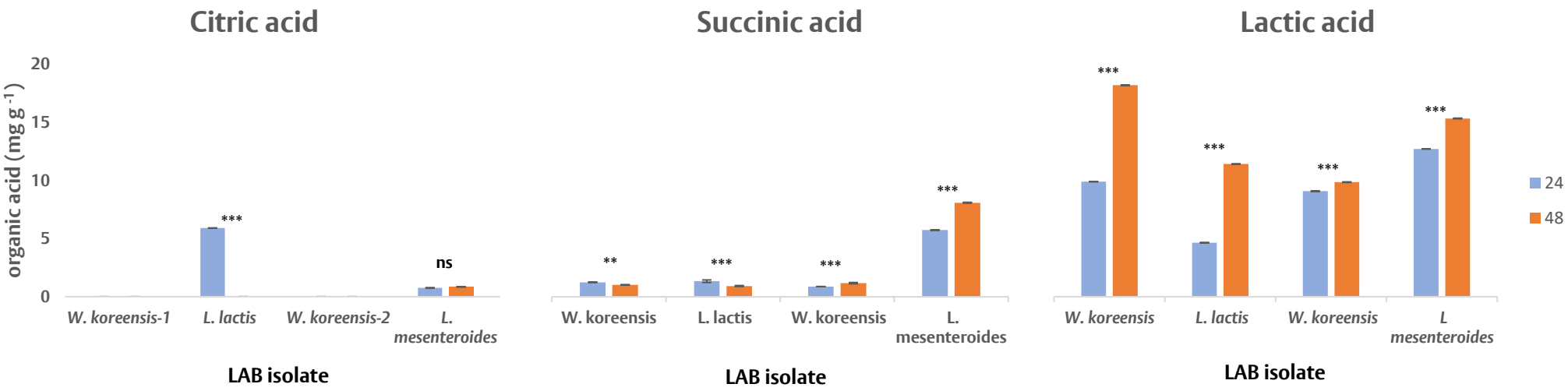

Figure 4. 2: Concentration of non-volatile organic acids in lafun produced by fermentation for 24 h and 48 h; the error bars indicate the range between the flavour compounds concentration of 3 replicates. Probability as obtained from ANOVA that there is a difference between means of 24 h and 48 h flavour compound concentration: ns, no significant difference ($p > 0.05$); * significant at 5% level, ** significant at 1% level and *** significant at 0.1% level

4.3.4 Monitoring of lactic acid bacteria strains acidification rate

In chapter 3, the production of some non-volatile flavour compounds was used in selecting four LAB strains as the starter for the fermentation of cassava roots to produce lafun. Strains would be combined by pairing a strain that is responsible for desirable flavour production with one that has rapid acidification rate. *W. koreensis-1* had the highest acidification rate reducing the pH from 7.68 ± 0.04 to 5.13 ± 0.17 within 12 h of fermentation but with the highest final pH by 48 h (Table 4.3). The remaining three strains maintained a consistent reduction of pH from 0 h to 48 h with *L. lactis* being the fastest acidifier among them (7.65 ± 0.08 to 5.16 ± 0.12 in 12 h). Chen et al. (2017) opine that the ability to produce acid is strain dependent and found *L. lactis* among strains with higher producing ability. The capability of *L. lactis* to reduce < 6 pH in 6 hours was demonstrated in the work of Turhan and Öner (2014) which is in agreement with the result of this experiment.

Acid formation is vital for preservation and flavour generation in food products (Widyastuti et al., 2014). Lactic acid fermentation inhibits the growth of several spoilage and pathogenic bacteria through their ability to initiate rapid decrease in pH of a fermenting raw material (Rault et al., 2009). The degree of acidification achieved among other factors like levels of hygiene and sanitation would determine the extent to which food contamination and spoilage are controlled. Gunsalus and Niven (1942) reported that the higher the pH, the lower the formation of non-volatile compounds. Therefore, it would be necessary to combine strains with highest acidification with those of moderate capability. Both strains of *W. koreensis* showed a good ability of rapid pH reduction of the fermenting medium while also maintaining a low pH between 12 h and 48 h even though they are yet to be recognised as GRAS (Generally Recognized

Table 4.3: Acidification rate during cassava fermentation by LAB strains

LAB strain	pH at 0 h	pH at 12 h	pH at 24 h	pH at 36 h	pH at 48 h
W. koreensis-1	7.68^{a}± 0.04	5.13^{b}±0.17	4.80^{b}±0.29	4.90^{b} ±0.43	5.02^{b} ±0.51
L. lactis	7.65^{a} ±0.08	5.16^{b} ±0.12	4.72^{c} ±0.06	4.47^{d} ±0.03	4.43^{d} ±0.03
W. koreensis-2	7.65^{a} ±0.36	5.43^{b}±0.60	4.83^{b}±0.07	4.71^{b}±0.14	4.68^{b}±0.21
L. mesenteroides	7.65^{a} ±0.10	5.76^{b}±0.22	4.92^{c}±0.16	4.76^{c} ±0.19	4.72^{c} ±0.21

*abcd means with different letters across the rows indicate significant statistical differences (Tukey's Test, $p < 0.05$); Values are Mean ± SD

As Safe) (Fessard & Remize, 2017). The latter authors noted that the acidification rate of *W. koreensis* in food fermentation is similar to those observed for *Leuconoctoc* which is in agreement with the result obtained in this study at 12 h. Weak acidification power is commonly observed in *L. mesenteroides* as it is for *Leuconostoc sp.* especially in milk fermentation. However, it competes well with other strains in this study, which could be attributed to the fact that sucrose (the main carbohydrate of cassava) enhance Leuconostoc growth more than other carbon sources (Abekhti et al., 2014). As an obligate heterofermentative lactic acid bacterium, *L. mesenteroides* produces carbon dioxide which replaces oxygen, making the fermenting medium anaerobic and suitable for the growth of successive species of LAB. Then, combining this strain with other LAB could increase acidification rate.

4.4 Conclusion

Blast freezing and frozen storage of prepared cassava roots is an effective process to maintain the quality of the roots for up to 3 months, facilitating further research. The findings of this study suggest that 48 h of fermentation could enhance the desired flavour wanted in lafun as higher levels of lactic acid were produced by LAB cultures by this time. While the introduction of a pressing step may reduce the drying time of lafun from 96 h to 24 h, it is not recommended as it may reduce the concentration of lactic acid in the finished product. This present study lays the groundwork for future research into more efficient drying processes for lafun production. Acidification performance is a major criterion in the selection of starters in food fermentation. Considering the inability of *W. koreensis-1* to consistently reduce pH up to 48h, combining it with the other LAB is thus recommended.

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Chapter Five

5.0 Impact of Selected Starters and Cassava Varieties on the Quality and Safety of Lafun

Abstract

Lactic acid bacteria (LAB) have a pivotal role in the production of lafun due to their role in acidification and cyanide reduction. The lafun samples derived from cassava varieties (bitter: IBA30527; fortified: IBA011371; and sweet: TMEB117) were fermented with selected LAB (*Weissella koreensis* (2 strains); *Lactococcus lactis*; and *Leuconostoc mesenteroides*) and assessed for proximate quality, rheological properties, volatile favour profile and metabolite fingerprinting. The quality of lafun regarding cyanide content and microbial load was also evaluated. LAB fermentation of fortified cassava variety gave the highest nutritional values with the composition of the lafun produced using *Leuconostoc mesenteroides* (Ash: being 4.37 ± 0.05%; Protein: 3.08 ± 0.23% and Fibre: 7.43 ± 0.18%). The results of rheological analysis indicated *Weissella koreensis-2* fermentation of fortified variety resulted in lafun with the highest viscoelastic behaviour. The volatile flavour compounds identified in lafun included a total of 21 aldehydes, 2 ketones, 5 organic acids, 1 alcohol, 1 furan, 3 enones, and 2 cresol. The metabolites identified using ^{1}H-NMR were ethanol, alanine, lactate, succinate and sucrose. During the microbiological assessment of lafun no *Salmonella* spp. was detected in 25 g of samples and microbial enumeration of samples were satisfactory. The cyanide contents of the final products were as low as 5.59 ± 1.44 mg kg^{-1} (bitter cassava); 4.48 ± 0.30 mg kg^{-1} (fortified cassava) and 2.58 ± 0.21 mg kg^{-1} (sweet cassava).

The use of LAB in the control fermentation of fortified cassava could be a sustainable alternative to improve lafun physical, nutritional, and flavour properties

***Keywords*:** Lactic acid bacteria, lafun, proximate, rheology, cyanide, volatile flavour profile

5.1 Introduction

Fermentation typically infers the necessary action of food-grade microorganisms, and it occurs when these organisms, as part of their metabolic processes, consume susceptible organic substrate. It is formerly known as a chemical reaction or process of converting sugars to alcohol and carbon dioxide by yeast under anaerobic condition. Recent definitions have extended it to the aerobic or anaerobic conversion of organic materials into various biomolecules, biochemicals and biofuels by the actions of a multiple range of microorganisms (yeasts, bacteria, moulds, plant cells or animal cells), singly or in combination (Terefe, 2016; Ibrahim, 2015). The application of fermentation includes the reduction of cyanogenic glycosides in food raw materials that contain the phytotoxins.

Cyanogenic glycosides (cyanoglycosides) are natural plant toxins stored in some plant vacuoles in an inactive state. They are glycosides of sugar(s), and cyanide with a glycone. About 25 forms had been identified in at least 2,500 plants. Among these cyanoglycosides are linamarin, lotaustralin, phaseolunatin, prunasin, sambunigrin, amygdalin, dhurrin and vicianin (Cressey & Reeve, 2019; Barceloux, 2008). They are released into the cytoplasm and activated there by enzymes when the plant is attacked during processing or consumption. This results in the enzymes and glycosides coming together to produce toxic, volatile hydrogen cyanide (HCN) gas (Emire et al., 2013; Bolarinwa et al., 2016). Cassava is a root crop that contains mainly linamarin and lotaustralin (the methyl derivative of linamarin) (Moriasi et al., 2017; Burns et al., 2012). Both cyanoglycosides and the linamarase enzyme that degrades them by hydrolysis are separated in the cassava tissues. Thus, the formation of cyanide is prevented until there is a physical disruption to the plant preventing toxic effects to the plant.

When cassava roots are cut, soaked, boiled or processed in any form, linamarase is released to hydrolyse linamarin to hydrogen cyanide, cyanohydrin, glucose, and acetone, which helps to reduce cassava toxicity. Barceloux, (2008) reported that fermented cassava products in Africa, such as lafun, gari and fufu, have their cyanoglycoside content reduced by 80-85%. These food products are derived from bitter cassava roots that originally contain cyanide between 10-500 mg kg^{-1} of dry matter. The knowledge of the concentration levels of cyanide in cassava roots as well as the specific processing technique (e.g. fermentation) that reduces cyanide in the roots has been useful to resolve accidental cyanide toxicity problems (Ifeabunike et al., 2017; Bolarinwa et al., 2016).

The rapid spoilage of fresh cassava root is a major problem and it can occur because of its high-water activity, making it a susceptible organic substrate supporting the metabolic activity of undesirable microorganisms. The food poisoning or spoilage organisms (undesirable microorganisms) growing in cassava belong to, but are not limited to the genera *Staphylococcus, Salmonella, Yersiniae, Pseudomonas, Bacillus,* and the species *Escherichia coli,* as well as osmophilic moulds and yeasts (Ihenetu et al., 2017; Adesokan et al., 2008; Pepe et al., 2003). Fermentation of cassava has played a vital role in preventing the growth of undesirable organisms in cassava.

The preservative role of food fermentation is complex and it is mediated by the activity of the microorganisms that produce antimicrobial compounds inhibiting spoilage and pathogenic bacteria (Li et al., 2014). The effectiveness of the use of defined strain starter systems, especially lactic acid bacteria (LAB), that produce antimicrobial metabolic compounds that inhibit the growth or eliminate food pathogens has been extensively reported (Adesokan et al, 2008; Modzelewska-Kapitola & Marin–Iniestal,

2005; Ogunbanwo et al., 2004; Ross et al, 2002). Organic acids and carbon dioxide are examples of the antagonistic substances produced and are used successfully in many food applications (Ross et al., 2002). LAB have been the focus of the earliest starter culture development for fermented food products. They are also reported as the most prevalent microorganisms in the traditionally fermented cassava products (Kostinek et al., 2005). They deliver rapid and consistent acidification of the raw material and produce aroma compounds (Edward et al., 2011; Franz et al., 2005; Leroy & De Vuyst, 2004). This group of bacteria, due to their dominant role in cassava roots fermentation, have become the subject of many studies on the development of functional starter cultures (Anyogu et al., 2014; Yao et al., 2009; Kostinek et al., 2005)

Fermentation makes a valuable contribution to the development of flavour and overall acceptability of fermented products. The concentration and composition of volatile flavour compounds can be key to consumer acceptability and preference (Chiang & Ismail, 2006; Banigo et al., 1974). Fermentation by microorganisms could also result in a significant increase in the quality of food protein, vitamins and other essential nutrients. Ojokoh and Orekoya (2016) have shown that fermentation improves the proximate values of the epicarp of watermelon (*Citrullus lanatus*) and transformed it into a potential food supplement. Unlike other preservation methods with only one objective of retarding the growth of microbes in food or at least reducing their numbers, fermentation encourages the growth of desired organisms whose metabolic activities improve the product sensory and physical attributes (Franz et al., 2005; Leroy & De Vuyst, 2004).

Rheological evaluation of food is also an important tool for the assessment of product quality. Rheological measurement is mainly concerned with how food

materials respond to an external (applied) force (stress) and deformation (strain) as a function of time (Daubert & Foegeding, 2010). Oscillatory rheology, in which a small strain is applied, is well suited to the evaluation of the physical properties of food products, such as hydrated lafun, that are viscoelastic (Tabilo-Munizaga & Barbosa-Cánovas, 2005). The rheological measurement of food is closely correlated with its texture when restricted within thin films between two shearing surfaces. This is because the deformation of a food item that is squeezed by hand and the flow of the bolus of chewed food, fluid, or semifluid in the mouth are all textural as well as rheological properties (Bourne, 2002). Rheological analysis is important in the study to monitor the effects of compositional and processing parameters that are needed in developing lafun with the desired texture property.

Lafun is a cassava product traditionally processed by steeping peeled cassava root pieces in water followed by fermentation for 2-5 days. The fermented roots are sun-dried on surfaces such as cement floors, tarred roads, and rocks, and subsequently milled into powder (Padonou et al., 2010). Drying can take days or weeks, depending on the weather (Kuye & Sanni, 1999). The coarse flour obtained is sieved and made into a gruel in boiling water and consumed with a stew of vegetable and protein source. If properly stored, the shelf-life of the cassava flour can be six months or more.

Since lafun is a product of spontaneous fermentation, the microbiological composition is of a mixed culture that includes *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia scutulata*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Bacillus cereus* (Padonou et al., 2009a; Padonou et al., 2009b). Some work has been done to understand the predominant microorganisms in the fermentation of cassava to produce lafun and the role they play in the process (Adebayo-Oyetoro et al.,2013; Oyewole & Odunfa, 1988). Padonou et al. (2010) in their work showed the effect of

combining yeast and LAB as a mixed starter culture to produce lafun. Still, there is little or no information on the impact of LAB strains and cassava varieties on the rheological properties, proximate composition and volatile flavour compounds of lafun. The current work was undertaken to determine the impact of selected LAB strains as monoculture or co-culture on the physical properties and flavour profile of lafun produced using three varieties of cassava (bitter, sweet and fortified forms). In addition, to ensure that the selected LAB strains do not compromise the safety of lafun its cyanide content and microbiological safety was determined. Lafun metabolite fingerprinting by ^{1}H-NMR was also analysed to detect the metabolites that happen to be present in lafun samples, discriminate between these samples and identify the most important regions of the spectra.

5.2 Materials and Methods

5.2.1 Collection of raw cassava roots and lafun market samples

Cassava roots, bitter cassava (IBA30527), fortified cassava (IBA011371) and sweet cassava (TMEB117), were imported from the International Institute of Tropical Agriculture (IITA), Nigeria to produce lafun. These cassava roots were harvested and waxed on the day of export and shipped by air freight to the University of Reading, arriving on the third day after harvest. Lafun samples produced by indigenous food manufacturers were collected from three different markets in Ibadan, Nigeria: Bodija, Songo and Mokola, to be used as market samples M1, M2 and M3 respectively.

5.2.2 Preparation of cassava roots

The cassava roots were prepared according to good manufacturing practices in the food processing pilot plant at the University. The samples were washed thoroughly, peeled and rewashed after cutting into pieces of approximately 2 cm. The pieces were packed into Ziploc polyethylene bags and blast frozen before holding at -18 ^{o}C. For lafun production, the frozen roots were initially blanched for 2 min to reduce microbial loads and cooled under a UV light cabinet with a fan before fermentation. Samples of the raw cassava roots from each variety were freeze-dried without fermentation, milled, packed in 250 ml sterile bottles and kept at -18 ^{o}C until further analysis.

5.2.3 Preparation of lactic acid bacteria starter cultures

The four strains identified and selected as the most efficient strains for lactic acid production in chapter 3 were used in this study. They were *Weissella koreensis-1*; *Lactococcus lactis*; *Weissella koreensis-2* and *Leuconostoc mesenteroides*. Each strain was preserved in 2 ml cryo vials of 1860 µl cultured broth and 140 µl Dimethyl sulfoxide (DMSO) at -80 ^{o}C. They were reactivated by first culturing on either De Man, Rogosa and

Sharpe broth (MRS) (*W. koreensis -1 and -2* and *L. mesenteroides*) or M17 broth (*L. lactis*) before plating on the media agar. Incubation was performed anaerobically at 37 ± 1°C for 48 h.

5.2.4 Lafun production

The selected LAB starter cultures were used in control fermentation of the prepared cassava roots. The procedure used for the three cassava varieties was the same. Samples of prepared roots (300 g) were steeped in 300 ml of sterile water. Samples were inoculated singly with a 3000 µl cultured broth of each strain (8.0 log CFU ml^{-1}). In the co-starter culture, 1500 µl cultured broth each of the two LAB strains that were combined was used. These were placed in an anaerobic workstation (MG1000, Don Whitley Scientific, UK) at 37 ± 1°C for 48 h to ferment**.** The samples were taken out at 48 h to freeze-dry (CoolSafe 4-15LFreeze Dryer, Labogene, UK) for 4 days and were milled with a coffee blender. The flour obtained was packed into a 250 ml sterile bottle and stored at -18 °C for further analyses. The experiment was repeated in duplicate.

5.2.5 Proximate analysis of lafun samples and raw cassava roots

The laboratory samples (n=24), market sample and raw cassava roots were analysed for moisture, ash, crude protein, crude lipid, crude fibre and digestible carbohydrates. The proximate analysis was carried out in duplicate and on a dry weight basis.

5.2.5.1 Moisture content

Moisture content was determined via a thermogravimetric approach; that is, the oven-dry method (AOAC, 2000). Crucibles were dried in the oven at 100 °C for 1 h and subsequently cooled in a desiccator. These crucibles were weighed before adding 3 g

of sample and placed in the oven at 105 ºC. Samples were dried to constant weight for 3 h. Percentage moisture content was calculated by the difference in wet and dry weight as follows (Eq. 5.1):

$$\% \text{ Moisture } = \frac{(W1-W2)100}{W1} \ \% \qquad \text{Eq. 5.1}$$

Where:

W_1 = Initial weight in g of sample

W_2 = Final weight in g of sample after drying

5.2.5.2 Ash content

Samples (3 g) were weighed into porcelain crucibles that had been dried and weighed. Samples were placed in a drying oven at 100 ºC for 3 h. The organic matter was charred by igniting the material on a hot plate in the fume cupboard until no smoke was visible (pre-ash) and transferred to a muffle furnace. Samples were maintained at 550 ºC in the furnace for 8 h to burn off all organic matter. The furnace was allowed to cool below 200 ºC and maintained at this temperature for 20 min. The inorganic materials which do not volatilise at that temperature were obtained as white ash. Crucibles were moved into a desiccator with stopper top and weighed soon after cooling (modified method of AOAC, 1995). Percentage of ash was calculated as follows (Eq. 5.2):

$$\% \text{ Ash} = \frac{\text{wt.crucible and ash} - \text{wt.crucible}}{\text{wt.crucible and sample} - \text{wt.crucible}} \ x100 \qquad \text{Eq. 5.2}$$

5.2.5.3 Crude protein

Crude protein was carried out using the Kjeldahl method (AOAC, 2000). Sucrose of 1.0 g (Blank), 0.2 g of glycine (standard) and 3.0 g of each sample were weighed in

separate nitrogen free paper digested together with the sample. Each was done in a clean 300 ml digesting tube. Concentrated sulphuric acid (25 ml; Fisher Scientific, UK) was added with two tablets of kjeltabs S (Thompson and Capper Ltd, UK). The mixture was placed on a heater to digest for 90 min in a fume cupboard. Fifty millilitres of 2 % boric acid (Sigma-Aldrich, UK) was poured in a 250 ml Erlenmeyer flask, and three drops methyl red indicator was added. The sample was distilled into the flask and 0.05 M (0.1 N) sulphuric acid was poured in the burette. The distillate in the Erlenmeyer flask was titrated with 0.05 M sulphuric acid until a light-pink endpoint was reached. The percentage of nitrogen and protein in lafun were calculated (Eq. 5.3)

$$\%\ Nitrogen = (V1 - V2)\ x\ T\ x \frac{0.014}{m}\ x\ 100 \qquad \text{Eq. 5.3}$$

Where:

V1- is the volume, in millilitres, of the sulfuric acid solution required for the test portion;

V2- is the volume, in millilitres, of the sulfuric acid solution needed for the blank test;

T -is the normality of the sulfuric acid solution used for the titration;

m- is the mass, in grams, of the test portion;

0,014 -is the value, in grams, of the quantity of nitrogen equivalent to the use of 1 ml of a 0.05 mol l^{-1} sulfuric acid solution.

The crude protein content of the product was calculated by multiplying the value obtained at the time of determination of the nitrogen content (% Kjeldahl N) by a conversion factor F, which is 6.25 for flour, that is:

$$\%\ \text{Crude Protein} = \%\ \text{Kjeldahl N x F} \qquad \text{Eq. 5.3b}$$

5.2.5.4 Crude lipid

Crude lipid content was determined via solvent extraction (AOAC, 2006). Petroleum Ether B.P. 40-60 °C (Fisher Scientific, UK) was used. Five grams of lafun sample was weighed into a Soxhlet extraction thimble (28 x 80). The thimble was plugged with a cotton wool bung at the top. Extraction flask was cooled in a desiccator after drying in an oven at 100 °C for 1 h, then weighed soon after cooling. The thimble of the sample was placed in a barrel and connected to the pre-weighed extraction flask. Petroleum ether was poured into the barrel until it siphons over into flask twice (2 barrels). Soxhlet was connected to the condenser and the condenser water turned on. The heating unit was turned on for 8 h extraction. The unit was turned off at a point when all the ether was in the flask. Ether was evaporated in an oven at 80 °C for 30 min and the process repeated until a constant weight was achieved. The percentage of ether extract was calculated as follows (Eq. 5.4):

$$\% \text{ crude lipid} = \frac{\text{wt.of the flask+E.E.}-\text{wt.of the flask}}{5} x100 \qquad \text{Eq. 5.4}$$

5.2.5.5 Crude fibre

The analysis of crude fibre was determined using Acid Detergent Fibre (ADF) method according to the batch procedure outlined by ANKOM Technology Corp., Fairport, NY, USA (Kanani et al., 2014). The concept behind the acid detergent fibre analysis is that plant cells can be divided into less digestible cell walls containing mainly cellulose and lignin, and mostly digestible cell contents containing starch and sugars. When samples are digested using cetyl trimethyl ammonium bromide in 1 N H2SO4 (an acid detergent solution), there is a notion that the mass fraction of residue left after the use of the detergent is crude fibre consisting of cellulose, lignin, cutine and insoluble minerals in the cell wall (Obregón-Cano et al., 2019). Crude fibre gives an

indication of bulkiness of a feed. The ADF method is a semi-automated method that involves the use of filter bag technology and fibre analyser. Lafun samples (0.5 g) were weighed into pre-weighed filter bags (ANKOM Corp., USA). The bags were heat-sealed to encapsulate the sample. An empty bag was included as a blank correction (C_1). The bags were placed evenly on bag suspender trays and trays inserted into a fibre analyser (ANKOM 200 fibre analyser, ANKOM Corp., USA) containing 2000 ml of Acid Detergent Solution (20g cetyl trimethylammonium bromide (CTAB) to 1 L 1.00 *N* H_2SO_4). The bags were incubated for 1 h with agitation for ADF extraction. After that, filter bags containing indigestible fibre were rinsed with 2000 ml hot distilled water (90 ^oC) for 5 min. To confirm that the rinsing was thorough, the water drained from the exhaust pipe was checked to be at the neutral pH 7. The bags were pressed to release excess water and soaked in acetone for 5 min. Then the filter bags were spread out on a wire screen to air-dry and placed under a ventilation hood for 30 min to evaporate the acetone. The bags were completely dried in an oven at 102 ± 2 ^oC for 4 h. Bags were cooled to ambient temperature in a collapsible desiccant pouch and weighed. Percentage crude fibre is calculated, as shown below (Eq. 5.5):

$$\% \text{ Crude fibre} = 100 \text{ x} \frac{W3-(W1 \text{ x } C1)}{W2} \qquad \text{Eq. 5.5}$$

Where: W_1 = Bag tare weight

W_2 = Sample weight

W_3 = Dried weight of bag with fibre after extraction process

C_1 = Blank bag correction (running average of final oven-dried weight divided by original blank bag weight)

5.2.5.6 Carbohydrate

The estimation of carbohydrate content in lafun was calculated by difference after analysis of all the other items in the proximate analysis. Calculations were done by using the formula below (Eq. 5.6):

$$\% \text{ Carbohydrate} = 100\% - (\% \text{ moisture} + \% \text{ crude lipid} + \% \text{ crude protein} + \% \text{ ash} + \% \text{ crude fibre}) \quad \text{Eq. 5.6}$$

5.2.6 Lafun rheological measurements

Lafun was prepared from laboratory samples (n=24) and the market sample (M1) by stirring 7 g of the samples in 30 ml of boiling water until a gruel is formed. The gruel was prepared immediately prior to analysis. The analysis was a dynamic oscillatory test performed using a controlled stress-strain rheometer (Anton Paar MCR 102, Germany) and a 50 mm serrated parallel plate. The lafun gruel samples were placed between the serrated plates and the gap set at 1 mm; the edges were trimmed with a spatula. The samples were rested between the plates for 1 min before testing. The measurements were performed at a constant temperature of 25 ^{o}C using a Peltier plate. For all measurements to be done within a specific viscoelastic region, amplitude sweeps were done between the range of 0.0001 and 100% strain. Oscillation stresses for lafun gruels were then selected from the result of the amplitude sweeps test. Frequency sweep tests were set up at frequencies between 0.1 and 10 Hz with a percentage strain of 0.1 in the viscoelastic region. Storage modulus (G') and loss modulus (G'') were then calculated using the manufacturer's software Each sample was analysed in triplicate with the results presented as means.

5.2.7 Volatile compounds analysis of lafun samples and raw cassava roots

5.2.7.1 Sample preparation

For gas chromatography–mass spectrometry (GC-MS) analysis, samples (n=54) were prepared by adding 3 ml of standard solution (200 ppm thymol in saturated sodium chloride (NaCl)) to 0.5 g of lafun in 20 ml glass SPME vials. Samples were then mixed thoroughly using a vortex mixer (MS1, Minishaker, UK) for 30 s. Prepared samples were then placed onto the sample tray. The samples analysed were: two batches of lafun processed in the laboratory, raw cassava roots of each variety and the three market samples. The analysis was run in triplicate. For gas chromatography–olfactometry (GC-O), one of the lafun samples collected from the markets and one processed in the laboratory (0.5 g) were used. Selection was based on the PCA analysis of the GC-MS results. Each sample was prepared by adding 3 ml of saturated NaCl and mixed thoroughly using a vortex. Detection and verbal description of the odour active components were done in duplicate by two assessors.

5.2.7.2 Gas chromatography–mass spectrometry analysis

The volatile compounds were extracted by Solid-phase microextraction (SPME) using a 50/30 µm DVB/ CAR/ PDMS Stableflex fibre (Supelco, Poole, UK). Prepared samples were equilibrated for 10 min at 35 ^{o}C with agitation (500 rpm). The SPME fibre was then exposed to the headspace for 30 min, followed by desorption in the GC injection port (splitless) at 250 ^{o}C. An Agilent 5975C Series GC/MSD coupled to an Agilent 7890A Gas Chromatograph was used, equipped with a Zeborn ZB5-MSi column (30 m x 250 µm x 1.0 µm). The oven was held at 40 ^{o}C for 5 min, subsequently the temperature was increased to 260 ^{o}C at a rate of 4 ^{o}C min^{-1}, and then held at 300 ^{o}C for 5 min. The carrier gas was helium at a flow rate of 0.9 ml min^{-1}. Mass spectra were

recorded in electron impact mode at an ionisation voltage of 70 eV and source temperature of 230 ^{o}C. A scan range of m/z 20-280 with a scan time of 0.69 s was employed, and the data was collected and stored by the ChemStation system. Volatiles were identified by comparison of spectra and linear retention indices (based on C5-C26 alkane series) from authentic compounds.

5.2.7.3 Gas chromatography–olfactometry analysis

The prepared sample was equilibrated with agitation at 35 ^{o}C for 10 min. The SPME fibre was then exposed to the headspace, just above the sample, for 30 min by penetrating the sample bottle liner with the stainless-steel needle housing the fibre. The extract (SPE) was analysed, using a DB-Wax column (30 m length x 0.25 mm diameter x 0.25 µm thickness). Analysis was performed by releasing the fibre into the injection port of an Agilent 7890B gas chromatograph fitted with an ODO II GC–O system (SGE Ltd, Ringwood, Australia). The outlet was split (ratio of 1:1) between a sniffing port with an FID/ODP and a flame ionisation detector. The injector temperature was maintained at 250 ^{o}C, that of the detector at 250 ^{o}C and that of the oven started at 40 ^{o}C but increased gradually with the run. Helium (carrier gas) had a flow rate of 1.2 mL min^{-1}. A 2 µL volume from the extract were injected each time. n-Alkanes C5–C25 were analysed to find linear retention index (LRI) values for the active odour components.

5.2.8 Metabolite fingerprinting of lafun and raw cassava roots by ^{1}H-NMR analysis

5.2.8.1 Extraction of sample

Each sample (15 mg) was weighed into a 2 ml Eppendorf tube and 1 mL of 80:20 D_2O:CD_3OD (deuterium oxide: deuterated methanol) containing 2.32 mM of TSP-d4 (d4-trimethylsilylpropionate) was added as an internal standard. After thorough

mixing, samples were extracted at 50 °C for 10 min in a water bath. The content of the tube was allowed to cool and then centrifuged at 13,000 rpm for 5 min. The supernatant was pipetted into a new tube and heated to 90 °C in a water bath for 2 min to stop any enzyme activity. It was further centrifuged after cooling at 4 °C for 45 min and 650 µl of the supernatant transferred into a 5 mm NMR tube for analysis (Shewry et al., 2017; Baker et al., 2006; Ward et al., 2003).

5.2.8.2 Data collection

^{1}H NMR spectra were acquired automatically at a temperature of 297 °K on a Bruker Avance II+ (Coventry, UK) spectrometer operating at 500 MHz (11.7 T) and equipped with a 5mm z-gradient direct observation (1H/13C/19F) - BBFO probe. Spectra were collected using a NOESYPR1D pulse sequence with a relaxation delay of 3 s. Each spectrum was acquired using 64 scans plus 8 dummy scans of 24,036 data points with a spectral width of 7,002.80 Hz. The spectra were automatically Fourier-transformed after the application of an exponential window function with a line broadening value of 0.3 Hz. Phasing and baseline correction were carried out manually using the TopSpin software program (Bruker), and spectra were references to TSP-d4 signal at δ0.0.

5.2.9 Microbial analysis of lafun samples

Samples of lafun produced in the laboratory were sent to SYNLAB Analytics and Services, UK Limited for microbial analysis. The procedures used for the enumeration of coagulase-positive S*taphylococci*, viable yeasts and moulds, presumptive *Enterobacteriaceae*, β-glucuronidase positive *Escherichia coli* and detection of *Salmonella* in lafun are the same as those for products intended for human consumption and animal feeding.

5.2.9.1 Enumeration of selected microorganisms

Plates of appropriate agar (Table 5.1) were inoculated with 0.5 ml of an initial suspension of the sample for *coagulase-positive Staphylococci*, yeasts and moulds or 1ml for *presumptive Enterobacteriaceae and β-glucuronidase positive Escherichia coli*. The inoculation were repeated for further with the decimal dilutions prepared from the initial sample. The suspension was spread quickly with a sterile spreader over the surface of the agar plates or pour plated in case of *Enterobacteriaceae and E. coli*. The plates of yeasts and moulds were incubated upright at 25 ^{o}C ± 1 ^{o}C for 120 h ± 3 h except for MY-40G plates that were incubated for 7-21 days. Other plates were inverted and incubated at 37 ^{o}C ± 1 ^{o}C for 24 h ± 2 h. The plates for *Staphylococci* were further re-incubated at 37 ^{o}C ± 1 ^{o}C for 24 h ± 2 h for. The incubation temperature for *E. coli* was 44 ^{o}C ± 1 ^{o}C. All typical colonies were counted using dilutions that were between 150 and 300 typical colonies. The results were reported as the number of CFU per gram of the test sample (Manual for Microbiological Methods Method 4.1, 2015).

5.2.9.5 Detection of Salmonella

Samples (25 g) were diluted in Buffered Peptone Water [BPW (1:10 dilution)] and incubated at 37 ^{o}C ± 1 ^{o}C for 24 h. Enrichment cultures (100 µl) were then added to Rappaport-Vassiliadis medium (10 ml) with soya (RVS) and incubated for 24 h ± 3 h at 41.5 ^{o}C ± 1 ^{o}C. Enrichment culture (1 ml) is added to 10 ml Muller-Kauffmann tetrathionate and novobiocin broth (MKTTn) and incubated for 24 h ± 3 h at 37 ^{o}C ± 1 ^{o}C. Using a sterile loop to spread the culture, the RVS culture medium enrichment was used to inoculate XLD plate and BG plate. The procedure was repeated with MKTTn broth enrichment to inoculate both XLD and BG media. All the inoculated plates were incubated at 37 ^{o}C ±1 ^{o}C for 24 h ±3 h. The typical colonies of *Salmonella* and atypical

Table 5.1: Agars used for microbial enumerations

Agar	Organisms enumerated
Baird Parker agar	coagulase-positive Staphylococci
Dichloran 18 % glycerol agar (DG18)	Osmophilic Yeasts and Moulds
Malt Extract – Yeast Extract 40 % w/w glucose agar (MY 40 G)	Xerotolerant Yeasts and Moulds
Orange Serum Agar (OSA)	Acidophilic Yeast and Moulds
Dichloran Rose Bengal Chloramphenicol Agar	General purpose
Violet Red Bile Glucose Agar (VRBGA)	presumptive Enterobacteriaceae
Tryptone-bile-glucuronide agar (TBX)	β-glucuronidase positive E. coli

colonies that may be Salmonella were counted. The result was reported as detected or not detected (Manual for Microbiological Methods, 2008).

5.2.10 Cyanide content determination of lafun samples and raw cassava roots

All lafun samples (2 batches (n=48) plus market samples (n=3)) and raw cassava roots were analysed for total cyanide contents. Analysis was carried out in duplicate and on dry weight basis. The method of determination of the cyanogenic potential of cassava and lafun involves the use of an automated enzymic method using an Auto-Analyzer (Rao & Hahn, 1984). Linamarin and lotaustralin are the cyanogenic glycosides presents in bound form in cassava; they were hydrolysed by the enzymes linamarase when the tissue cellular structure is damaged to form acetone cyanohydrins and hydrogen cyanide. An exogenous linamarase was added to the samples after extraction with cold orthophosphoric acid to form cyanide ions which reacts with chloramines T, Isonicotinate and dimethyl barbiturate to produce a dye colour. The intensity of the colour produced is proportional to the cyanide ions in the sample expressed as milligram HCN per 100 gram and calculated as shown below:

$$mg\ HCN\ per\ 100g = (mg/L)xDFxvolx100\ /\ wtx1000 \quad \text{Eq. 5.7}$$

Where:

mg/L = conc. obtained from the colorimeter using the Linamarin standard curve

DF = dilution factor

Vol = Ration of total volume to volume taken

Wt (g) = weight of sample

5.2.10.1 Extraction procedure

Orthophosphoric acid (0.1M H_3PO_4) was prepared and 250 ml measured into each sample cup. Each sample (30 g) was added to the orthophosphoric acid and the

mixture was homogenised in a blender. The resulting mixture was vortexed to ensure uniformity and centrifuged at 3500 rpm for 40 min at 4 ^{o}C. The supernatant was decanted into a new centrifuge tube and preserved in the refrigerator until analysed.

5.2.10.2 Cyanogenic potential procedure on auto-analyser

Forty vials of the sampling tray were filled halfway in respective order. The first to the seventh vials were filled with 40 ppm linamarin standard, pH 6 buffer, pH 6 buffer again, 10 ppm, 20 ppm 30 ppm and 40 ppm linamarin standards correspondingly. The remaining vials were filled with the samples extract. The system was run using the highest standard (recognised as Primer by the TC software connected to a Colorimeter) first. Serial dilutions of the standards were then run in duplicate and lastly the experimental samples.

5.2.11 Statistical analysis

Data of the proximate, ^{1}H NMR and cyanide analyses, and rheological properties evaluation were expressed as means ± standard deviation and the differences in means were compared by one-way analysis of variance (ANOVA) where $p < 0.05$ was taken to indicate a significant difference. Principal component analysis (PCA) was used to analyse the data from the GC-MS experiment. The PCA method showed similarities between samples projected on a plane and makes it possible to identify which variables determine these similarities. For all the statistical analysis done, XLSTAT 2018.1 software package was used.

5.3 Results and Discussion

5.3.1 Proximate analysis of lafun samples and raw cassava roots

Fermented cassava products are important sources of carbohydrates; this was reflected in the percentage of carbohydrate estimated in all the samples (Table 5.2). Cassava root has more carbohydrates than some food like potatoes (Montagnac et al., 2009). According to literature, about 72% of this carbohydrates is starch in the form of amylopectin and amylose. The presence of sucrose, fructose and dextrose was also reported (Okigbo, 1980). Cassava contain mainly glucose as sugar with small level of galactose, xylose, rhamnose, arabinose and mannose according to Hermiati et al. (2011). The proximate composition of lafun samples from the fermentation process revealed a higher level of crude fibre (highest being 7.43%) compared to the market sample (1.43%). Their crude fibre contents are also generally higher than the raw cassava from which they were produced. The higher crude fibre contents indicate that the method of lafun processing in this study could be used to produce lafun that are better consumed for good functioning of the digestive tract. Crude fibre can have positive effects on health through healthy intestine as it influences the digesta pH and transit rate through the digestive tract (Panaite et al., 2015). Food transit is crucial for digestion and absorption of nutrients, appetite regulation, gut hormone signalling and gut microbiota metabolism (Müller et al., 2018). Thus, a good level of fibre in diets improves glucose tolerance, prevents constipation and reduces cholesterol level (Bello et al., 2008).

There was no significant difference ($p>0.05$) in the moisture content among 76% of the samples (Table 5.2). However, there were significant differences ($p<0.05$) in ash, protein, lipid and fibre across the tested samples. The ash content of the raw

bitter cassava (1.83%) is significantly higher than that of all the lafun samples produced from this variety. The same trend was seen in the sweet variety (2.33%) and its products except the samples produced with *L. mesenteroides* (2.43%), and the co-cultures of the two *W. koreensis* strains (3.00%) and *W. koreensis-1* with *L. lactis* (3.17%). However, the values of ash in the lafun produced from fortified cassava were significantly higher than that of the raw material (3.00%). Eromosele et al. (2017) reported that the increase in ash level after fermentation could be as a result of an incomplete utilisation of the nutrients present in the raw material by the fermenting organisms.

Generally, the lipid contents of the raw bitter and sweet cassava (0.5% and 0.69% respectively) were slightly higher than that of their lafun products, however an inverse was observed for the fortified cassava(1.39%). The reduction in lipid content after fermentation is in conformity with the study of Onuoha et al. (2017) who reported that the reduction could be an advantage in decreasing the chance of rancidity in fermented food. Chikwendu et al. (2014) fermented pearl millet like the former author but recorded an increase in lipid content after fermentation. The opposing observation in both studies could suggest that the proximate composition of products of a fermented plant is influenced by the type of variety of that plant used. This suggests that the fatty acid profile of the varieties are likely to be different. Sample with an increased lipid level might require additional storage controls to extend its shelf-life.

A similar impact of cassava variety on lipid content was observed for protein content. There was a significant increase ($p<0.05$) in the protein content of lafun from fortified cassava relative to the raw material (1.78%). Previous studies had proven that fermentation can increase protein content in food (Oduah et al., 2015; Ahaotu et al., 2013; Irtwange & Achimba, 2009). However, the contrary results (reduction in protein

Table 5. 2: Percentage proximate composition of lafun produced with LAB starter cultures, 3 cassava varieties (raw) and Bodija market sample

		% Proximate					
LAB Strain	Cassava variety	Moisture Content	Ash	Crude Protein	Crude Lipid	Crude Fibre	Carbohydrate
Not fermented (Raw)	Bitter	2.33 ± 0.00^{bc}	1.83 ± 0.24^{fghij}	1.75 ± 0.05^{de}	0.5 ± 0.14^{ghi}	2.11 ± 0.04^{hi}	91.47^{cdefg}
	Fortified	2.33 ± 0.47^{bc}	3.00 ± 0.00^{de}	1.78 ± 0.06^{de}	1.39 ± 0.01^{bcd}	3.43 ± 0.03^{efg}	88.06^{hi}
	Sweet	2.17 ± 0.24^{bcd}	2.33 ± 0.00^{efg}	2.19 ± 0.00^{cd}	0.69 ± 0.14^{fgh}	1.43 ± 0.01^{k}	91.19^{defg}
W. koreensis-1	Bitter	1.37 ±0.06^{bcd}	1.27 ±0.09^{hij}	1.11 ±0.02^{defg}	0.40 ±0.00^{gh}	3.13 ±0.05^{g}	92.72^{abc}
	Fortified	2.33 ±0.47^{bc}	3.83 ±0.24^{abc}	2.99 ±0.15^{ab}	2.20 ±0.00^{a}	3.46 ±0.10^{efg}	85.18^{hi}
	Sweet	0.90 ±0.14^{cd}	1.54 ±0.18^{ghi}	1.37 ±0.02^{cdef}	0.20 ±0.00^{h}	2.45 ±0.26^{h}	93.53^{a}
L. lactis	Bitter	1.17 ±0.24^{bcd}	1.47 ±0.19^{hij}	0.86 ±0.02^{fg}	0.50 ±0.14^{fgh}	3.62 ±0.25^{defg}	92.39^{abcd}
	Fortified	1.87 ±0.66^{bcd}	3.80 ±0.28^{abc}	2.89 ±0.05^{ab}	1.70 ±0.14^{b}	6.63 ±0.11^{b}	83.11^{ij}
	Sweet	1.03 ±0.90^{bcd}	1.53 ±0.19^{ghi}	1.39 ±0.07^{cdef}	0.40 ±0.00^{gh}	2.12 ±0.07^{hi}	93.53^{a}
W. koreensis-2	Bitter	0.94 ±0.38^{bcd}	1.27 ±0.09^{hij}	1.50 ±0.05^{cde}	0.40 ±0.00^{gh}	1.89 ±0.00^{ij}	94.01^{a}
	Fortified	2.33 ±0.47^{bc}	3.63 ±0.05^{bcd}	2.91 ±0.06^{ab}	1.50 ±0.14^{b}	4.75 ±0.08^{c}	84.87^{hi}
	Sweet	1.50 ±0.71^{bcd}	1.37 ±0.05^{hij}	1.34 ±0.17^{cdef}	1.09 ±0.13^{cd}	3.69 ±0.22^{def}	91.02^{bcde}
L. mesenteroides	Bitter	1.47 ±0.19^{bcd}	1.47 ±0.19^{hij}	0.93 ±0.04^{fg}	0.59 ±0.00^{fg}	3.21 ±0.21^{fg}	92.33^{abcd}
	Fortified	1.80 ±0.28^{bcd}	4.37 ±0.05^{a}	3.08 ±0.23^{a}	1.40 ±0.00^{bc}	7.43 ±0.18^{a}	81.93^{j}
	Sweet	0.73 ±0.09^{d}	2.43 ±0.33^{ef}	1.50 ±0.05^{cde}	0.60 ±0.00^{fg}	3.44 ±0.05^{efg}	91.3^{bcde}
L. lactis and	Bitter	1.23 ±0.61^{bcd}	1.74 ±0.10^{fgh}	1.05 ±0.01^{efg}	0.49 ±0.14^{fgh}	3.43 ±0.01^{efg}	92.06^{abcd}
L. mesenteroides	Fortified	1.71 ±0.42^{bcd}	3.92 ±0.12^{ab}	2.91 ±0.05^{ab}	1.70 ±0.14^{b}	6.66 (±0.13)^{b}	83.1^{ij}
	Sweet	1.10 ±0.14^{bcd}	2.20 ±0.28^{fg}	1.58 ±0.10^{cde}	0.49 ±0.14^{fgh}	4.14 ±0.09^{d}	90.49^{de}
W. koreensis (both strains)	Bitter	2.17 ±0.24^{bcd}	0.83 ±0.24^{j}	0.79 ±0.06^{g}	0.40 ±0.00^{gh}	2.30 ±0.07^{hi}	93.51^{a}
	Fortified	2.20 ±0.28^{bcd}	3.63 ±0.05^{bcd}	2.62 ±0.09^{ab}	1.00 ±0.00^{de}	3.85 ±0.25^{de}	86.71^{gh}
	Sweet	1.97 ±0.52^{bcd}	3.00 ±0.00^{de}	1.84 ±0.15^{c}	0.40 ±0.00^{gh}	2.19 ±0.01^{hi}	90.6^{cde}
W. koreensis-1	Bitter	1.67 ±0.00^{bcd}	1.00 ±0.00^{ij}	0.60 ±0.25^{g}	0.50 ±0.14^{fgh}	2.06 ±0.05^{hi}	94.18^{a}
and *L. lactis*	Fortified	1.81 ±0.27^{bcd}	3.92 ±0.11^{ab}	2.53 ±0.33^{b}	0.80 ±0.00^{def}	3.31 ±0.07^{efg}	87.63^{fg}
	Sweet	1.27 ±0.09^{bcd}	3.17 ±0.23^{cd}	1.81 ±0.01^{c}	0.70 ±0.14^{efg}	3.67 ±0.13^{defg}	89.39^{ef}
W. koreensis-2 and	Bitter	2.50 ±0.24^{bcd}	0.83 ±0.24^{j}	0.76 ±0.13^{g}	0.50 ±0.14^{fgh}	2.25 ±0.06^{hi}	93.16^{ab}
L. mesenteroides	Fortified	2.37 ±0.05^{bc}	3.47 ±0.19^{bcd}	2.76 ±0.10^{ab}	0.79 ±0.00^{def}	3.70 ±0.04^{def}	86.91^{gh}
	Sweet	1.17 ±0.24^{bcd}	1.73 ±0.09^{gh}	1.64 ±0.12^{cd}	0.60 ±0.00^{fg}	3.63 ±0.19^{defg}	91.23^{bcde}
Spontaneous fermentation	Market sample	10.50 ±0.24^{a}	1.33 ±0.00^{hij}	0.87 ±0.22^{fg}	0.49 ±0.14^{fgh}	1.43 ±0.04^{j}	85.37^{h}

*^{abcdefghij} means with different letters in the columns indicate significant statistical differences (Tukey's Test, $p < 0.05$); Values are Mean ± SD. Bitter (IBA30527), Fortified (IBA011371) and Sweet (TMEB117)

level after fermentation) recorded for both bitter and sweet cassava had been noticed by Oyewole & Odunfa, (1989). The reduction in protein content after fermentation could be an indication that the varieties affected have more sulphur amino acid content that might have been metabolised by the LAB starters (Çabuk et al., 2018).

It was interesting to note that the highest values in the ash (4.37%), protein (3.08%), and fibre (7.43%) contents were in fortified samples fermented with *L. mesenteroides*. There is not much data regarding the role of *L. mesenteroides* as a starter in the improvement of proximate values of fermented cassava products or food in general. Tefera et al. (2014) reported its ability to reduce cyanide in fermented cassava flour. There are also some reports on its excellent roles in probiotic and antimicrobial activities and its impact in texture and flavour quality of some other fermented foods (Vyas et al., 2017; Jung et al., 2012; Johanningsmeier et al., 2007).

5.3.2 Lafun rheological properties

Frequency sweep studies (a dynamic test) were conducted to characterise the rheological properties of the experimentally produced lafun samples (Table 5.3). The storage modulus *G'* and the loss modulus *G''* were obtained from a rheological test in the linear viscoelastic range. *G'* is an amount of the deformation energy stored in the sample during the shear process (elastic behaviour of the sample) and *G''* indicates the deformation energy sapped during shear but lost to the sample afterwards (viscous behaviour of the sample) (Tabilo-Munizaga & Barbosa-Cánovas, 2005). The plots of frequency against both *G'* and *G''* produced a linear relationship in all the samples (*G'*, $R^2 \geq 0.80$ and *G''*, $R^2 \geq 0.90$) (results not shown). Regardless of the treatment used, *G'* was always higher than *G''*, signifying lafun gruels exhibited more solid-like behaviour (Tabilo-Munizaga & Barbosa-Cánovas, 2005). Also, the values of the two moduli

increased with increasing frequency from 0.1 Hz to 10 Hz. To further understand the inherent attribute of the bonds within each sample, tan δ (G''/G') was plotted against frequency. Figure 5.1 shows the impact of cassava variety on the tan δ of lafun sample prepared using *L. mesenteroides* as the starter culture and a similar trend was observed for all other samples. Tan δ values for all the samples were hardly affected by frequency. However, tan δ values for both bitter and sweet cassava were higher, which indicates that they are more viscous in nature than the fortified variety and have a greater number of weak-bond interactions within the gruel (Harbourne et al., 2011).

The more elastic the samples are, the higher the consumer acceptability, as lafun is a food product expected to behave like an elastic gruel based on the characteristics mentioned by He and Hoseney (1991). Thus, the behaviour of the laboratory lafun samples were compared with that of the market sample. The *G'* values obtained for the market sample were higher only in the lafun samples produced with bitter cassava and *W. koreensis-2* and combination of both *W. koreensis* strains starter, as well as sample produced with sweet cassava and *L. mesenteroides*. It can, therefore, be inferred that 88% of the laboratory samples had higher deformability and thus exhibit elastic-like behaviour. These results suggest that majority of the laboratory samples have better quality regarding rheological properties than the market sample studied.

Additionally, rheological measurement revealed different trends in the behaviour of lafun gruel made with different cassava varieties. Thus, rheology of lafun is dominated by the type of cassava used. This behaviour is in agreement with the findings of Hüttner et al., (2010) who reported that different flour characteristics influence rheological properties. The products of fortified cassava are more elastic than the

Table 5.3: Rheological properties of lafun samples produced from control fermentation of LAB and market sample

LAB strain(s)	Cassava variety	Storage modulus	Loss modulus	δ value
W. koreensis-1	Bitter	1378.73^{fgh}±21.19	287.89^{cdef}±4.29	0.21
	Fortified	2864.80^{l}±105.32	480.90^{ij}±11.34	0.17
	Sweet	1467.57^{gh}±170.91	356.27^{fgh}±41.47	0.24
L. lactis	Bitter	1215.97^{def}±84.35	284.56^{cdef}±12.29	0.23
	Fortified	2775.27^{kl}±234.98	489.91^{ij}±39.32	0.18
	Sweet	1283.63^{efg}±77.14	300.27^{def}±17.78	0.23
W. koreensis-2	Bitter	869.46^{a}±61.49	205.25^{a}±10.73	0.24
	Fortified	4589.87^{n}±466.45	804.57^{l}±66.35	0.18
	Sweet	999.64^{abcd}±62.95	241.09^{abcd}±9.92	0.24
L. mesenteroides	Bitter	1260.83^{efg}±58.77	274.69^{abcde}±12.09	0.22
	Fortified	2324.40^{i}±334.78	382.09^{ghi}±58.3	0.16
	Sweet	926.81^{abc}±86.34	228.18^{abcd}±15.34	0.25
L. lactis and *L. mesenteroides*	Bitter	1197.10^{cdef}±19.77	268.96^{abcde}±1.28	0.22
	Fortified	2340.33^{ij}±82.14	390.51^{ghi}±9.79	0.17
	Sweet	1329.67^{efgh}±29.93	288.32^{cdef}±3.79	0.22
W. koreensis (both strains)	Bitter	908.65^{ab}±41.81	226.29^{abc}±9.49	0.25
	Fortified	2561.83^{jk}±76.51	439.02^{ij}±13.45	0.17
	Sweet	1275.67^{efg}±61.29	282.60^{cde}±9.74	0.22
W. koreensis-1 and *L. lactis*	Bitter	1093.03^{abcde}±22.05	239.95^{abcd}±3.24	0.22
	Fortified	3480.77^{m}±159.46	621.58^{k}±16.47	0.18
	Sweet	1543.57^{h}±61.08	322.29^{efg}±10.36	0.21
W. koreensis-2 and *L. mesenteroides*	Bitter	1303.20^{efg}±28.9	281.44^{bcde}±8.3	0.22
	Fortified	2292.30^{i}±57.57	397.48^{hi}±7.69	0.17
	Sweet	1470.27^{gh}±29.28	335.22^{efgh}±2.87	0.23
Spontaneous fermentation	Market sample	1130.13^{bcde}±8.2	208.07^{ab}±0.99	0.18

*^{abcdefghijklm} means with different letters in the columns indicate significant statistical differences (Tukey's Test, $p < 0.05$); Values are Mean ± SD of three replicates. Bitter (IBA30527), Fortified (IBA011371) and Sweet (TMEB117)

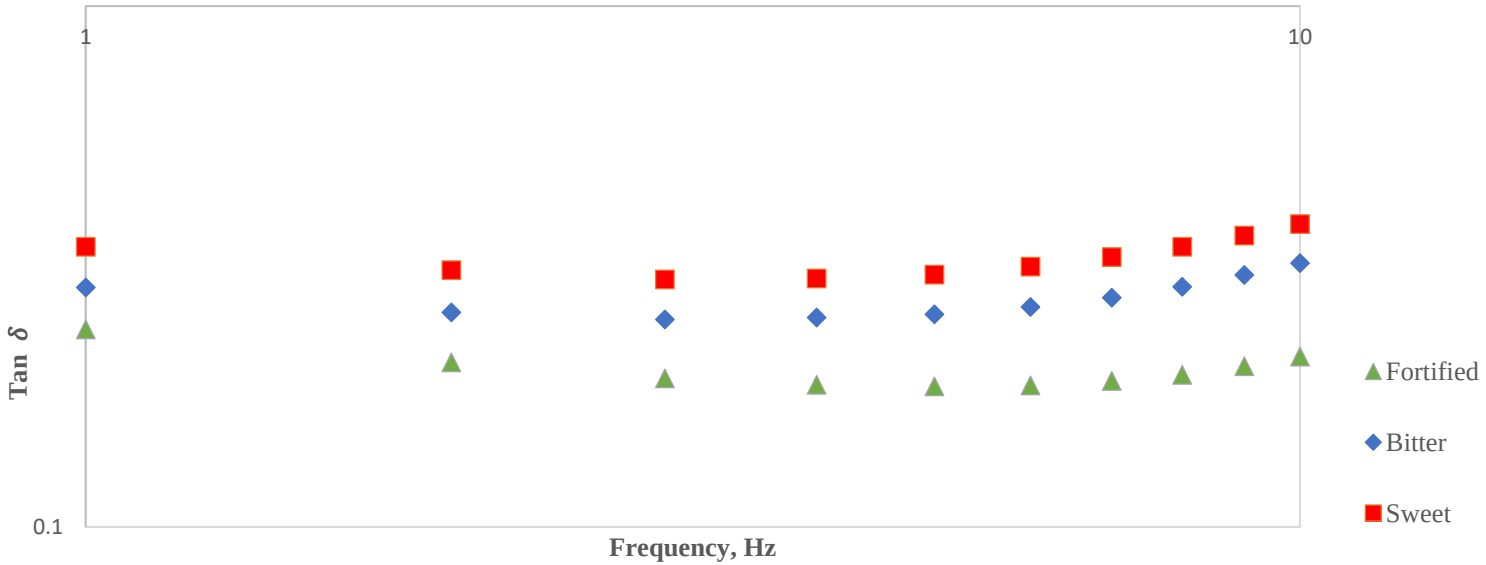


Figure 5. 1: Tan δ as a function of frequency of lafun gruel produced using *L. mesenteroides*, typical of fortified (green), bitter (blue) and sweet (red) cassava varieties

products of other two varieties. This solid-like behaviour of fortified cassava products could be an indication of a better water holding capacity.

5.3.3 Volatile compounds analysis of lafun samples and raw cassava roots

5.3.3.1 Gas chromatography–mass spectrometry analysis

In this study, the application of SPME-gas chromatography-mass spectrometry (GC–MS) for the characterisation of key volatile flavour compounds in lafun was described. Identification and quantitation of 35 volatile organic compounds (VOCs) from lafun samples (n=51) and raw cassava roots (n=3) on a dry weight basis was possible using Solid Phase Microextraction (SPME). The VOCs include a total of 21 aldehydes, 2 ketones, 5 organic acids, 1 alcohol, 1 furan, 3 enones, 2 cresol (only found in Sango market sample) and 1 unknown volatile compound (Table 5.4). The peaks obtained in full scan mode had enough resolution and were used for quantification analysis.

PCA analysis was performed to determine the impact of the starter cultures and cassava varieties on the identified volatile compounds. PC1 and PC2 accounts for 69.86% of the total variance in the VOCs (Figure 5.2). The principal components separates samples into three groups: one composed by cassava varieties bitter and sweet projected in the right side, the second by the fortified cassava variety on the left side and the market samples at the left-bottom side. The variables which determine this separation are aldehydes, ketones and volatile acids. The lafun samples produced with both bitter and sweet cassava varieties have more abundant aldehydes compounds while those from fortified cassava are richer in ketones and the market samples are mainly driven by volatile acids.

Table 5. 4: Volatile compounds identified and quantified in lafun produced with 3 cassava varieties fermented with selected LAB cultures

S/N	Compound	Key Ion	Means of Identification		
			LRI[A]	ID[B]	CID[C]
	Aldehydes				
1	Acetone	43.1	849	A	180
2	2-Butanone	43	936	A	6569
3	Pentanal	44	1012	A	8063
4	1-Penten-3-one	55	1048	A	15394
5	Hexanal	56	1102	A	6184
6	3-Penten-2-one	69	1144	A	637920
7	E-2-pentenal	55.05	1147	A	5364752
8	Heptanal	70.1	1196	A	8130
9	2-Hexenal	69.1	1232	A	5281168
10	Octanal	43.05	1298	A	454
11	2,3-Octanedione	99	1330	A	11449
12	E-2-heptenal	83.05	1335	A	5283316
13	Nonanal	57	1401	A	31289
14	2-Octenal, (E)-	70	1440	A	5283324
15	2,4-Heptadienal, (E,E)-	81	1474	A	5283321
16	2,4-Heptadienal, (E,E)-	81	1506	A	5283321
17	2-Nonenal, (E)-	55	1549	A	5283335
18	2-Decenal, (E)-	70	1663	A	5283345
19	2,4-Nonadienal, (E,E)-	81	1723	A	5283339
20	2-Undecenal	70	1773	A	5283356
21	2,4-Decadienal	81	1834	A	5283349
	Ketones				
1	5-Hepten-2-one, 6-methyl-	108	1344	A	9862
2	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z	107	1863	A	19633
	Organic acids				
1	Acetic acid	43	1458	A	176
2	Butanoic acid	60	1652	A	264
3	Pentanoic acid	60	1761	A	7991
4	Hexanoic acid	60	2005	A	8892
5	Heptanoic acid	107	2044	A	8094
	Furan				
1	Furan, 2-pentyl-	81	1239	A	19602
	Enones				
1	3-Octen-2-one	55	1416	A	5363229
2	3,5-Octadien-2-one	95	1516	B	5352876
3	3,5-Octadien-2-one, (E,E)-	95	1567	B	5352876
	Alcohol				
1	1-Octen-3-ol	57	1455	A	18827
	Cresol				
1	p-cresol	107	2079	A	2879
2	m-cresol	107	2079	A	342
	Unknown				
1	55/70	70.1	1296		

[A]Linear retention index on ZB –WAX column, calculated from a linear equation between each pair of straight chain alkanes C5-C26. [B]A, mass spectrum and LRI agree with those of authentic compound; B, mass spectrum agrees with reference spectrum in the NIST mass spectra database and LRI agree with those in the literature. [C] PubChem CID.

The PCA score plot of volatile compounds in Figure 5.2 shows that the samples from fortified cassava were considerably different from the others along PC1 (explaining 57.29% of the variance). The PCA plot of volatile compounds in Figure 5.3 shows that 5-hepten-2-one, 6-methyl-, 5,9-undecadien-2-one, 6,10-dimethyl-, (Z), acetic acid, acetone and 2-butanone were closely correlated with lafun from fortified cassava. The correlation of the ketones (5-hepten-2-one, 6-methyl-, 5,9-undecadien-2-one, 6,10-dimethyl-, (Z)) with the fortified cassava could be as a result of the carotenoids present in this variety. This is reasonable as some ketones are known to be degradation products or oxidative by-products derived from carotenoids. Carotenoid pigmentation has been reported to affect the volatile composition of some plants (Lewinsohn et al., 2005). On the other hand, butanoic acid correlated with all market lafun samples, while majority of the identified aldehydes were closely correlated to lafun from both sweet and bitter cassava. Organic acids such as heptanoic, hexanoic and pentanoic acids were related to the positive axis of the PC1 and were correlated to most of the bitter cassava products.

The flavour profile of lafun fermented with LAB cultures varied greatly among strains and with cassava varieties. Aldehydes are prevalent components of food products, and this is evident by the highest occurrence of this group of compounds in this study. The aldehyde compounds identified in lafun could originate either from the enzymatic or chemical oxidation of lipids (Grosch, 1967). As pointed out by Kazeniac and Hall (1968), enzymatic oxidation of lipids yields a wide range of carbonyl compounds among which are acetone, pentanal, hexanal, E-2-pentenal, heptanal, 2-hexenal, octanal, E-2-heptenal, 2-octenal, nonanal, 2,4-heptadienal, (E,E)-, 2-nonenal, (E)-, 2-decenal, (E)-, 2,4-nonadienal, (E,E)- and 2-undecenal also found in this study (Table 5.5 and 5.6).

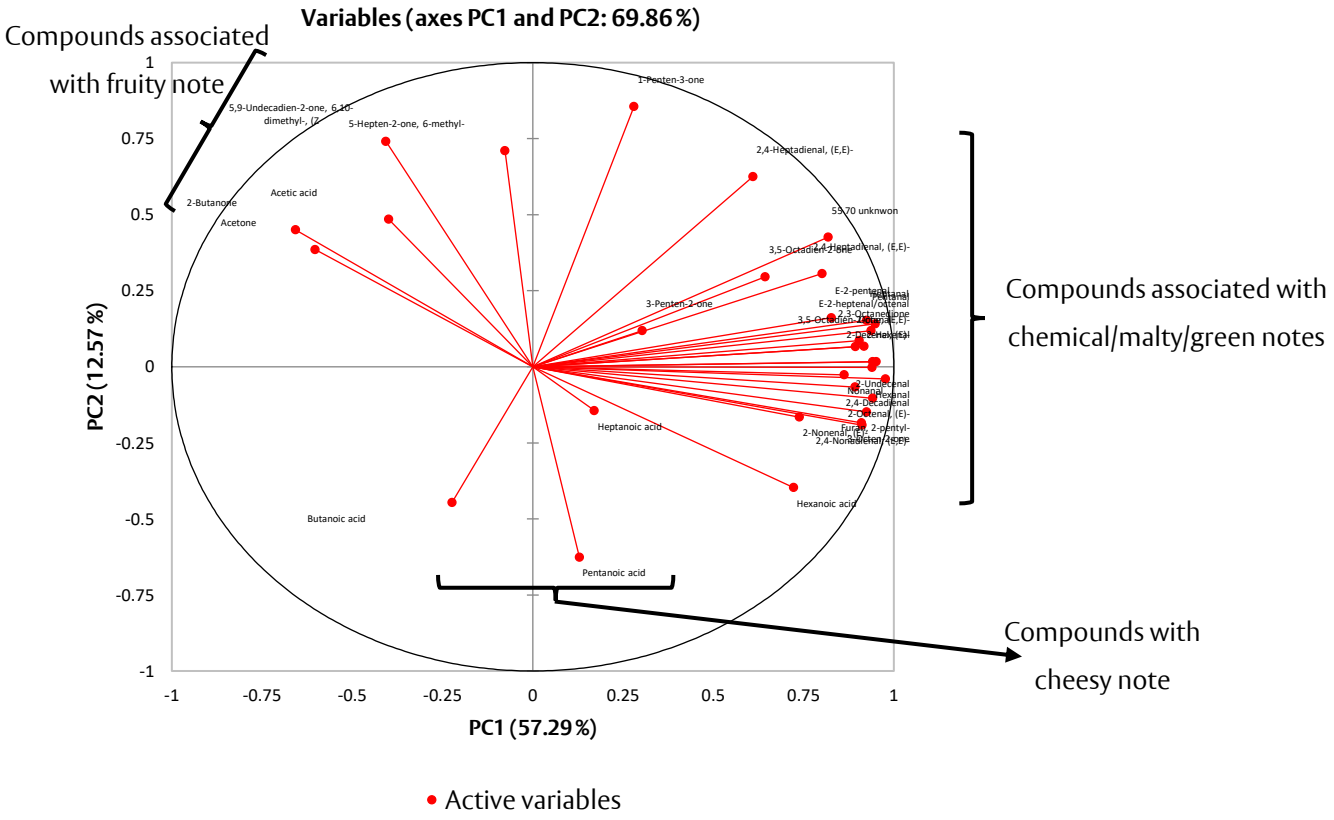


Figure 5.2: Projection of volatile compounds (variables: aldehydes, ketones, organic acids, furan, enones and unknown-55,70 compounds) into the plan composed by the two principle axes PC1 and PC2. The two plans contain 69.86% of the total variance

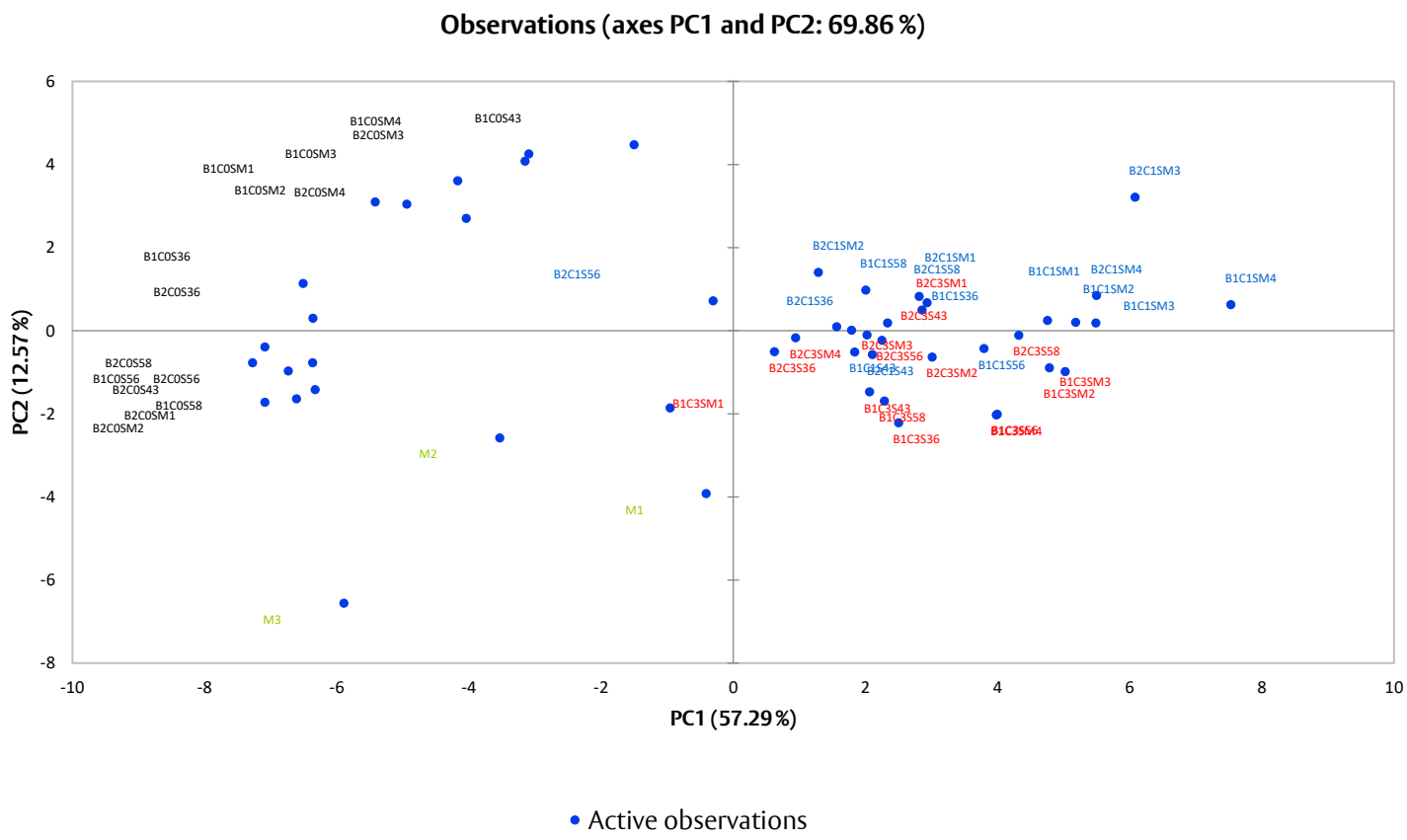


Figure 5. 3: Projection of batch 1(B1) and 2 (B2) samples of lafun produced with bitter cassava (red), sweet cassava (blue), fortified cassava (black) with market samples (green) into the two principal components

Key: Lafun and cassava samples

C3S36: Bitter + *W. koreensis-1*;
C0S43: Fortified + *L. lactis*;
C1S56: Sweet + *W. koreensis-2;*
C3SM1: Bitter + *L. lactis* and *L. mesenteroides*;
C0SM2: Fortified + *W. koreensis* (both);
C1SM3: Sweet + *W. koreensis-1* and *L. lactis*;
C1SM4: Sweet + *W. koreensis-2* and *L. mesenteroides*
M1: Bodija market Sample;
C0S36: Fortified + *W. koreensis-1*;
C1S43: Sweet + *L. lactis*;
C3S58: Bitter + *L. mesenteroides*;
C0SM1: Fortif. + *L. lactis* and *L. mesenteroides*;
C1SM2: Sweet + *W. koreensis* (both);
C3SM4: Bitter + *W. koreensis-2* and *L. mesenteroides*
C305: Bitter cassava;
M2: Sango market Sample;
C1S36: Sweet + *W. koreensis-1*;
C3S56: Bitter + *W. koreensis-2*;
C0S58: Fortified + *L. mesenteroides*;
C1SM1: Sweet + *L. lactis* and *L. mesenteroides*;
C3SM3: Bitter + *W. koreensis-1* and *L. lactis*;
C011: Fortified cassava
M3: Mokola market sample
C3S43: *Bitter + L. lactis*;
C0S56: Fortified + *W. koreensis-2*;
C1S58: Sweet + *L. mesenteroides*;
C3SM2: Bitter + *W. koreensis* (both);
C0SM3: Fortified. + *W. koreensis-1 and L. lactis;*
C0SM4: Fortified + *W. koreensis-2* and *L. mesenteroides*
C117: Sweet cassava

Among the volatile compounds, hexanal has the highest level in all the samples, especially in the sample of bitter cassava produced with the combination of *W. koreensis-1* and *L. lactis*. Hexanal is one of the many well-documented aromatic compounds in the literature. It contributes to flavour and aroma and it is used in the measure of the oxidative status of foods; and antimicrobial properties (Elisia, And, & Kitts, 2011; Kerry & Ledward, 2011). Like pentanal and 3,5-octadien-2-one, hexanal was considered products of autoxidation. Hexanal was found to be formed during the termination phase of oxidation of specific fatty acids (linoleic and arachidonic acids). Therefore, it is used as the measure of oxidative stability (García et al., 2007).

Oxidation of lipids is the cause of rancidity in food and thereby its reduction in quality (Fuentes et al., 2014; Jeremiah, 2001). The lipid content of lafun from fortified cassava is generally higher than those of the other two varieties as noted in the proximate analysis, but their hexanal, pentanal and 3,5-octadien-2-one are much lower. The quantitative difference in the level of hexanal formation between lafun samples from different cassava varieties could suggest that there are varying amounts of specific fatty acid that is oxidised to this compound in different varieties. Among organic acids identified, acetic acid had the highest concentration in lafun. This could be related to the high content of acetic acid produced during fermentation (Choi et al., 2019).

Cresols were found only in Sango market sample (Table 5.7). Cresols have a characteristic medicinal or coal-tar smell, released to the environment through fuel combustion (wood and trash burning, and vehicle exhaust). A small amount of cresols has been detected in foods like tomatoes, asparagus, butter, smoked foods, and some drinks such as coffee, brandy, black tea and wine (ATSDR, 2015). The presence of cresols in the market sample could be due to lack of hygiene during processing, especially at the drying stage. Some indigenous lafun producers spread it to dry on a tarred road

(Chapter 2, Figure 2.13) that could have cresol as part of its content and where automobile exhaust is heavy. Cresols are poisonous, eating contaminated food (with p-cresol or a combination of m- and p-cresol) for 28 days minimum had caused lesions inside the nose, lungs, and thyroid gland of rats and mice (ATSDR, 2015; NTP Technical Report, 2008).

The quantitation of the raw cassava roots showed that bitter cassava is the only variety with 1-octen-3-ol although in small amount compared with the concentration found in Sango and Mokola markets samples. This compound (1-octen-3-ol) is the fresh or dried mushroom flavour. It is prominently produced by fungi such as *Aspergillus sp.*, *Fusarium sp.* and *Penicillium sp.* (Combet et al., 2006; Kaminski, 1974). It is a product of enzymatic oxidation of linoleic acid, just like hexanal. The two market samples that contained 1-octen-3-ol were products of spontaneous fermentation. The occurrence of fungi in wild fermentation of cassava has been widely reported (Ihenetu et al., 2017; Ahaotu et al., 2013; Oboh & Elusiyan, 2007; Akinrele, 1964).

5.3.3.2 Gas chromatography–olfactometry analysis

A total of 49 odorants were identified by both assessors from the odorants yielded in the chromatogram of the GC-O analysis of the samples (Table 5.8). Aldehydes appeared to be the highest contributors to the aroma of lafun contributing mainly green, fried and coriander odours. The notes contributed by aldehydes is expected of plant products. However, 3-hexanal and 6-methyl-5-hepten-2-one contributed to the desirable sweet and orange notes in both samples. It was clearly observed that E,E-2,4-decadienal (fried note) had the highest intensity in both samples. Quantitative differences were observed between the market sample and the laboratory sample. The notable one was the contribution of an off-note from pyrazine found only in the market sample. Also, butanoic acid was not detected in the GC-O analysis of the LAB lafun.

Twenty-one of the odorant compounds were not identified in the GC-MS analysis. Out of these (n=21), fifteen were recognised by their characteristic aroma and confirmed by comparison of their LRI with that of the authentic samples. There is no data in the literature regarding odour description in lafun and the identified odorants were detected for the first time in this study.

To sum up, aldehydes occurred mostly among the odour active compounds found in lafun as mentioned earlier. Aldehydes (very potent flavour compounds) are important contributors to the characteristic 'fresh green' odour of vegetables. They are widely used as food additives because of this fresh green odour (Schwab et al., 2008). However, when the aldehydes become oxidised, they cause rancidity in foods and reduce their keeping quality. The results indicate the need for an effective storage method that will lower the rate of oxidation of aldehydes in lafun. The oxidation of aldehydes leads to the corresponding organic acids (Smit et al., 2005). The high concentration of organic acids in the market samples could result from the oxidation of aldehydes. Butanoic acid, an unpleasant odour of rancid butter, is very high in both Sango (6097 mg kg^{-1}) and Mokola (5653 mg kg^{-1}) markets samples. The GC-MS results of butanoic acid showed that the method of production of the laboratory samples is a good tool for reducing unwanted volatile organic compounds like butanoic acid to a very low level. In addition, the cresols and pyrazine absence in the laboratory samples could be related to the fact that they were not dried on the road like the market samples.

5.3.4 Metabolite fingerprinting of lafun and raw cassava roots by ^{1}H-NMR analysis

Nuclear magnetic resonance analysis was successfully applied to measure the presence and concentrations of metabolites present in lafun. The identification of

metabolites was made by comparing the NMR chemical shifts and coupling constants with the Chenomx NMR Database (Chenomx NMR suite 8.4) which contains hundreds of NMR spectra of authentic chemical standards, as well as by reference to metabolites published in the work of Kim et al., (2010). Overall, the NMR spectra obtained showed peaks corresponding to abundant carbohydrates, from δ3.0 to δ6.0 (Figure 5.4). Sucrose is the carbohydrate identified as observed by its doublet signal at δ5.40 and δ4.17. There were well-defined signals in the aliphatic region of the spectra arising from organic acids and aliphatic amino acids. The organic acids identified were lactic acid at δ1.33 and succinic acid at δ2.53/2.56, while alanine at δ1.47/1.48 was the only aliphatic amino acid. The signal detected at δ1.18 (triplet) was identified as ethanol.

Baseline resolved peaks were integrated to show the levels in arbitrary unit (a.u.) of the identified metabolites. Quantitative differences were evident in the samples relating to variances in cassava roots and LAB culture(s) used for fermentation (Figure 5.5). Succinate was not detected in the raw cassava roots or Bodija or Sango markets samples. Generally, fortified-cassava lafun samples had the highest concentration of ethanol, lactate and succinate. The maximum values detected for sucrose were seen in raw cassava of fortified variety. Thus, it could be inferred that the fortified cassava had the highest carbohydrate content for the metabolism of the LAB strains, which could have led to the higher metabolites yield in its products. The highest values of acetone found in lafun of fortified variety could be as a result of sucrose fermentation. The lactate value of the 3 raw materials was 0.05 a.u. After fermentation, there was an increase to 9.83 ± 0.34 a.u. in fortified cassava fermented by *L. mesenteroides*.

A significant ($p<0.05$) upward trend was found in the other two varieties up to 4.39 ± 0.14 a.u. in bitter cassava with *L. mesenteroides* and 3.52 ± 0.04 a.u. in sweet

Table 5. 5: Relative mg kg^{-1} of volatile compounds quantified in lafun produced with three cassava varieties and fermented with LAB monocultures

	W. koreensis-1			*L. lactis*			*W. koreensis-2*			*L. mesenteroides*		
Volatile Compound	**B**	**F**	**S**	**B**	**F**	**S**	**B**	**F**	**S**	**B**	**F**	**S**
Aldehydes	**Concentration (mean ± SD)* (mg kg^{-1})**											
Acetone	$928^{abc} \pm 70$	$1910^{abc} \pm 488$	$142^{c} \pm 28$	$1034^{abc} \pm 485$	$1709^{abc} \pm 352$	$294^{bc} \pm 127$	$653^{abc} \pm 636$	$1469^{abc} \pm 825$	$160^{bc} \pm 54$	$320^{bc} \pm 128$	$2190^{ab} \pm 1280$	$61^{c} \pm 18$
2-Butanone	$619^{bcde} \pm 50$	$2259^{ab} \pm 694$	$147^{de} \pm 33$	$731^{bcde} \pm 169$	$1957^{abc} \pm 84$	$280^{cde} \pm 18$	$472^{cde} \pm 305$	$1724^{abcde} \pm 1053$	$221^{cde} \pm 4$	$243^{cde} \pm 50$	$899^{bcde} \pm 596$	$19^{e} \pm 27$
Pentanal	$416^{abcde} \pm 93$	$95^{def} \pm 48$	$441^{abc} \pm 30$	$420^{abcd} \pm 7$	$269^{abcdef} \pm 297$	$427^{abcd} \pm 88$	$503^{a} \pm 110$	$72^{f} \pm 8$	$446^{abc} \pm 60$	$436^{abc} \pm 32$	$82^{ef} \pm 23$	$443^{abc} \pm 17$
1-Penten-3-one	$63^{a} \pm 22$	$89^{a} \pm 12$	$110^{a} \pm 27$	$76^{a} \pm 8$	$139^{a} \pm 139$	$82^{a} \pm 8$	$79^{a} \pm 6$	$14^{a} \pm 20$	$65^{a} \pm 92$	$80^{a} \pm 21$	$22^{a} \pm 1$	$151^{a} \pm 10$
Hexanal	$12520^{a} \pm 2890$	$1078^{b} \pm 16$	$11117^{a} \pm 11$	$11816^{a} \pm 1207$	$3581^{b} \pm 4286$	$11641^{a} \pm 1006$	$13352^{a} \pm 1314$	$315^{b} \pm 153$	$11375^{a} \pm 2453$	$12982^{a} \pm 323$	$245^{b} \pm 96$	$12154^{a} \pm 2864$
3-Penten-2-one	$48^{a} \pm 68$	$116^{a} \pm 106$	$262^{a} \pm 318$	$47^{a} \pm 54$	$32^{a} \pm 28$	$41^{a} \pm 12$	$108^{a} \pm 4$	$102^{a} \pm 122$	$171^{a} \pm 82$	ND	$19^{a} \pm 8$	$124^{a} \pm 133$
E-2-pentenal	$139^{bcd} \pm 3$	$49^{bcd} \pm 24$	$240^{abc} \pm 133$	$188^{abcd} \pm 40$	$82^{bcd} \pm 85$	$156^{abcd} \pm 23$	$180^{abcd} \pm 6$	$13^{bcd} \pm 18$	$176^{abcd} \pm 40$	$181^{abcd} \pm 40$	$25^{bcd} \pm 1$	$216^{abcd} \pm 21$
Heptanal	$398^{abc} \pm 0$	$10^{c} \pm 13$	$510^{abc} \pm 102$	$349^{abc} \pm 62$	$248^{abc} \pm 350$	$602^{ab} \pm 120$	$475^{abc} \pm 100$	$17^{c} \pm 24$	$493^{abc} \pm 178$	$490^{abc} \pm 23$	ND	$487^{abc} \pm 111$
2-Hexenal	$130^{abcd} \pm 38$	$16^{cd} \pm 7$	$109^{abcd} \pm 47$	$129^{abcd} \pm 17$	$55^{bcd} \pm 66$	$100^{abc} \pm 25$	$155^{abcd} \pm 35$	$15^{d} \pm 8$	$106^{abcd} \pm 60$	$156^{abcd} \pm 9$	$11^{d} \pm 6$	$95^{abcd} \pm 23$
Octanal	$462^{abc} \pm 167$	$57^{c} \pm 15$	$736^{abc} \pm 137$	$405^{abc} \pm 171$	$348^{bc} \pm 204$	$793^{abc} \pm 8$	$593^{abc} \pm 182$	$20^{c} \pm 28$	$605^{abc} \pm 406$	$648^{abc} \pm 83$	$31^{c} \pm 6$	$608^{abc} \pm 229$
2,3-Octanedione	$79^{abc} \pm 4$	$18^{c} \pm 0$	$64^{abc} \pm 33$	$75^{abc} \pm 13$	$29^{bc} \pm 18$	$59^{abc} \pm 19$	$94^{abc} \pm 7$	$19^{c} \pm 6$	$79^{abc} \pm 19$	$93^{abc} \pm 25$	$25^{c} \pm 8$	$77^{abc} \pm 40$
E-2-heptenal	$932^{abcdef} \pm 21$	$119^{fg} \pm 34$	$818^{abcdefg} \pm 80$	$1114^{abc} \pm 256$	$348^{cdefg} \pm 414$	$828^{abcdefg} \pm 37$	$1110^{abc} \pm 88$	$36^{g} \pm 33$	$794^{abcdefg} \pm 110$	$1145^{abc} \pm 311$	$56^{g} \pm 18$	$953^{abcde} \pm 96$
Nonanal	$542^{abc} \pm 233$	$81^{c} \pm 17$	$711^{abc} \pm 136$	$499^{abc} \pm 205$	$242^{abc} \pm 243$	$424^{abc} \pm 599$	$731^{abc} \pm 180$	$150^{bc} \pm 126$	$640^{abc} \pm 476$	$722^{abc} \pm 89$	$40^{c} \pm 1$	$588^{abc} \pm 67$
2-Octenal, (E)-	$1018^{a} \pm 163$	$64^{cde} \pm 17$	$722^{abcde} \pm 154$	$1011^{a} \pm 45$	$183^{bcde} \pm 212$	$783^{abcd} \pm 60$	$1178^{a} \pm 218$	$17^{e} \pm 24$	$663^{abcde} \pm 240$	$1313^{a} \pm 373$	$33^{e} \pm 9$	$799^{abc} \pm 142$
2,4-Heptadienal, (E,E)-	$42^{a} \pm 18$	$31^{a} \pm 6$	$67^{a} \pm 18$	$57^{a} \pm 31$	$48^{a} \pm 42$	$58^{a} \pm 10$	$53^{a} \pm 28$	$13^{a} \pm 18$	$72^{a} \pm 21$	$55^{a} \pm 32$	$17^{a} \pm 1$	$84^{a} \pm 23$
2,4-Heptadienal, (E,E)-	$153^{a} \pm 99$	$43^{a} \pm 4$	$171^{a} \pm 25$	$221^{a} \pm 163$	$78^{a} \pm 78$	$152^{a} \pm 7$	$183^{a} \pm 94$	$18^{a} \pm 25$	$168^{a} \pm 2$	$197^{a} \pm 140$	$21^{a} \pm 1$	$233^{a} \pm 13$
2-Nonenal, (E)-	$305^{a} \pm 213$	ND	$91^{a} \pm 31$	$263^{a} \pm 151$	$66^{a} \pm 93$	$175^{a} \pm 148$	$344^{a} \pm 221$	ND	$204^{a} \pm 246$	$314^{a} \pm 86$	ND	$66^{a} \pm 31$
2-Decenal, (E)-	$485^{abcdef} \pm 85$	$13^{ef} \pm 18$	$677^{abcde} \pm 75$	$469^{abcdef} \pm 72$	$93^{cdef} \pm 132$	$778^{ab} \pm 118$	$597^{abcdef} \pm 83$	$6^{ef} \pm 8$	$567^{abcdef} \pm 293$	$716^{abc} \pm 203$	ND	$600^{abcdef} \pm 54$
2,4-Nonadienal, (E,E)-	$63^{abcd} \pm 6$	ND	$52^{abcd} \pm 16$	$73^{a} \pm 12$	$6^{cd} \pm 8$	$57^{abcd} \pm 17$	$76^{a} \pm 4$	ND	$47^{abcd} \pm 23$	$92^{a} \pm 33$	ND	$51^{abcd} \pm 14$
2-Undecenal	$146^{abcde} \pm 30$	ND	$215^{abcd} \pm 25$	$150^{abcde} \pm 11$	$19^{de} \pm 27$	$240^{abc} \pm 45$	$188^{abcde} \pm 29$	ND	$180^{abcde} \pm 104$	$225^{abcd} \pm 61$	ND	$193^{abcde} \pm 8$
2,4-Decadienal	$99^{ab} \pm 28$	ND	$82^{ab} \pm 4$	$77^{ab} \pm 40$	$5^{b} \pm 7$	$82^{ab} \pm 6$	$77^{ab} \pm 35$	ND	$76^{ab} \pm 38$	$94^{ab} \pm 44$	ND	$84^{ab} \pm 58$

Table 5.5 (*continued*)

Compound	*W. koreensis-1*			*L. lactis*			*W. koreensis-2*			*L. mesenteroides*		
	B	F	S	B	F	S	B	F	S	B	F	S
Ketones	**Concentration (mean ± SD)* (mg kg^{-1})**											
5-Hepten-2-one, 6-methyl-	$90^{a} \pm 9$	$126^{a} \pm 30$	$85^{a} \pm 4$	$104^{a} \pm 25$	$166^{a} \pm 132$	$97^{a} \pm 16$	$108^{a} \pm 13$	$104^{a} \pm 7$	$83^{a} \pm 26$	$106^{a} \pm 6$	$81^{a} \pm 4$	$113^{a} \pm 4$
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z	$7^{b} \pm 3$	$17^{ab} \pm 2$	$4^{b} \pm 1$	$5^{b} \pm 0$	$16^{ab} \pm 10$	$4^{b} \pm 0$	$6^{b} \pm 2$	$8^{ab} \pm 3$	$6^{b} \pm 1$	$10^{ab} \pm 5$	$12^{ab} \pm 6$	$7^{b} \pm 0$
Organic acids												
Acetic acid	$1233^{a} \pm 238$	$2330^{a} \pm 777$	$1855^{a} \pm 97$	$1088^{a} \pm 10$	$1859^{a} \pm 171$	$1507^{a} \pm 74$	$1073^{a} \pm 30$	$3241^{a} \pm 727$	$1503^{a} \pm 124$	$1897^{a} \pm 95$	$2263^{a} \pm 127$	$1472^{a} \pm 1113$
Butanoic acid	$9^{a} \pm 1$	$79^{a} \pm 82$	$44^{a} \pm 43$	$13^{a} \pm 3$	$26^{a} \pm 5$	$18^{a} \pm 1$	$8^{a} \pm 0$	$25^{a} \pm 2$	$10^{a} \pm 1$	$12^{a} \pm 4$	$32^{a} \pm 1$	$11^{a} \pm 4$
Pentanoic acid	$36^{a} \pm 22$	$11^{a} \pm 7$	$20^{a} \pm 6$	$30^{a} \pm 11$	$13^{a} \pm 9$	$23^{a} \pm 0$	$38^{a} \pm 22$	$10^{a} \pm 2$	$19^{a} \pm 13$	$35^{a} \pm 4$	$7^{a} \pm 1$	$19^{a} \pm 0$
Hexanoic acid	$549^{a} \pm 327$	$77^{a} \pm 30$	$196^{a} \pm 129$	$455^{a} \pm 156$	$109^{a} \pm 59$	$378^{a} \pm 79$	$581^{a} \pm 212$	$88^{a} \pm 23$	$260^{a} \pm 224$	$551^{a} \pm 43$	$76^{a} \pm 21$	$258^{a} \pm 35$
Heptanoic acid	$79^{a} \pm 103$	$41^{a} \pm 16$	$73^{a} \pm 103$	$94^{a} \pm 124$	$23^{a} \pm 25$	$2^{a} \pm 2$	$109^{a} \pm 147$	$24^{a} \pm 27$	$2^{a} \pm 3$	$180^{a} \pm 247$	$43^{a} \pm 9$	$115^{a} \pm 75$
Furan												
Furan, 2-pentyl-	$214^{abc} \pm 62$	$20^{de} \pm 8$	$158^{abcde} \pm 27$	$191^{abcd} \pm 52$	$49^{cde} \pm 63$	$140^{abcde} \pm 21$	$235^{ab} \pm 45$	$2^{e} \pm 3$	$137^{abcde} \pm 64$	$233^{ab} \pm 41$	$10^{e} \pm 3$	$148^{abcde} \pm 8$
Enones												
3-Octen-2-one	$185^{abc} \pm 95$	$8^{c} \pm 11$	$169^{abc} \pm 59$	$191^{abc} \pm 94$	$32^{bc} \pm 39$	$212^{abc} \pm 64$	$219^{abc} \pm 98$	ND	$162^{abc} \pm 114$	$212^{abc} \pm 54$	ND	$161^{abc} \pm 44$
3,5-Octadien-2-one	$60^{a} \pm 3$	$18^{a} \pm 13$	$111^{a} \pm 79$	$78^{a} \pm 17$	$57^{a} \pm 81$	$112^{a} \pm 41$	$59^{a} \pm 13$	$7^{a} \pm 10$	$114^{a} \pm 13$	$65^{a} \pm 6$	$7^{a} \pm 4$	$131^{a} \pm 85$
3,5-Octadien-2-one, (E,E)-	$100^{bcdef} \pm 28$	$7^{ef} \pm 1$	$130^{abcde} \pm 46$	$124^{abcdef} \pm 21$	$25^{def} \pm 35$	$146^{abcd} \pm 21$	$114^{abcdef} \pm 26$	$2^{ef} \pm 2$	$144^{abcd} \pm 29$	$119^{abcdef} \pm 21$	$1^{f} \pm 0$	$179^{ab} \pm 86$
Unknown												
55/70	$58^{abc} \pm 4$	$15^{bc} \pm 7$	$57^{abc} \pm 2$	$66^{abc} \pm 1$	$50^{abc} \pm 59$	$60^{abc} \pm 6$	$67^{abc} \pm 4$	$6^{c} \pm 3$	$62^{abc} \pm 1$	$62^{abc} \pm 5$	$12^{c} \pm 6$	$69^{abc} \pm 3$

*^{abcdef} means with different letters across the row indicate significant statistical differences (Tukey's Test, $p < 0.05$); Values are Means ± SD of two biological replicates (n=6); ND: Not detected.

Table 5. 6: Relative mg kg^{-1} of volatile compounds quantified in lafun produced with three cassava varieties and fermented with LAB co-culture

	L. lactis* and *L. mesenteroides			***W. koreensis* (both strains)**			***W. koreensis-1* and *L. lactis***			***W. koreensis-2* and *L. mesenteroides***		
Compound	**B**	**F**	**S**	**B**	**F**	**S**	**B**	**F**	**S**	**B**	**F**	**S**
Aldehydes	**Concentration (mean ± SD)* (mg kg^{-1})**											
Acetone	776^{abc} ± 954	1438^{abc} ± 347	63^{c} ± 18	783^{abc} ± 305	479^{abc} ± 445	226^{bc} ± 89	1005^{abc} ± 405	2508^{a} ± 692	264^{bc} ± 106	551^{abc} ± 656	2073^{abc} ± 495	88^{c} ± 42
2-Butanone	349^{cde} ± 189	1448^{abcde} ± 1114	178de ± 86	538^{bcde} ± 32	1860^{abcd} ± 899	385^{cde} ± 179	767^{bcde} ± 218	2747a ± 141	366^{cde} ± 152	297^{cde} ± 156	1167^{abcde} ± 195	166de ± 84
Pentanal	449^{abc} ± 3	138^{bcdef} ± 79	522^{a} ± 20	484^{a} ± 76a	125^{cdef} ± 108	474^{ab} ± 45	538^{a} ± 1	283^{abcdef} ± 57	524^{a} ± 28	452^{abc} ± 27	242^{abcdef} ± 18	550^{a} ± 81
1-Penten-3-one	83^{a} ± 30	94^{a} ± 122	134^{a} ± 3	78^{a} ± 3	101^{a} ± 117	141^{a} ± 45	98^{a} ± 36	140^{a} ± 4	133^{a} ± 55	87^{a} ± 38	194^{a} ± 53	130^{a} ± 1
Hexanal	11876^{a} ± 1382	871^{b} ± 1059	13408^{a} ± 602	14112^{a} ± 1707	1158^{b} ± 1523	12518^{a} ± 2838	14851^{a} ± 942	3927^{b} ± 1498	14164^{a} ± 392	12809^{a} ± 2082	3202^{b} ± 377	14985^{a} ± 2024
3-Penten-2-one	20^{a} ± 11	32^{a} ± 29	46^{a} ± 65	159^{a} ± 93	16^{a} ± 3	234^{a} ± 29	90^{a} ± 6	50^{a} ± 31	138^{a} ± 71	32^{a} ± 45	48^{a} ± 20	101^{a} ± 78
E-2-pentenal	143^{abcd} ± 50	7^{cd} ± 10	231^{abcd} ± 9	182^{abcd} ± 8	ND	195^{abcd} ± 4	242^{abc} ± 50	110^{bcd} ± 21	248^{ab} ± 4	158^{abcd} ± 35	106^{bcd} ± 1	372^{a} ± 168
Heptanal	342^{abc} ± 178	85^{bc} ± 120	592^{ab} ± 121	507^{abc} ± 91	102^{bc} ± 136	543^{abc} ± 238	474^{abc} ± 113	356^{abc} ± 91	655^{a} ± 126	476^{abc} ± 144	317^{abc} ± 8	794^{a} ± 156
2-Hexenal	120^{abcd} ± 52	24^{cd} ± 23	120^{abcd} ± 62	171^{abcd} ± 14	17^{cd} ± 23	137^{abcd} ± 54	178^{abcd} ± 28	50^{abcd} ± 4	179^{ab} ± 11	153^{abcd} ± 40	47^{abcd} ± 6	190^{a} ± 20
Octanal	382^{abc} ± 194	184^{c} ± 107	757^{abc} ± 255	625^{abc} ± 115	126^{c} ± 19	716^{abc} ± 321	585^{abc} ± 233	420^{abc} ± 70	977^{ab} ± 99	576^{abc} ± 247	375^{c} ± 52	1090^{a} ± 221
2,3-Octanedione	67^{abc} ± 50	25^{c} ± 4	97^{abc} ± 4	97^{abc} ± 18	19^{c} ± 6	92^{abc} ± 31	87^{abc} ± 8	33^{abc} ± 13	114^{ab} ± 39	87^{abc} ± 1	26^{c} ± 6	117^{a} ± 11
E-2-heptenal	966^{abcde} ± 673	150^{efg} ± 180	1057^{abc} ± 42	1313^{a} ± 148	157^{defg} ± 222	974^{abcd} ± 87	1187^{ab} ± 61	475^{bcdefg} ± 74	1200^{ab} ± 102	1010^{abc} ± 35	427^{bcdefg} ± 76	1239^{ab} ± 110
Nonanal	443^{abc} ± 223	113^{bc} ± 99	767^{abc} ± 285	773^{abc} ± 116	151^{bc} ± 165	784^{abc} ± 350	704^{abc} ± 292	319^{abc} ± 53	1088^{ab} ± 106	699^{abc} ± 337	401^{abc} ± 107	1154^{a} ± 257
2-Octenal, (E)-	888^{ab} ± 347	55de ± 61	1003^{a} ± 127	1265^{a} ± 104	57de ± 80	799^{abc} ± 252	1251^{a} ± 228	209^{bcde} ± 39	1082^{a} ± 32	1088^{a} ± 327	207^{bcde} ± 8	1189^{a} ± 190
2,4-Heptadienal, (E,E)-	58^{a} ± 17	32^{a} ± 30	78^{a} ± 23	51^{a} ± 8	23^{a} ± 33	85^{a} ± 13	46^{a} ± 1	68^{a} ± 8	90^{a} ± 27	41^{a} ± 15	64^{a} ± 17	76^{a} ± 8
2,4-Heptadienal, (E,E)-	166^{a} ± 139	40^{a} ± 41	206^{a} ± 28	189^{a} ± 65	42^{a} ± 59	215^{a} ± 13	157^{a} ± 23	104^{a} ± 18	258^{a} ± 84	128^{a} ± 30	95^{a} ± 9	248^{a} ± 21
2-Nonenal, (E)-	235^{a} ± 52	40^{a} ± 56	258^{a} ± 202	348^{a} ± 179	49^{a} ± 69	226^{a} ± 278	321^{a} ± 216	119^{a} ± 28	297^{a} ± 169	333^{a} ± 288	111^{a} ± 3	341^{a} ± 255
2-Decenal, (E)-	352^{abcdef} ± 356	24^{def} ± 33	812^{a} ± 83	654^{abcdef} ± 48	27^{def} ± 37	687^{abcd} ± 362	604^{abcdef} ± 256	136^{bcdef} ± 40	981^{a} ± 120	566^{abcdef} ± 255	133^{bcdef} ± 5	978^{a} ± 228
2,4-Nonadienal, (E,E)-	60^{abcd} ± 42	ND	61^{abcd} ± 5	90^{a} ± 8	2^{d} ± 3	50^{abcd} ± 28	79^{a} ± 15	9^{bcd} ± 5	72^{ab} ± 5	68^{abc} ± 17	8^{cd} ± 4	77^{a} ± 10
2-Undecenal	109^{bcde} ± 107	ND	238^{abc} ± 33	198^{abcde} ± 19	ND	199^{abcde} ± 112	187^{abcde} ± 84	32^{cde} ± 11	288^{ab} ± 48	176^{abcde} ± 92	30^{cde} ± 1	325^{a} ± 56
2,4-Decadienal	42^{ab} ± 46	ND	91^{ab} ± 0	111^{ab} ± 67	ND	101^{ab} ± 41	105^{ab} ± 78	5^{b} ± 6	155^{a} ± 40	94^{ab} ± 53	6^{b} ± 1	136^{ab} ± 23

Table 5.6 (*continued*)

Compound	*L. lactis* and *L. mesenteroides*			*W. koreensis* (both strains)			*W. koreensis-1* and *L. lactis*			*W. koreensis-2* and *L. mesenteroides*		
	B	F	S	B	F	S	B	F	S	B	F	S
Ketones	**Concentration (mean ± SD)* (mg kg^{-1})**											
5-Hepten-2-one, 6-methyl-	99^{a} ± 8	149^{a} ± 103	121^{a} ± 13	118^{a} ± 21	160^{a} ± 138	95^{a} ± 19	129^{a} ± 30	243^{a} ± 8	171^{a} ± 29	101^{a} ± 18	226^{a} ± 64	139^{a} ± 20
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z	9^{ab} ± 6	14^{ab} ± 5	7^{b} ± 2	7^{b} ± 1	13^{ab} ± 11	5^{b} ± 2	8^{ab} ± 1	25^{a} ± 3	11^{ab} ± 8	7^{b} ± 0	25^{a} ± 1	7^{b} ± 1
Organic acids												
Acetic acid	1596^{a} ± 379	3217^{a} ± 1416	1344^{a} ± 631	1587^{a} ± 173	3496^{a} ± 2405	1806^{a} ± 120	1777^{a} ± 66	2242^{a} ± 361	2070^{a} ± 231	1732^{a} ± 631	2281^{a} ± 69	1814^{a} ± 193
Butanoic acid	11^{a} ± 1	29^{a} ± 4	11^{a} ± 3	11^{a} ± 1	20^{a} ± 8	11^{a} ± 0	13^{a} ± 4	22^{a} ± 8	18^{a} ± 0	10^{a} ± 3	22^{a} ± 1	11^{a} ± 0
Pentanoic acid	42^{a} ± 27	11^{a} ± 4	26^{a} ± 8	38^{a} ± 8	10^{a} ± 8	19^{a} ± 11	41^{a} ± 16	12^{a} ± 1	29^{a} ± 8	37^{a} ± 22	12^{a} ± 1	33^{a} ± 8
Hexanoic acid	532^{a} ± 183	110^{a} ± 21	371^{a} ± 88	572^{a} ± 106	68^{a} ± 42	280^{a} ± 223	656^{a} ± 209	97^{a} ± 20	406^{a} ± 155	559^{a} ± 336	94^{a} ± 23	459^{a} ± 129
Heptanoic acid	106^{a} ± 142	29^{a} ± 35	82^{a} ± 109	146^{a} ± 198	10^{a} ± 8	38^{a} ± 48	146^{a} ± 197	25^{a} ± 31	4^{a} ± 1	54^{a} ± 66	3^{a} ± 1	5^{a} ± 2
Furan												
Furan, 2-pentyl-	153^{abcde} ± 103	1^{e} ± 1	172^{abcde} ± 12	242^{a} ± 14	24de ± 32	159^{abcde} ± 58	236^{ab} ± 32	67^{bcde} ± 23	205^{abc} ± 6	227^{ab} ± 74	67^{bcde} ± 4	241^{a} ± 26
Enones												
3-Octen-2-one	199^{abc} ± 62	9^{c} ± 13	223^{abc} ± 54	274^{ab} ± 110	10^{c} ± 14	242^{abc} ± 41	268^{ab} ± 100	36^{bc} ± 11	303^{a} ± 27	228^{abc} ± 91	32^{bc} ± 4	285^{ab} ± 24
3,5-Octadien-2-one	104^{a} ± 40	24^{a} ± 34	132^{a} ± 48	83^{a} ± 1	39^{a} ± 54	119^{a} ± 10	76^{a} ± 12	91^{a} ± 16	145^{a} ± 33	61^{a} ± 13	85^{a} ± 22	120^{a} ± 16
3,5-Octadien-2-one, (E,E)-	98^{bcdef} ± 43	6^{ef} ± 8	189^{ab} ± 13	148^{abcd} ± 13	10^{ef} ± 13	160^{abc} ± 41	140^{abcd} ± 28	33^{cdef} ± 13	231^{a} ± 62	105^{abcdef} ± 7	24^{def} ± 1	215^{ab} ± 23
Unknown												
55/70	61^{abc} ± 23	24^{abc} ± 27	72^{abc} ± 11	66^{abc} ± 11	18^{bc} ± 21	76^{abc} ± 6	72^{abc} ± 13	68^{abc} ± 11	90^{a} ± 19	69^{abc} ± 4	63^{abc} ± 29	84^{ab} ± 13

*^{abcdef} means with different letters across the row indicate significant statistical differences (Tukey's Test, $p < 0.05$); Values are Means ± SD of two biological replicates (n=6); ND: Not detected.

Table 5. 7: Relative mg kg^{-1} of volatile compounds quantified in raw cassava roots and market samples

	Cassava varieties (raw)			Market samples		
	Bitter	Fortified	Sweet	M1	M2	M3
Compound	Concentration (mean ± SD)* (mg kg^{-1})					
Aldehydes						
Acetone	1503 ± 32	6211 ± 582	1513 ± 152	29 ± 2	12 ±0	40 ± 2
2-Butanone	1394 ± 39	4746 ± 629	1278 ± 149	23 ±1	ND	ND
Pentanal	27 ± 1	ND	30 ± 3	256 ± 19	216 ± 10	37 ± 1
1-Penten-3-one	15 ± 1	ND	72 ± 9	22 ± 3	23 ± 2	ND
Hexanal	420 ±20	162 ± 23	514 ± 60	6500 ± 396	2563 ± 130	481 ± 19
3-Penten-2-one	ND	ND	ND	52 ± 1	ND	ND
E-2-pentenal	ND	ND	ND	38 ± 2	170 ± 127	ND
Heptanal	42 ± 2	ND	54 ± 8	346 ± 18	328 ± 12	65 ± 4
2-Hexenal	14 ± 1	ND	28 ± 4	55 ± 3	18 ± 1	ND
Octanal	31 ± 2	ND	31 ± 4	596 ± 40	498 ± 24	157 ± 1
2,3-Octanedione	14 ± 1	3 ± 1	6 ± 0	19 ± 1	14 ± 1	3 ± 0
E-2-heptenal	27 ± 2	ND	37 ± 5	236 ± 14	217 ± 8	22 ± 1
Nonanal	125 ± 6	126 ± 18	130 ± 16	541 ± 27	810 ± 52	309 ± 28
2-Octenal, (E)-	ND	ND	28 ± 5	376 ± 11	119 ± 7	24 ± 0
2,4-Heptadienal, (E,E)-	7 ± 1	ND	20 ± 2	28 ± 4	8 ± 0	ND
2,4-Heptadienal, (E,E)-	10 ± 1	ND	20 ± 3	87 ± 1	ND	ND
2-Nonenal, (E)-	ND	ND	ND	101 ± 3	137 ± 28	29 ± 3
2-Decenal, (E)-	ND	ND	ND	151 ± 15	93 ± 3	0
2,4-Nonadienal, (E,E)-	ND	ND	ND	73 ± 2	16 ± 0	7 ± 1
2-Undecenal	ND	ND	ND	ND	ND	ND
2,4-Decadienal	ND	ND	ND	8 ± 0	19 ± 1	0
Ketones						
5-Hepten-2-one, 6-methyl-	27 ± 2	58 ± 9	31 ± 5	69 ± 2	252 ± 14	23 ± 1
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z	ND	ND	ND	ND	8 ± 1	4 ± 0
Organic acids						
Acetic acid	7 ± 1	36 ± 6	6 ± 0	580 ± 83	490 ± 14	686 ± 24
Butanoic acid	ND	ND	ND	90 ± 3	6097 ± 99	5653 ± 82
Pentanoic acid	ND	ND	ND	61 ± 6	46 ± 2	210 ± 2
Hexanoic acid	ND	ND	ND	819 ± 16	149 ± 5	129 ± 7
Heptanoic acid	ND	ND	ND	421 ± 73	ND	ND
Furan						
Furan, 2-pentyl-	ND	ND	ND	223 ± 20	93 ± 9	17 ± 1
Enones						
3-Octen-2-one	ND	ND	ND	284 ± 4	30 ± 2	11 ± 1
3,5-Octadien-2-one	5 ± 1	ND	10 ± 2	159 ± 23	26 ± 1	29 ± 1
3,5-Octadien-2-one, (E,E)-	2	ND	ND	122 ± 22	14 ± 1	7 ± 0
Alcohol						
1-Octen-3-ol	9	ND	ND	ND	104 ± 5	33 ± 1
Cresol						
p-cresol	ND	ND	ND	ND	9 ± 1	ND
m-cresol	ND	ND	ND	ND	6 ± 0	ND
Unknown						
55/70	2 ± 0	ND	4 ± 0	21 ± 0	56 ± 0	5 ± 0

Values are Means ± SD of three replicates; ND: Not detected. M1, Bodija market sample; M2, Sango market sample; M3, Mokola market sample.

Table 5. 8: Odour description and intensity of the volatile compounds detected by GC-O in headspace of lafun

No	Odour description	Compound	SPME GC-O[A]	ID[B]	Variety-intensity[C] M	L	P[D]
1	green	hexanal	800	A	5	5	ns
2	sweet	3-hexenal	803	A	5	4.5	ns
3	odd green	3-hexenal	807	A	4	3	ns
4	cheese	butanoic acid	810	A	5.5	ND	***
5	meat	2-methyl-3-furanthiol	865	A	4	ND	**
6	odd tinny fruity	2-heptanone	898	A	4.5	ND	***
7	lamb fat	cis-4-heptenal	902	A	5	6.5	ns
8	potato	methional	906	A	ND	4	**
9	cats pee		941		ND	5	***
10	fried	E-2-heptenal	955	A	ND	5	***
11	greenhouse	2-methoxy-3-methylpyrazine	973	A	3	ND	**
12	geranium	1,5-octadien-3-one	983	A	3.5	6	**
13	orange	6-methyl-5-hepten-2-one	986	A	5	5	ns
14	orange	octanal	1006	A	6	8	ns
15	fried	2,4-heptadienal	1011	A	ND	6	***
16	sharp green fuity viney	(E)-2-Hexenyl acetate	1018	A	ND	3.5	**
17	fruity ald	phenylacetaldehyde	1039	A	1.5	ND	**
18	pyr hot	2-Ethyl-3-methoxypyrazine	1054	A	4.5	ND	**
19	fried	Z-2-octenal	1059	A	5.5	7	ns
20	fried	E-2-octenal	1064	A	5	6	ns
21	manure	2-methylphenol	1077	A	5	ND	***
22	cooked coffee odd	diethylpyrazine	1081	A	4	4	ns
23	dry pyrazine	3,5-dimethyl-2-ethylpyrazine	1087	A	ND	3	**
24	medicinal	guaiacol	1091	A	4	ND	**
25	fruity		1095		ND	5	***
26	odd pyr	2-Isopropyl-3-methoxypyrazine	1096	A	4	ND	**
27	sheets	2-nonanone	1100	A	2.5	ND	**
28	fatty aldehyde	nonanal	1106	A	3.5	ND	**
29	fried	2,4-octadienal	1112	A	ND	4	**
30	coriander	Z-2-nonenal	1152	A	2.5	4.5	*
31	violets	2,6-nonadienal	1155	A	6	5	ns
32	sheets + medicinal	E-2-nonenal	1161	A	6	6.5	ns
33	parma violets		1169		6	ND	***
34	medicinal	2,4-dimethylphenol	1173	A	3	ND	**
35	meat	2-methyl-3-furyl methyl disulfide	1174	A	4.5	ND	**
36	green house	2-Isobutyl-3-methoxypyrazine	1183	A	4	ND	**
37	fries	2,4-nonadienal isomer	1194		ND	5	***
38	fries	E,E-2,4-nonadienal	1214	A	6	6.5	ns
39	minty	2-(2-Methylbutyl)-3-methylpyrazine	1246	A	5	4	ns
40	dry pyr		1248		ND	4	**
41	coriander	2-decenal	1264	A	4	6.5	**
42	tea		1273		4	2	*
43	dry hot		1279		ND	6	***
44	fatty	2,4-decadienal isomer	1296		ND	3	**
45	fried	E,E-2,4-decadienal	1318	A	8	8	ns
46	fried	Z,Z-2,4-decadienal isomer	1332	A	ND	3.5	**
47	coriander	Z-2-undecenal	1352		ND	5.5	***
48	coriander	E-2-undecenal	1367	A	4.5	5.5	ns
49	hot dry	pyrazine	1380		5.5	ND	***

[A] SPME GC–O: Linear retention index on DB-5 column, calculated from a linear equation between each pair of straight chain alkanes C5–C25. [B] A, LRI agree with those of authentic compound. [C] The average of intensities observed by two assessors for each sample (M, Market sample; L, LAB sample); ND, not detected. [D] Probability as obtained from ANOVA that there is a difference between means: ns, no significant difference ($p > 0.05$); * significant at 5% level, ** significant at 1% level and *** significant at 0.1% level.

cassava with W. koreensis-1. The market samples showed a lactate concentration very close to the sweet cassava samples: Bodija sample- 1.03 a.u; Sango sample- 3.00 a.u.; and Mokola sample- 2.37 a.u. The results of this experiment confirm, support and help to further our understanding of the previous findings regarding the impacts of the selected LAB cultures and cassava roots types on the chemical properties of lafun and how laboratory samples are different from the market samples.

5.3.5 Microbial analysis of lafun samples

In this experiment, the safety evaluation of lafun produced with LAB strains and three cassava varieties was conducted. It has been documented in the literature that in lactic acid fermentation, especially when it involves the lactic acid bacteria, the pH is lowered to the point that most spoilage and pathogenic organisms are eradicated or inactivated. However, the process of drying, milling and packaging are usually not done in a sterile environment. Osmophilic moulds and yeast (spoilage organisms), presumptive *Enterobacteriaceae* and *E. coli* (indicator organisms), with coagulase-positive *Staphylococci* (pathogenic organisms), were enumerated in the lafun samples and *Salmonella spp*. (food infection and food intoxication organisms) detection test was conducted. These undesirable microbes could grow in food to cause infection, produce enough toxins to cause intoxication, or develop undesirable attributes like change in colour, odour and texture.

These organisms are examples of the most sought microorganisms used as a reference to determine food quality and safety and to certify that a food sample is acceptable, good, potentially hazardous or unsatisfactory. There were no *Salmonella spp*. detected in 25 g of samples. The values obtained from the total plate count of the spoilage and pathogenic organisms were satisfactory (Table 5.9). Lafun has the advantage of

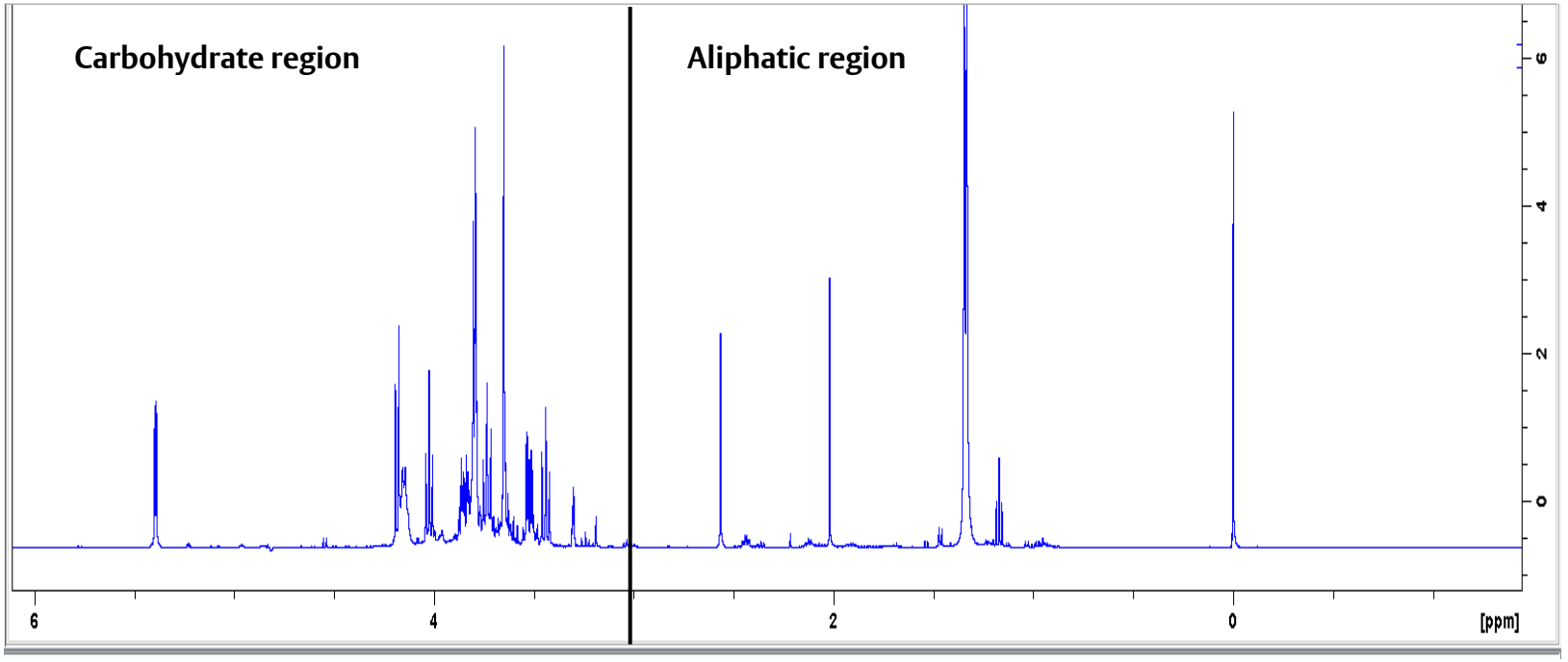
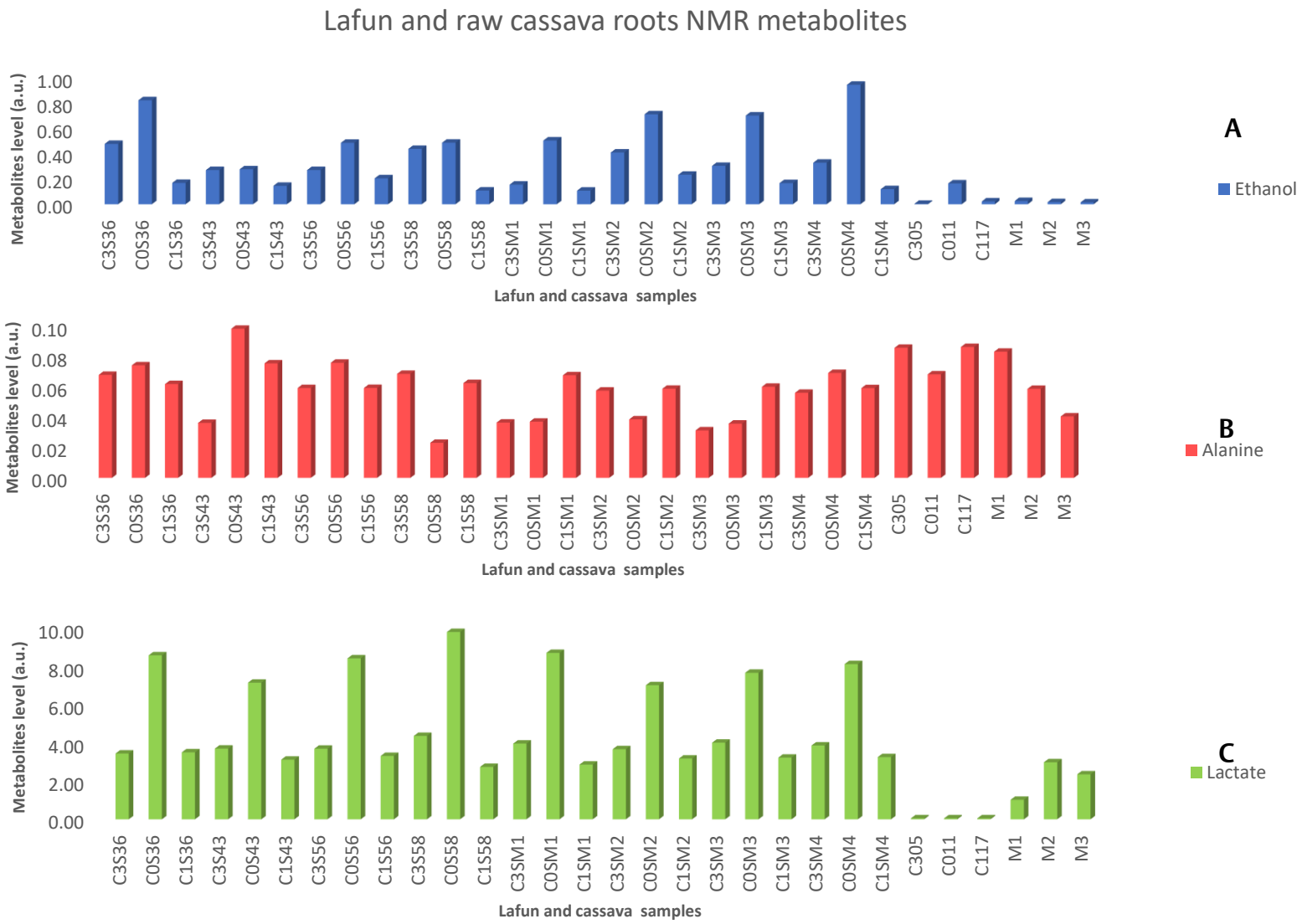
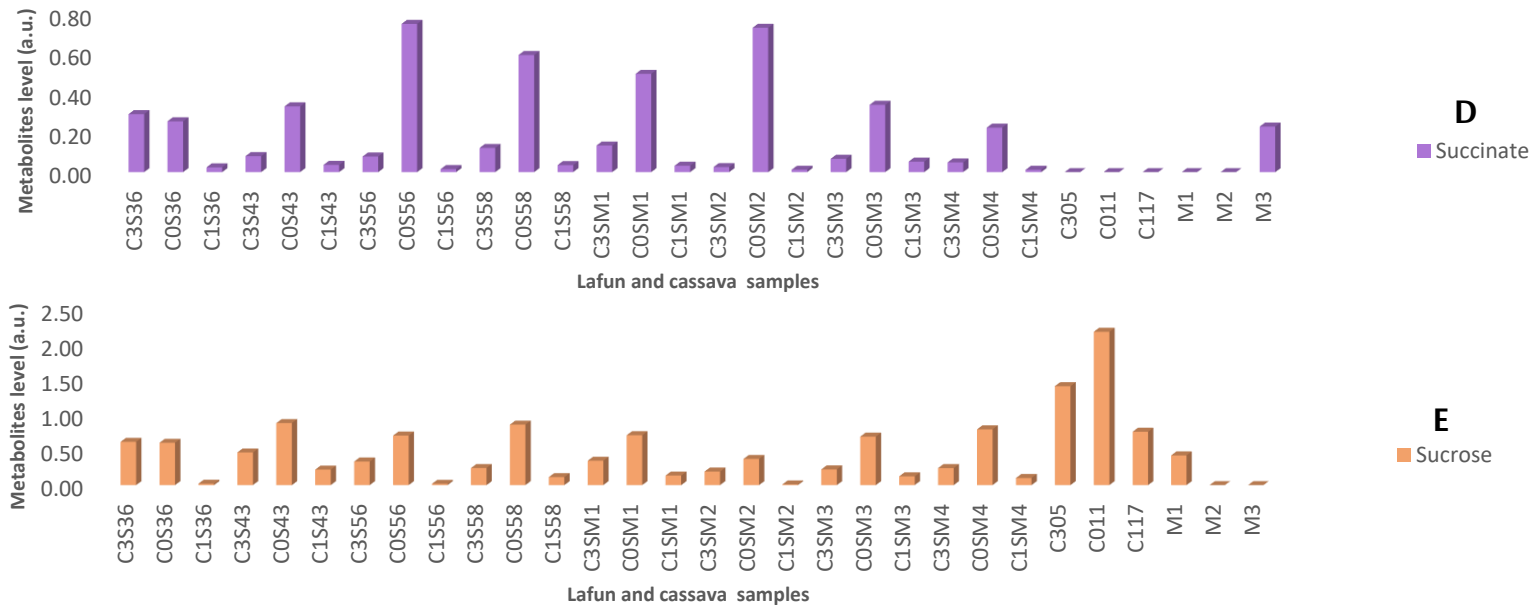


Figure 5. 4: A typical lafun NMR spectrum showing carbohydrate and aliphatic regions


Lafun and raw cassava roots NMR metabolites
Metabolites level (a.u.)
1.00
0.80
0.60
0.40
0.20
0.00
C3S36 C0S36 C1S36 C3S43 C0S43 C1S43 C3S56 C0S56 C1S56 C3S58 C0S58 C1S58 C3SM1 C0SM1 C1SM1 C3SM2 C0SM2 C1SM2 C3SM3 C0SM3 C1SM3 C3SM4 C0SM4 C1SM4 C305 C011 C117 M1 M2 M3
Lafun and cassava samples
A
Ethanol
Metabolites level (a.u.)
0.10
0.08
0.06
0.04
0.02
0.00
C3S36 C0S36 C1S36 C3S43 C0S43 C1S43 C3S56 C0S56 C1S56 C3S58 C0S58 C1S58 C3SM1 C0SM1 C1SM1 C3SM2 C0SM2 C1SM2 C3SM3 C0SM3 C1SM3 C3SM4 C0SM4 C1SM4 C305 C011 C117 M1 M2 M3
Lafun and cassava samples
B
Alanine
Metabolites level (a.u.)
10.00
8.00
6.00
4.00
2.00
0.00
C3S36 C0S36 C1S36 C3S43 C0S43 C1S43 C3S56 C0S56 C1S56 C3S58 C0S58 C1S58 C3SM1 C0SM1 C1SM1 C3SM2 C0SM2 C1SM2 C3SM3 C0SM3 C1SM3 C3SM4 C0SM4 C1SM4 C305 C011 C117 M1 M2 M3
Lafun and cassava samples
C
Lactate



Key: Lafun and cassava samples

C3S36: Bitter + *W. koreensis-1*; C0S36: Fortified + *W. koreensis-1*; C1S36: Sweet + *W. koreensis-1*; C3S43: *Bitter + L. lactis*;
C0S43: Fortified + *L. lactis*; C1S43: Sweet + *L. lactis*; C3S56: Bitter + *W. koreensis-2*; C0S56: Fortified + *W. koreensis-2*;
C1S56: Sweet + *W. koreensis-2;* C3S58: Bitter + *L. mesenteroides*; C0S58: Fortified + *L. mesenteroides*; C1S58: Sweet + *L. mesenteroides*;
C3SM1: Bitter + *L. lactis* and *L. mesenteroides*; C0SM1: Fortif. + *L. lactis* and *L. mesenteroides*; C1SM1: Sweet + *L. lactis* and *L. mesenteroides*; C3SM2: Bitter + *W. koreensis* (both);
C0SM2: Fortified + *W. koreensis* (both); C1SM2: Sweet + *W. koreensis* (both); C3SM3: Bitter + *W. koreensis-1* and *L. lactis*; C0SM3: Fortified. + *W. koreensis-1 and L. lactis;*
C1SM3: Sweet + *W. koreensis-1* and *L. lactis*; C3SM4: Bitter + *W. koreensis-2* and *L. mesenteroides* C0SM4: Fortified + *W. koreensis-2* and *L. mesenteroides*
C1SM4: Sweet + *W. koreensis-2* and *L. mesenteroides* C305: Bitter cassava; C011: Fortified cassava C117: Sweet cassava
M1: Bodija market Sample; M2: Sango market Sample; M3: Mokola market sample

Figure 5. 5: Lafun and cassava metabolite content: A, Ethanol; B, Alanine; C, Lactate, D, Succinate and E, Sucrose

maintaining the low counts of the enumerated organisms because it is a dried food substance (Begum, 1985). The results of lafun microbial analysis indicates that the laboratory samples are of good quality regarding food safety.

5.3.6 Cyanide content determination of lafun samples and raw cassava roots

The concentration of cyanide in the raw cassava, experimentally produced lafun samples and the market samples were determined. Lafun from the markets contained by far the highest concentrations of cyanide with the one collected from Sango market having the highest value of 47.95 mg kg^{-1}. JECFA (1992) reported that a level of up to 10 mg kg^{-1} cyanide in cassava flour is not associated with acute toxicity in humans. The World Health Organisation (WHO) thus set a safe limit of 10 mg kg^{-1} total cyanide for cassava flour (FAO/WHO, 1995) which has been adopted by other food regulatory bodies in various countries. Although the cyanide content of the cassava used in processing the market samples was not known, the cyanide content is very high relative to the set limit. Thus, a calculation using Sango market sample value of 47.95 mg kg^{-1} and based on the lethal dose of cyanide for humans expresses that a child of 20 kg would need to consume no more than 200 g of the lafun sample to stay within the set limit.

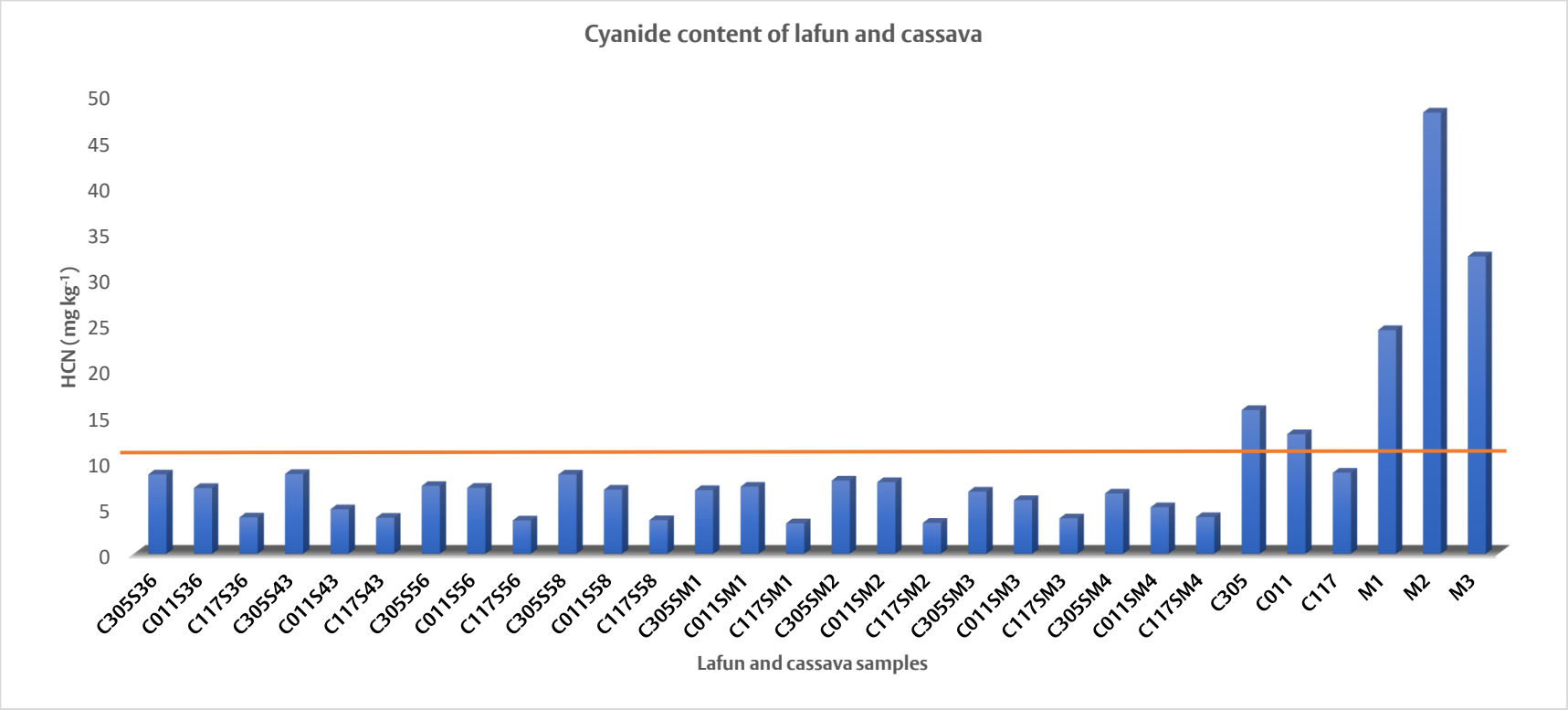
Cassava was originally classified as either bitter (HCN up to 400 mg kg^{-1}) or sweet (HCN $<$ 50 mg kg^{-1}) based on their cyanide content (Ifeabunike et al., 2017; Hahn et al., 1992). However, due to advance in technology, modern techniques have been employed by researchers to bring the level of cyanide in cassava root to as low as 10 mg kg^{-1} in raw samples (William, 2011). These modern techniques include the production of transgenic cassava root through inhibition of cyanogenic glycoside synthesis in cassava leaves by antisense expression of CYP79D1/D2 gene fragments (Siritunga &

Sayre, 2004). Although the results presented here for raw cassava roots were from sliced, dried and milled roots (processing reduce cyanide level), it was observed that the varieties are types of such transgenic cassava roots: bitter- 15.09 ± 1.11-16.11 ± 0.75 mg kg^{-1}; fortified- 13.45 ± 0.41-12.51± 0.40 mg kg^{-1}; and sweet- about 8.87± 0.11 mg kg^{-1}. The levels of hydrogen cyanide found in the laboratory samples were all below the recommended 10 mg kg^{-1}.

Table 5. 9: Microbial loads of lafun samples produced from control fermentation of three cassava varieties with selected LAB, singly and in combination

LAB strain(s)	**Cassava variety**	**Osmophilic Moulds**	**Osmophilic Yeasts**	***Enterobacteriaceae* (presumptive)**	**Coagulase + *Staphylococci***	***E. coli* (presumptive)**	***Salmonella spp.* (detection)**
		Microbial count in 25g of lafun (CFU g^{-1})					
W. koreensis-1	Bitter	40	<20	<10	<20	<10	ND in 25g
	Fortified	<20	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND
L. lactis	Bitter	<20	<20	<10	<20	<10	ND
	Fortified	<20	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND
W. koreensis-2	Bitter	<20	<20	<10	<20	<10	ND
	Fortified	<20	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND
L. mesenteroides	Bitter	<20	<20	<10	<20	<10	ND
	Fortified	<20	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND
L. lactis and *L. mesenteroides*	Bitter	<20	<20	<10	<20	<10	ND
	Fortified	40	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND
W. koreensis (both strains)	Bitter	<20	<20	<10	<20	<10	ND
	Fortified	<20	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND
W. koreensis-1 and *L. lactis*	Bitter	<20	<20	<10	<20	<10	ND
	Fortified	20	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND
W. koreensis-2 and *L. mesenteroides*	Bitter	<20	<20	<10	<20	<10	ND
	Fortified	<20	<20	<10	<20	<10	ND
	Sweet	<20	<20	<10	<20	<10	ND

Bitter (IBA30572), Fortified (IBA011371) and Sweet (TMEB117)



Key: Lafun and cassava samples

C305: Bitter cassava; C011: Fortified cassava C117: Sweet cassava

C305S36: Bitter + *W. koreensis-1*; C011S36: Fortified + *W. koreensis-1*; C117S36: Sweet + *W. koreensis-1*; C305S43: *Bitter + L. lactis*;

C011S43: Fortified + *L. lactis*; C117S43: Sweet + *L. lactis*; C305S56: Bitter + *W. koreensis-2*; C011S56: Fortified + *W. koreensis-2*;

C117S56: Sweet + *W. koreensis-2*; C305S58: Bitter + *L. mesenteroides*; C011S58: Fortified + *L. mesenteroides*; C117S58: Sweet + *L. mesenteroides*;

C305M1: Bitter + *L. lactis* and *L. mesenteroides*; C011SM1: Fortif. + *L. lactis* and *L. mesenteroides*; C117SM1: Sweet + *L. lactis* and *L. mesenteroides*; C305M2: Bitter + *W. koreensis* (both);

C011SM2: Fortified + *W. koreensis* (both); C117SM2: Sweet + *W. koreensis* (both); C305M3: Bitter + *W. koreensis-1* and *L. lactis*; C011SM3: Fortif. + *W. koreensis-1 and L. lactis;*

C117SM3: Sweet + *W. koreensis-1* and *L. lactis*; C305M4: Bitter + *W. koreensis-2* and *L. mesenteroides* C011SM4: Fortified + *W. koreensis-2* and *L. mesenteroides*

C117SM4: Sweet + *W. koreensis-2* and *L. mesenteroides* M1: Bodija market Sample; M2: Sango market Sample; M3: Mokola market sample

Figure 5. 6: Lafun and cassava cyanide content with line showing WHO safe limit of 10 mg kg^{-1} total cyanide intake

5.4 Conclusion

Lactic acid fermentation of cassava has excellent potential to produce safe, high quality lafun. The result of proximate analysis supports the conclusion that the inoculation of fortified cassava (IBA011371) with *L. mesenteroides* starter culture strain results in higher crude ash, protein and fibre content in the lafun. Bitter cassava is traditionally used to produce lafun, however the results indicate that lafun from fortified cassava roots had more desirable rheological properties, suggesting the use of fortified cassava could improve consumer acceptability. Ketones found in the headspace of lafun were found by GC-MS to be generally higher in the fortified variety products than the other two varieties. The fermentation process should be controlled by parameters that would increase the yield of ketones among the volatile compounds for better lafun flavour and quality. Hexanal level in food during storage should be monitored to determine the onset of rancidity. Storage study should be conducted to check the effect of oxidation on the changes of lafun flavour profile. It could be decided that the characteristics of the variety of cassava employed in the production of lafun determine what product metabolite fingerprints generated. All lafun samples produced with selected LAB cultures in this study passed basic microbiological testing.

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Chapter Six

6.0 Overall Conclusions and Future Work

6.1 Overall conclusions

The purpose of this study was to improve the production of lafun, a fermented cassava flour, by standardising LAB cultures as starters for cassava fermentation. The literature reviewed justified the study objectives, the strategies used to decide the best process conditions and the choice of analyses employed. Lafun is mostly produced at a cottage level with traditional procedures even though it should be processed in licensed industries, whether at a small or large scale. The traditional technologies lack process control that favours physical, sensory and microbiological quality of lafun. Traditionally produced lafun were found by past studies to have spoilage and pathogenic organisms including *Bacillus cereus, Staphylococcus aureus, Salmonella spp., Escherichia coli, Klebsiella spp., Citrobacter spp., Enterobacter spp.* and *Aspergillus spp.* The dominance of enteric and spoilage organisms in lafun could be attributed to the spontaneous fermentation of cassava and the lack of good hygiene practices during production. It, therefore, became necessary to develop LAB starter cultures for its production under good manufacturing practice.

Isolating LAB from spontaneous fermentation of cassava led to the use of strains that are fast in inducing fermentation in a controlled system such that the fermentation process was effective in producing the desired flavour compounds after 48 h. Acidification rate of LAB strains was high as expected, dropping the pH of the fermenting medium by approximately 42% (7.65 to 4.43). Acidification helped to maintain a selective environment against undesirable microorganisms. A taxonomic polyphasic system that combined both phenotypic and genotypic identification used

in this study compared isolates metabolic capabilities and resolved ambiguities from growth requirements, respectively. The selection of four LAB strains (2 strains of *W. koreensis* and one of *L. lactis* and *L. mesenteroides*) that were used as starters in the production of lafun was based on the highest production of desired flavour compounds and safety measured by rapid acidification capability.

The processing conditions that were followed through the study were supported by the initial demonstration of the effect of pressing of fermented roots and the length of fermentation on the concentration of flavour compounds in the final product. The concentration of flavour components of lafun were estimated at both 24 h and 48 h. For the mechanical pressing effect, water were squeezed out of a portion of the fermented roots before drying. The processing conditions that favoured better concentration of non-volatile organic compounds were found to be 48 h fermentation time with no pressing stage production.

This was the first time that the effect of cassava varieties on the quality of lafun was studied. The advantage of fortified cassava over the conventional bitter cassava used in lafun production was justified by the results of this research. The fortified cassava gave better quality lafun regarding proximate and rheological properties, and flavour profile regardless of which strain of LAB was used.

No data is available in the literature on the volatile organic compounds (VOCs), causing the characteristic aroma of lafun. These VOCs were identified in the study as aldehydes, ketones, organic acids, alcohol, furan, enones and cresols. The concentration of aldehydes that accounts for lipid content rancidity in foods was low in the lafun samples of fortified cassava. This low concentration and the earlier-mentioned qualities

of fortified variety demonstrated that using the variety is an innovative matrix for improving the keeping quality of lafun and enhancing a desirable flavour profile.

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy used in this study was initially developed and evaluated for plant metabolomic study by some researchers. ^{1}H-NMR spectroscopic fingerprinting of lafun extracts showed identification and quantification of primary metabolites responsible for differences in sample composition and quality. The effects of processing on lafun components and metabolites generated by microbiological fermentation were also determined. Comparison of the ^{1}H-NMR spectra of polar metabolites extracted with deuterated methanol-water showed a stronger influence of the type of raw material used in the production of lafun, that is, cassava varieties. Differences were spotted in the peaks of sugars, organic acids and amino acid (the carbohydrate and aliphatic regions) and they contributed to the differences in the sample set. The technique has proven to be a valuable tool for unbiased metabolite fingerprinting of lafun. It also provided sufficient knowledge about lafun characteristics that will ease the standardisation of the cassava fermentation process.

This research determined the bacteriological quality of lafun produced with LAB cultures and the three cassava varieties for food safety purpose. Osmophilic moulds and yeast (spoilage organisms), presumptive *Enterobacteriaceae* and *E. coli* (indicator organisms), with coagulase-positive *Staphylococci* (pathogenic organisms), were enumerated in the lafun samples and *Salmonella spp*. (food infection and food intoxication organisms) detection test was also conducted. The results obtained from microbiological analysis of LAB lafun checking the indicator organisms mentioned were

satisfactory. Thus, the selected LAB starters have a great potential in their use as starter cultures to produce lafun for human consumption.

An unprocessed cassava plant contains potentially toxic levels of a cyanogen called linamarin. Thus, the World Health Organisation (WHO) set a safe limit of 10 mg kg^{-1} to which a proper processing of cassava should effectively reduce cassava's total cyanide content. All lafun samples produced with selected LAB cultures in this study are safe for consumption with respect to this safe limit for cassava flour. This was not the case with the market samples used as the control as they contained an average of 34.9 mg kg^{-1} of total hydrogen cyanide. The knowledge of the total cyanide content of a food sample helps to calculate the amount of food that can be consumed per meal.

Generally, the use of fortified cassava (IBA011371) resulted in flavour, physical and nutritional improvement in lafun. This cassava variety performed best when combined with *L. mesenteroides* with respect to nutritional content and the level of desired metabolites in lafun. The combination is therefore recommended for further research leading to scale up at both small and medium scales.

6.2 Future work

6.2.1 Sensory descriptive analysis and consumer studies

An exciting avenue for further research would be to discriminate and describe both qualitative and quantitative sensory factors of lafun produced with the treatments in this study by trained panels. By the descriptive analysis, it is possible to pinpoint differences in products as an impact of LAB starter used with the cassava varieties, identify drivers of consumer hedonic responses, and establish relationships between sensory and chemical characteristics. In addition, it would be important to understand

consumer behaviour regarding the adoption of lafun from fortified cassava as it is visually very different due to the carotenoids present.

6.2.2 Storage study on lafun from the fortified cassava varieties

Research should be carried out to elucidate the keeping quality of lafun of the fortified variety used in this work. Lafun, like any other flour, is hygroscopic, which could lead to a reduction in nutrient levels or their bioavailability. Proper lafun storage and preservation would help to preserve its quality and nutritional value. It is crucial to determine what technique is suitable to prevent the growth of spoilage and pathogenic microorganisms as well as retarding the oxidation of lipids that causes rancidity since lafun is practically stored in ambient temperature. The prevention technique will rely more on lafun packaging materials and methods such as atmosphere within and without the package. Shelf life study is therefore recommended and should include determination of chemical, physical, and microbiological properties to determine the the period over which lafun remains stable.

6.2.3 Societal and economic impact

In future study on cassava, the economic impact of moving to a more industrialised process that will incorporate the LAB starters and use a better drying procedure should be investigated. This will increase the utilisation of cassava, benefit cassava producers greatly, and improve the economy of the society at large. The research would also assess the impact on cottage producers and the potential of improvement in lafun security in the food chain, reduction in cassava roots wastage and an increase in employment opportunities.

Appendices

Table A 1: Biochemical profile of 41 strains on the apiwebTM identification software

	Serial number of biochemical strips																						
LAB ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
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Key: Biochemical strips

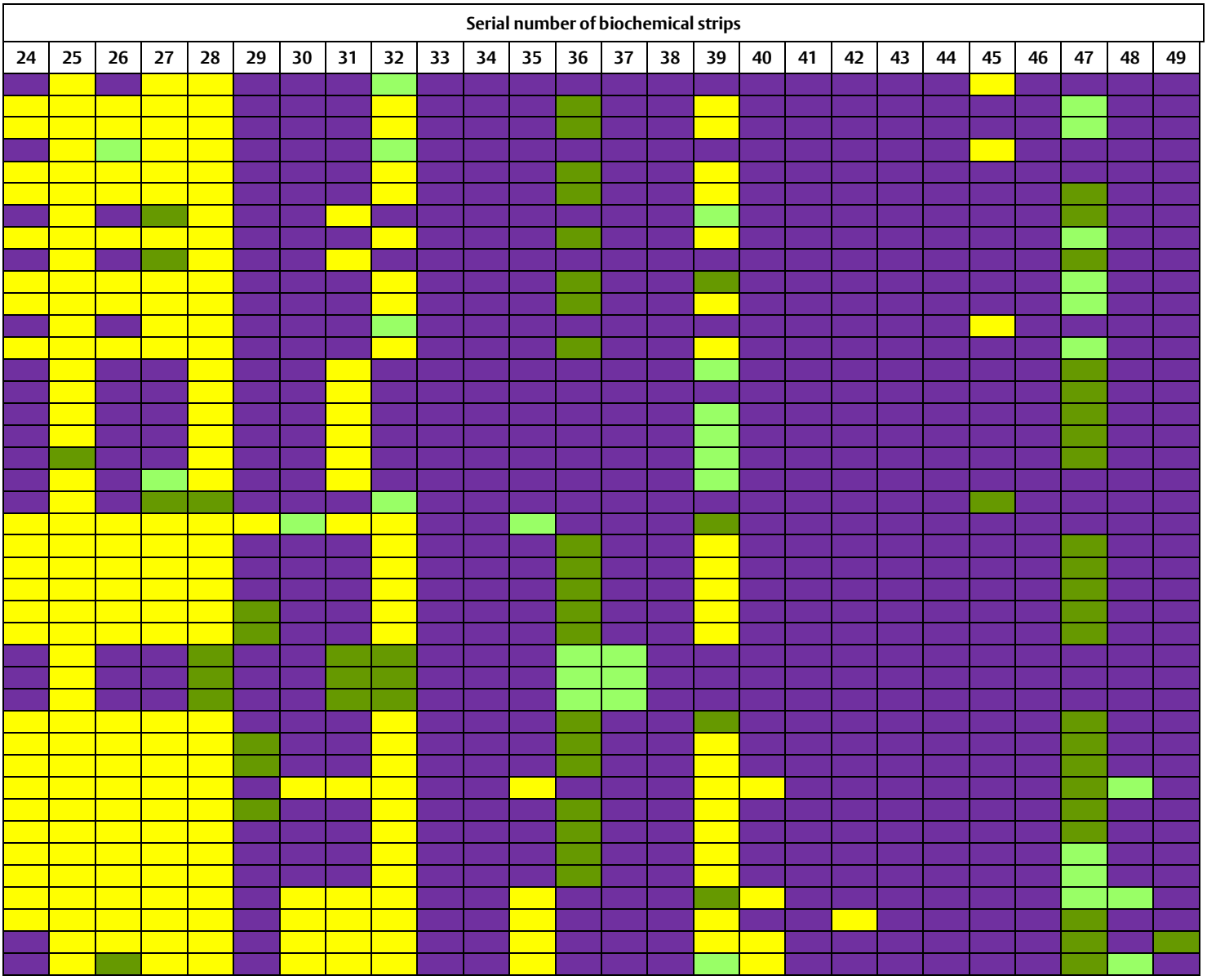
1 GLYcerol
2 ERYthritol
3 D-ARAbinose
4 L-ARAbinose
5 D-RIBose
6 D-XYLose
7 L-XYLose
8 D-ADOnitol
9 Methyl-βD-Xylopyranoside
10 D-GALactose
11 D-GLUcose
12 D-FRUctose
13 MaNnosE
14 L-SorBosE
15 L-RHAmnose
16 DULcitol
17 INOsitol
18 D-MANnitol
19 D-SORbitol
20 Methyl-αD-Mannopyranoside
21 Methyl-αD-Glucosamine
22 N-AcetylGlucosamine
23 AMYgdain
24 ARButin
25 ESCulin ferric citrate
26 SALicin
27 D-CELiobiose
28 D-MALtose
29 D-LACtose
30 D-MELibiose
31 D-SACcharose
32 D-TREhalose
33 INUlin
34 D-MeLeZitose
35 D-RAFfinose
36 AmiDON (starch)
37 GLYcoGen
38 XyLiTol
39 GENtiobiose
40 D-TURanose
41 D-LYXose
42 D-TAGatose
43 D-FUCose
44 L-FUCose
45 D-ARabitoL
46 L-ARabitoL
47 potassium GlucoNaTe
48 potassium 2-keto Gluconate
49 potassium 5-keto Gluconate

Negative

Positive

Weak positive

Very weak positive



Key: LAB ID

1- *L. plantarum*
2- *L. lactis*
3- *L. lactis*
6- *L. plantarum*
14- *L. lactis*
15- *L. lactis*
17- *L. collinoides*
18- *L. lactis*
19- *L. collinoides*
20- *L. lactis*
22- *L. lactis*
23- *L. plantarum*
24- *L. lactis*
31- *L. collinoides*
32- *L. collinoides*
33- *L. collinoides*
34- *L. collinoides*
35- *L. collinoides*
36- *L. collinoides*
37- *L. plantarum*
38- Unidentified
39- *L. lactis*
40- *L. lactis*
41- *L. lactis*
42- *L. lactis*
43- *L. lactis*
46- Unidentified
47- Unidentified
48- Unidentified
49- *L. lactis*
50- *L. lactis*
51- *L. lactis*
52- *L. mesenteroides*
53- *L. lactis*
54- *L. lactis*
55- *L. lactis*
56- *L. lactis*
57- *L. mesenteroides*
58- *L. brevis*
59- *L. mesenteroides*

Scientific publications

Oral presentation

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(a) 15th International Congress of Bacteriology and Applied Microbiology, the 15th International Congress of Mycology and Eukaryotic Microbiology and the 17th International Congress of Virology of the International Union of Microbiological Societies (IUMS), held at Singapore, Singapore, 17-21 July 2017: International Sharing, Cooperation and Open Discussions on Recent Advances and Perspectives in Microbiology Research.

Paper Presented: Production of Non-Volatile Flavour Compounds during Fermentation of Cassava by Selected Lactic Acid Bacteria

Poster presentations

A. O. Fawole, K. A. G. Karatzas and C. C. Fagan

Department of Food and Nutritional Sciences, University of Reading, PO Box 226, Whiteknights, Reading RG6 6AP, UK

(a) International Association for Food Protection (IAFP) European Symposium 2019 held at the La Cité des Congrès de Nantes in Nantes, France, 24-26 April 2019: Food Safety.

Paper Presented: Impact of Starter Culture and Cassava Variety on the Compositional and Rheological Properties of Lafun

(b) 26th Food Microbiology and Hygiene Conference (International ICFMH Conference) held at Freie University of Berlin, Berlin, Germany, 3-6 September 2018: Biodiversity of Foodborne Microbes.

Papers Presented:

- Impact of frozen storage on microbial quality of prepared cassava
- Determination of best process conditions for maximum flavour yield and safety in lafun produced with selected Lactic acid bacteria (LAB)

(c) 18th IUFoST World Congress of Food Science and Technology 2016, held at Dublin Ireland, 21-25 August 2016: Greening the Global Food Supply Chain through Innovation in Food Science and Technology.

Paper Presented: Selection of Starters for and Stabilisation of Flavour-enhancing Process in Lafun Production by Lactic acid bacteria

IUMS 2017 SINGAPORE
International Union of Microbiological Societies

University of Reading

PRODUCTION OF NON-VOLATILE FLAVOUR COMPOUNDS DURING FERMENTATION OF CASSAVA BY SELECTED LACTIC ACID BACTERIA

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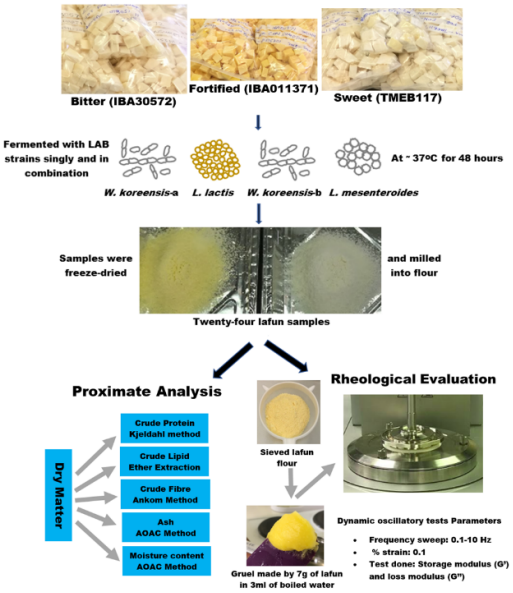
Impact of Starter Culture and Cassava Variety on the Compositional and Rheological Properties of Lafun

A. O. Fawole | K. A. G. Karatzas | C. C. Fagan

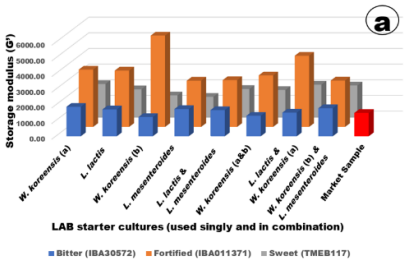
Introduction

Cassava has become a staple crop in Africa, consumed throughout society. There is also growing demand for traditional foods which are convenient and of high quality (1). Lafun, a fermented cassava flour, is one such product with industrial potential (2). However it is still produced by spontaneous fermentation resulting in variable quality and safety. Therefore, there is a requirement to optimise and standardise the production of lafun. The objective of this study is to determine the impact of selected lactic acid bacterial (LAB) cultures and cassava varieties on the nutritional and physical properties of lafun.

Methods



Results and discussion



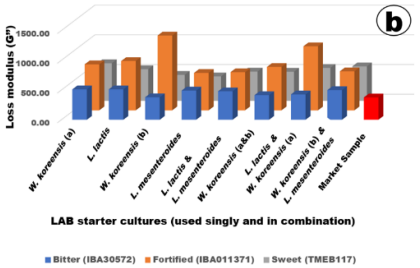


Fig 2 a and b: Rheological properties of optimised lafun and market sample

Table 1: Proximate analysis of optimised lafun samples

LAB Culture(s)	Cassava variety	% MC	% Ash	% C. Protein	% C. Lipid	% C. Fibre	% Digestible Carb
Weissella koreensis	Bitter (IBA30572)	1.37 (±0.06)	1.27 (±0.09)	1.11 (±0.02)	0.40 (±0.00)	3.13 (±0.05)	92.72
	Fortified (IBA011371)	2.33 (±0.47)	3.83 (±0.24)	2.99 (±0.15)	2.20 (±0.00)	3.46 (±0.10)	85.18
	Sweet (TMEB117)	0.90 (±0.14)	1.54 (±0.18)	1.37 (±0.02)	0.20 (±0.00)	2.45 (±0.26)	93.53
Lactococcus lactis	Bitter (IBA30572)	1.17 (±0.24)	1.47 (±0.19)	0.86 (±0.02)	0.50 (±0.14)	3.62 (±0.25)	92.39
	Fortified (IBA011371)	1.87 (±0.66)	3.80 (±0.28)	2.89 (±0.05)	1.70 (±0.14)	6.63 (±0.11)	83.11
	Sweet (TMEB117)	1.03 (±0.90)	1.53 (±0.19)	1.39 (±0.07)	0.40 (±0.00)	2.12 (±0.07)	93.53
W. koreensis (b)	Bitter (IBA30572)	0.94 (±0.38)	1.27 (±0.09)	1.50 (±0.05)	0.40 (±0.00)	1.89 (±0.00)	94.01
	Fortified (IBA011371)	2.33 (±0.47)	3.63 (±0.05)	2.91 (±0.06)	1.50 (±0.14)	4.75 (±0.08)	84.87
	Sweet (TMEB117)	1.50 (±0.71)	1.37 (±0.05)	1.34 (±0.17)	1.09 (±0.13)	3.69 (±0.22)	91.02
Leuconostoc mesenteroides	Bitter (IBA30572)	1.47 (±0.19)	1.47 (±0.19)	0.93 (±0.04)	0.59 (±0.00)	3.21 (±0.21)	92.33
	Fortified (IBA011371)	1.80 (±0.28)	4.37 (±0.05)	3.08 (±0.23)	1.40 (±0.00)	7.43 (±0.18)	81.93
	Sweet (TMEB117)	0.73 (±0.09)	2.43 (±0.33)	1.50 (±0.05)	0.60 (±0.00)	3.44 (±0.05)	91.30
L. lactis and *Leuconostoc mesenteroides*	Bitter (IBA30572)	1.23 (±0.61)	1.74 (±0.10)	1.05 (±0.01)	0.49 (±0.14)	3.43 (±0.01)	92.06
	Fortified (IBA011371)	1.71 (±0.42)	3.92 (±0.12)	2.91 (±0.05)	1.70 (±0.14)	6.66 (±0.13)	83.10
	Sweet (TMEB117)	1.10 (±0.14)	2.20 (±0.28)	1.58 (±0.10)	0.49 (±0.14)	4.14 (±0.09)	90.49
W. koreensis (2 strains)	Bitter (IBA30572)	2.17 (±0.24)	0.83 (±0.24)	0.79 (±0.06)	0.40 (±0.00)	2.30 (±0.07)	93.51
	Fortified (IBA011371)	2.20 (±0.28)	3.63 (±0.05)	2.62 (±0.09)	1.00 (±0.00)	3.85 (±0.25)	86.71
	Sweet (TMEB117)	1.97 (±0.52)	3.00 (±0.00)	1.84 (±0.15)	0.40 (±0.00)	2.19 (±0.01)	90.60
W. koreensis and L. lactis	Bitter (IBA30572)	1.67 (±0.00)	1.00 (±0.00)	0.60 (±0.25)	0.50 (±0.14)	2.06 (±0.05)	94.18
	Fortified (IBA011371)	1.81 (±0.27)	3.92 (±0.11)	2.53 (±0.33)	0.80 (±0.00)	3.31 (±0.07)	87.63
	Sweet (TMEB117)	1.27 (±0.09)	3.17 (±0.23)	1.81 (±0.01)	0.70 (±0.14)	3.67 (±0.13)	89.39
W. koreensis (b) and *Leuconostoc mesenteroides*	Bitter (IBA30572)	2.50 (±0.24)	0.83 (±0.24)	0.76 (±0.13)	0.50 (±0.14)	2.25 (±0.06)	93.16
	Fortified (IBA011371)	2.37 (±0.05)	3.47 (±0.19)	2.76 (±0.10)	0.79 (±0.00)	3.70 (±0.04)	86.91
	Sweet (TMEB117)	1.17 (±0.24)	1.73 (±0.09)	1.64 (±0.12)	0.60 (±0.00)	3.63 (±0.19)	91.23
Spontaneous fermentation	Market sample	10.50 (±0.24)	1.33 (±0.00)	0.87 (±0.22)	0.49 (±0.14)	1.43 (±0.04)	85.37

- There is a significant difference in both storage and loss moduli between most of the optimised lafun and the market sample
- Lafun from fortified cassava roots had the best viscoelastic gel-like behaviour
- Overall, the ash, protein, fibre and lipid contents of the lafun produced using Fortified (IBA011371) cassava were expectedly the highest

Conclusion

Taken together, the findings of this work suggest that standardisation of lafun production using the identified LAB strains in control fermentation of the fortified cassava will enhance both its physicochemical properties and quality.

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DEPARTMENT OF FOOD AND NUTRITIONAL SCIENCES

University of Reading

Impact of frozen storage on microbial quality of prepared cassava

A. O. Fawole | K. A. G. Karatzas | C. C. Fagan

Introduction

Cassava (Tapioca) had been cultivated in South America for nearly eight millennium before becoming an import to all other continents (2). It is cultivated mainly for its starchy roots but its leaves are eaten in some parts of Africa by humans and in parts of Asia by animals (3). Initially tagged 'the food of the poor,' cassava has become a 21st-century multiuse crop that can overcome climate change challenges (4). Though mostly a tropical crop, it has some similarities with potato regarding its inedible starchy state when fresh, bland flavour when cooked, and similar methods of preparation. However, the main challenge facing the widespread usage of cassava roots by processors is because it is bulky and highly perishable, deteriorating within three to four days of harvest raising the post-harvest losses of cassava to above 50% (1). Cassava, as a result of this, needs to be stored in an intermediate form prior to further processing. The objective of this study was to determine the impact of blast freezing at -20°C and holding at -18°C for three months on the microbial quality of cassava roots to be subsequently used for lafun production.

Methods

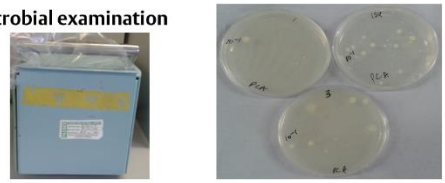
Sample collection

Bitter cassava (30572); Fortified cassava (1011371) and Sweet cassava (Isunikankiyan) were sourced from International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

- **Samples preparation**

- **Blast freezing (-20°C for 6h) ; Holding in Freezer (-18°C)**

- **Microbial examination**



Results and Discussion

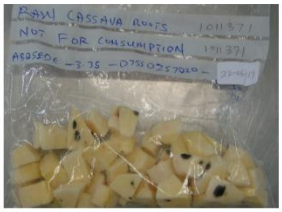
There was a decrease in the total microbial count from 30 days to 90 days in the varieties used which agrees with the findings of Wijesinghe and Sarananda (2010) except bacterial count in Isunikankiyan that increased (table 1). Physical examination regarding colour, odour and texture was consistent throughout the period of study and the frozen cassava roots were suitable and used for lafun production.

Table 1: Microbial counts of frozen cassava in 3 months

Sample	Microbe	30 days (cfu g^{-1})	60 days (cfu g-1)	90 days (cfu g-1)
30572	Bacteria	$3.0x10^4$	$1.0x10^4$	$1.8x10^3$
	Yeast	$6.9x10^3$	$3.0x10^3$	$1.0x10^3$
1011371	Bacteria	$6.0x10^2$	$8.0x10^2$	$3.0x10^2$
	Yeast	$5.0x10^2$	$1.0x10^3$	0
Isunikankiyan	Bacteria	$3.0x10^3$	$3.0x10^3$	$4.0x10^3$
	Yeast	$9.0x10^2$	$1.0x10^3$	0

Conclusion

Freezing at -18°C has the potential to maintain cassava quality for at least 3 months prior to further processing .



Contaminated cassava roots in 2°C for three months (Control)

Frozen cassava stored for three months

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DEPARTMENT OF FOOD AND NUTRITIONAL SCIENCES

University of Reading

Determination of best process conditions for maximum flavour yield and safety in lafun produced with selected Lactic acid bacteria (LAB)

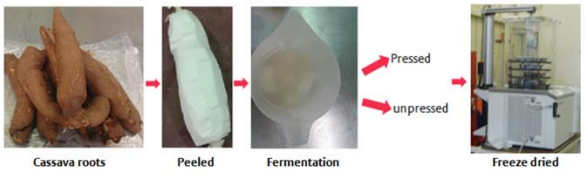
A. O. Fawole | K. A. G. Karatzas | C. C. Fagan

Introduction

Lafun is a fermented cassava flour produced mainly in the Republic of Nigeria, and the Benin Republic (1; 2). Lately, lafun has lost consumer acceptability due to its traditional production method that has not been optimised based on lack of advances in process technology. The overall aim of this study is to improve the flavour profile as well as ensuring that products have consistent quality regarding nutritional and sensory values.

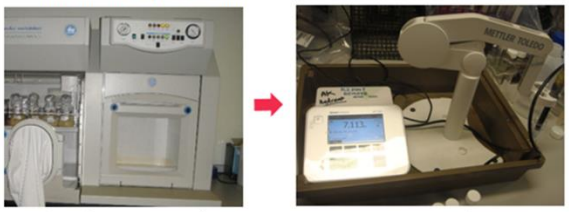
Methods and Experimental Design

- Experiment 1: Effect of pressing fermented cassava roots on flavour compounds quantity



- Experiment 2: Monitoring of quantity of non-volatile flavour compounds during fermentation

- Experiment 3: Rate of acidification monitored 12 hourly during cassava fermentation to produce lafun



Results and discussion

Table 1: Pressing effect on flavour compounds quantity in lafun

Cassava sample	Citric (mgg^{-1})	Malic (mgg^{-1})	Succinic (mgg^{-1})	Lactic (mgg^{-1})
Pressed	0.3±0.00	1.0±0.00	1.3±0.00	6.8±0.00
Unpressed	2.2±0.01	3.3±0.01	3.4±0.01	10.7±0.00

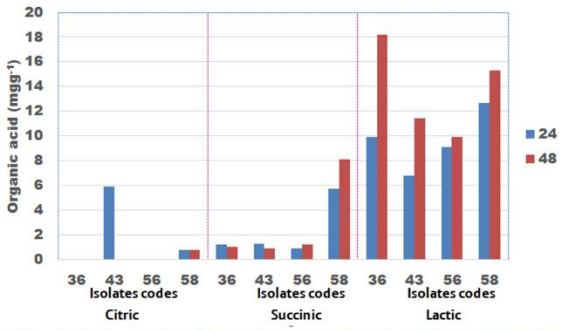


Fig. 1: Levels of citric, succinic and lactic acids produced after 24 and 48 hours fermentation by 4 LAB isolates (36, 43, 56 and 58)

Table 2: Acidification rate during cassava fermentation by 4 LAB isolates (36, 43, 56 and 58)

Treatment	pH at 0h	pH at 12h	pH at 24h	pH at 36h	pH at 48h
36	7.65	5.07	4.98	5.26	5.44
43	7.66	5.21	4.70	4.50	4.42
56	7.43	5.94	4.87	4.88	4.92
58	7.66	5.85	5.02	4.74	4.63

- The concentrations of flavour compounds were reduced substantially when pressing was applied.
- Overall, the quantity of flavour compounds increased over the period of 24h
- *L. collinoides*-36 had the fastest rate of acidification followed by *L. lactis*-43

Conclusion

- Though 'pressing' shortened drying time from 96h to 24h, it caused losses of flavour compounds.
- Fermentation up to 48 hours promotes high flavour profile.
- Fast rate of acidification gives opportunity to standardise the method for production of lafun that does not exist at the moment, in particular the standardisation of the starter culture for sensory and safety characteristics.

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Selection of starters for and stabilisation of flavour-enhancing process in lafun production by lactic acid bacteria

A. O. Fawole | K. A. G. Karatzas | C. C. Fagan

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Introduction

Various fermented cassava products are available which include garri, fufu, and lafun [1]. Despite being a traditional fermented food in the South-west, Nigeria, lafun lacks consumers appeal, and it is usually perceived by the new generation as an undesirable food that has an offensive odour [2]. This is mainly due to its processing which has not been standardised. In particular the starter culture needs to be standardised to control the desirable acidic sour flavour of lafun. There is a need to optimise process conditions in conjunction with starter culture to enhance product quality regarding stabilising flavour production along with high sensory acceptability. The objective of this study is to show the effect of temperature on the proximate content of lafun and identify the lactic acid bacteria involved in its production.

Methods

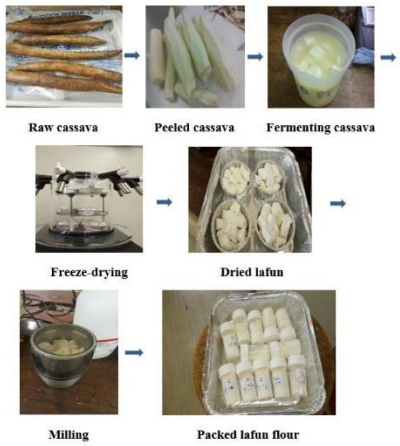

Figure 1: Flow diagram of lafun production

- Proximate analysis
- Colonial morphological study
- LAB identification on API fermentation galleries

Results

Figure 2: Traditional sun-dried versus freeze-dried

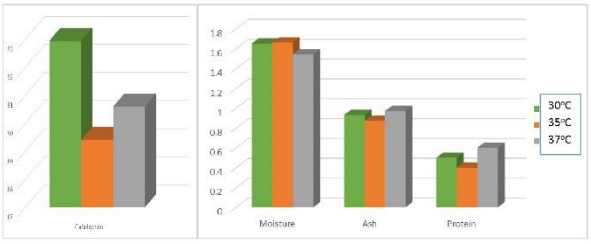

Figure 3: Proximate analysis of lafun samples as relates to fermentation temperature

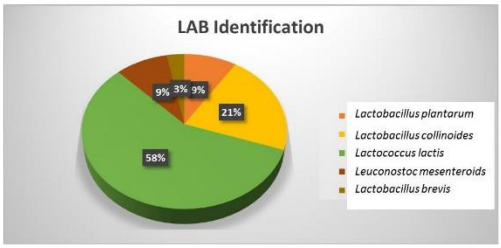

Figure 4: Percentage occurrence of LAB in fermented cassava

- The pH of the sample reduced from 7.47 to 4.30, 7.47 to 4.37 and 7.53 to 5.08 at 30°C, 35°C and 37°C respectively in 48h
- The lactic acid bacteria (LAB) isolated increased from $8.30X10^3$ cfu ml^{-1} at 6h to $1.74X10^8$ cfu ml^{-1} at 24h.
- The majority of isolates were *Lactococcus lactis* (Fig. 4)

Conclusions

Production of lafun was achieved using regulated cabinets for fermentation and Freeze dryer for drying. The moisture content went as low as 1.54% showing the effectiveness of freeze-drying method over the conventional sun drying that leaves lafun at about 13% [3]. Lactic acid bacteria is a major microbiota during fermentation of lafun and this was shown with the increase of colony count from $8.30X10^3$ cfu ml^{-1} at 6h to $1.74X10^8$ cfu ml^{-1} at 24h. Basically, the lafun produced at 35°C had an average proximate content (Fig. 3).

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