STORAGE PROTEINS IN CASSAVA AND SWEET POTATO ROOT TISSUES, QUALITY AND EFFECTS OF PROCESSING AND OTHER COMPOUNDS

By

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To the Faculty of Washington State University:

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STORAGE PROTEINS IN CASSAVA AND SWEET POTATO ROOT TISSUES, QUALITY AND EFFECTS OF PROCESSING AND OTHER COMPOUNDS

Abstract

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Cassava is a staple food in Africa with deficiency in protein content. Evaluation of protein fortified cassava was studied. Sporamin, the major protein of sweet potato, has potential for cassava fortification. One objective of this study was to evaluate effects on sporamin due to different parameters involved in cassava physiological postharvest deterioration (PPD). Model systems were designed to determine sporamin modification. Sporamin did not suffer any major degradation by PPD components: scopoletin (a hydroxycoumarin), cassava phenolic extract, peroxidase and hydrogen peroxide. Oxidation of methionine was observed at 0 and 24 h incubation with or without oxidation factors by matrix-assisted laser desorption/ionization (MALDI). Polymerization of sporamin A and B was observed by presence of a high molecular weight band in SDS-PAGE.

Physico-chemical parameters for cassava flours with zeolin, sporazein, sporazein

plus pro-vitamin A and pro-vitamin A, and a wild type sample were measured. Wild type flour had the greatest pasting temperature (90.4 °C) while pro-vitamin A fortified flour had the greatest final viscosity (269.94 RVU).

Fufu, a fermented cassava product, was prepared from flour with and without *Lactobacillus plantarum* 6710 starter for 96 h while monitoring lactic acid bacteria. Physico-chemical parameters of the intermediate uncooked products (wet fufu and fufu flour) were evaluated. Use of *Lactobacillus plantarum* allowed a rapid acidification of wet fufu reaching maximum acidity at 72 h. Thus, the use of starter cultures lead to a rapid acidification of the product inhibiting the growth of spoilage and pathogenic bacteria.

In general, fermentation caused a significant decrease of the protein content in the fortified samples.

Sensory evaluation of cooked fufu by a trained panel showed a significant influence of flour source in most attributes evaluated: brown color intensity, stale and fermented aromas; and hardness, adhesiveness and springiness ($p \leq 0.05$). However, no significant differences in the "fufu" aroma descriptor were found. Similar volatile compounds were identified in all cooked fufu samples.

Sporamin should be considered for protein fortification of cassava without expecting a major damage to the protein due to PPD. Based on "fufu"aroma sensory evaluation, fortified cassava flours offer consumers a product similar to that commonly consumed.

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DEDICATION

This dissertation is dedicated to my beloved husband, **Christopher M. Hartshorn**, who appeared in my life at the best moment. You have been with me every step of the way, through good times and bad. Thank you for your endless patience, faithful love, and support, especially throughout this tremendous endeavor. Thank you for helping me to succeed and instilling in me the confidence that I am capable of doing anything I put my mind to. I could not have done this without you.

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CHAPTER I
GENERAL INTRODUCTION

Cassava is the major source of calories for many in underdeveloped countries (Sheffield and others 2006). However, a cassava-based diet does not provide complete nutrition. My proposed field of research is the evaluation of the influence of post-harvest physiological deterioration and processing techniques, such as fermentation, on the protein characteristics of fortified cassava flour (*Manihot esculenta* Crantz). New cultivars are being developed in concert with our research that will provide complete and balanced nutrition in a readily marketable and higher yielding crop (Fargette and others 1996). The improved traits include enhanced bioavailable levels of zinc, iron, protein, vitamin A, and reduced quantities of toxic cyanogenic glycosides, improved post-harvest durability, and improved resistance to viral diseases. Fortified protein and pro-vitamin A cassava flours were used in this study. In addition, field and human feeding trials need to be conducted in close collaboration with scientists around the world to demonstrate efficacy and reduce malnutrition by delivering more nutritious, higher yielding, and more marketable cultivars of cassava.

The major world staple crops are seeds, with cereals and legumes in particular consumed in vast quantities. In addition, five types of root/tubers are considered to be staples. These five species accounted for almost 99% of the total world production of root/ tuber crops in 1999 with potato being the most important (approximately 45%) followed

by cassava (26%), sweet potato (20%), yams (6%) and taro (1%) (Shewry 2003).

Cassava (*Manihot esculenta* Crantz) is the staple root crop of over 800 million people (Nassar and others 2007) in the humid tropics and ranks sixth in terms of overall global crop production (Han and others 2001). The significant growth in urban populations in developing countries is bringing with it associated changes in consumer demand, especially for food and feed products, opening up new opportunities and challenges for the production of cassava. The opportunities lie in:

a) fresh and processed cassava for human consumption,

b) fresh and dried roots for animal feed,

c) flour and starch for food and non-food uses.

Cassava growers face many challenges in realizing these opportunities. Challenges include the provision of effective support services for farmers, processors and traders to improve competitiveness, through technological and organizational innovations in production and marketing (Best and others 2006). According to Fauquet and Tohme (2004), the application of new technologies in coordination with conventional plant breeding, can make cassava much more productive, nutritious, and profitable to grow.

Cassava processing operations in Nigeria have been described at 5 levels of capacity. These levels are household (or cottage), micro, small, medium and large.

Household level processing typically does not employ any outside labor. Most of the processed products are consumed and a small amount is sold to satisfy additional needs.

At the micro processing capacity, one or two laborers may be employed to process a variety of cassava products. Nigeria has few cassava processors in this level.

The small and medium processing operations typically employ three to ten workers and are small in number at present.

Large scale operations are defined as enterprises employing 10-30 or more laborers. Large-scale operations have the capacity for large processing scale processing that includes starch and syrup products (FAO 2004).

Cassava is produced mostly by smallholders on marginal and sub-marginal lands in the humid and semi-humid tropics. It is efficient in carbohydrate production, adapted to a wide range of environments and tolerant to drought and acidic soils. In Africa, an estimated 70 million people are dependent on cassava as a primary source of food contributing over 500 kcal per day per person. Cassava has a reputation as a poor person's crop, a crop of last resort (FAO 2010).

A major limitation is the potential toxicity of cassava roots or products. According to Nweke and Bokanga (1994), cassava produces the cyanogenic glycosides linamarin and lotaustralin. In Africa, improperly processed cassava is a major problem. It is associated with a number of cyanide-related health disorders, particularly among people who are already malnourished (Maziya-Dixon and others 2007). The cyanogenic glycosides begin to break down upon harvest into hydrocyanic acid, acetone and glucose by the action of the enzyme linamarase. The presence of hydrocyanic acid is easily recognized by a bitter taste. Hydrolysis of the glycoside by the enzyme can be accelerated by soaking the roots in water, by crushing, cutting or heating (FAO 1977).

As mentioned by Cabral and Carvalho (2001), systematic information on the biology, physiology and biochemistry of the cassava root is somewhat limited, resulting in a

lag in relation to other fields such as molecular genetics and plant transformation . Literature from the 1970's, referred to the search of efficient methods that help to determine the proteins in potato, which is different in origin but similar in function and appearance to a root tuber that is cassava.

Proteins are an important group of biomacromolecules, which are involved in a variety of physiological functions, such as a dietary source of amino acids in foods (Gorinstein and others 1999). Cassava is cultivated mainly for its edible storage root, which contains 85% starch and only 1-2% protein (Sheffield and others 2006). Cassava products faces a number of important constraints, including yield loss to biotic and abiotic stress, poor nutritional quality, and rapid deterioration of the harvested roots. Regarding the nutritional content of cassava roots, some improvements have been made. Stupak (2008) increased the protein content in cassava roots via the expression of an artificial storage protein (ASP1) based on zein from maize and sporamin from sweet potato.

A significant improvement of cassava root protein and amino acid content was achieved by gene transfer and expression of storage proteins such as ASP1, sporamin and zeolin. In addition, as a component of the BioCassava Plus Program (www.biocassavaplus.org), research has been developed in order to enhance the protein and the pro-vitamin A (beta-carotene) content of the cassava roots through biotechnology. Thus, researchers at the Danforth Center (St. Louis, MO, US) found that approximately 90% of the carotenoids that are present in the engineered roots are as all-trans-β carotene, which has the highest pro-vitamin A value of the different forms of carotenoids (Cahoon and others 2008).

Numerous processing methods have been devised, including sun drying, soaking and fermentation followed by drying or roasting, to reduce the toxicity and at the same time convert the highly perishable fresh roots of cassava into stable products (Vasconcelos and others 1990). Among the fermented products, fufu is a favorite cassava-based entrée popular in many parts of West Africa (Uyoh and others 2009). This is produced by steeping in water peeled cassava cubes. The fermented cassava is sieved to remove the fibers and allow to sediment. After sedimentation, the water is decanted and the sediment is dried, milled and the fufu flour is obtained (Sanni and others; Tomlins and others 2007). Oyewole and Odunfa (1989) reported that fermentation process during fufu production caused an increase of calcium and a decrease of magnesium, potassium and iron concentrations.

Fufu is a widely consumed fermented product throughout Africa. Complaints of processors are mainly related to the short shelf life of the fermented wet paste and ready to eat forms of fufu. Therefore, a simple drier that can operate with or without electricity has been developed in some areas to produce dried fufu and prolong its shelf life (FAO 2004).

When the majority of cassava consumption is in a fresh form, in 2001 in Nigeria, approximately 16 percent of cassava root production was utilized as an industrial raw material. Ten percent was used as chips in animal feed, 5 percent was processed into a syrup concentrate for soft drinks and less than 1 percent was processed into high quality cassava flour used in biscuits and confectionary, dextrin, pre-gelled starch for adhesives, starch and hydrolysates for pharmaceuticals, and seasonings. Cassava, a food staple with industrial potential, is finding a new place in the diets of both rural and urban populations. Cassava is no longer only grown by the poor. There is a need for new cassava processing

technologies that will contribute to extend its shelf life and increase yield of the mentioned commodity (FAO 2004).

Studies exist dealing with inclusion of a sweet potato protein by genetic manipulation into cassava to improve nutritional quality. In this study we attempted to elucidate the implications of the changes of cassava due to post-harvest physiological deterioration (PPD) on the major protein from sweet potato, sporamin. Therefore, model systems were designed in order to study the effects of the factors involved in PPD on sporamin. We hypothesized that oxidative condition as they occur during PPD will alter sporamin.

In addition, the present study characterized the cassava flours provided by the International Laboratory for Tropical Agricultural Biotechnology (ILTAB) in St. Louis (MO) named zeolin, sporazein, sporazein plus pro-vitamin A and pro-vitamin A fortified cassava. Moreover, processing technologies such as fermentation of the cassava flours were investigated to understand their potential as vehicles for the delivery of more nutritious cassava products. We hypothesized that fortified cassava flours and addition of *Lactobacillus plantarum* starter for cassava flour fermentation will affect physico-chemical characteristics and sensory attributes of fufu products.

This processing approach is suitable for large scale production of shelf stable products and promises a more stable nutritious cassava product.

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CHAPTER II
LITERATURE REVIEW

1. Cassava

1.1. Origin and distribution

Cassava (*Manihot esculenta* Crantz) has its origin in the Amazon in South America where it has been grown by the indigenous Indian population for at least 4000 years. The word cassava comes from "casabi", the name given by the Arawaks Indians to the root. It is known as "yuca" in Spanish, "manioc" in French, "mandioc" In Portuguese; "cassave" in Dutch and "maniok" in German. After the discovery of the Americas by Europeans, traders took the crop to Africa as a potentially useful food crop; later it was taken to Asia to be grown as a food security crop and for the extraction of starch (FAO 2000; Blagbrough and others 2010; Akinpelu and others 2011).

Cassava is a monoecious perennial shrub of the family Euphorbiaceae (Figure 1), cultivated mainly for its starchy roots, which grows to 1–4 m in height over the course of a year (Royer and others; Sayre and others 2011). It is one of the most important food staples in the tropics, where it is the fourth most important source of energy (Hillocks and others 2002). Worldwide, it is the sixth most important source of calories in the human diet (El-Sharkawy 2004) and the third most important in the tropics after rice and maize (Prakash 2006). Millions of people depend on cassava in Africa, Asia and Latin America. Given the crop's tolerance to poor soil and harsh climatic conditions, it is generally cultivated by small farmers and it is the basis for food security especially in those regions

prone to drought and with poor soils (Hillocks and others 2002; Prakash 2006). Other characteristics of this crop are its efficiency in producing carbohydrates and its high flexibility with respect to the timing of harvest (Prakash 2006).

Cassava can be propagated from stem cuttings (or stakes, 15-30 cm long) or sexual seed. Propagation from true seed occurs under natural conditions and is widely used in breeding programs. Sprouting and adventitious rooting occur after 1 week when propagation occurs by cuttings under favorable conditions (Hillocks and others 2002; El-Sharkawy 2004). The cassava roots are generally harvested 7-24 months after planting, depending on several conditions such as cultivar, use and growing conditions (El-Sharkawy 2004).

Figure 1. Cassava plant

1.2. Morphology

The morphological characteristics of cassava are highly variable, which indicate a high

degree of interspecific hybridization that implies the crossing between different species of the same genus (Jovanka 2004). There are many cassava cultivars in several germplasm banks held at both international and national research institutions. The cassava genotypes are usually characterized on the basis of morphological and agronomic descriptors. Among morphological descriptors, the following were defined as the minimum or basic descriptors that should be considered for identifying a cultivar: apical leaf color, apical leaf pubescence, central lobe shape, petiole color, stem cortex color, stem external color, phyllotaxis length, root peduncle presence, root external color, root cortex color, root pulp color, root epidermis texture, and flowering. It is difficult to make a precise description of the morphological descriptors due to the large number of cassava genotypes and the influence of environmental conditions (Hillocks and others 2002).

1.3. Nutritional profile

The principal parts of the mature cassava plant expressed as a percentage of the whole plant are leaves 6 percent; stem 44 percent and storage roots 50 percent. The roots and leaves of the cassava plant are the two nutritionally valuable parts, which offer potential as a feed source. The cassava storage root is essentially a carbohydrate source. Its composition shows 60-65 percent moisture, 20-31 percent carbohydrate, 0.2-0.6 percent ether extracts, 1-2 percent crude protein and a comparatively low content of vitamins and minerals. The root carbohydrate is made up of 64-72 percent starch. However, the roots are rich in calcium and vitamin C and contain a nutritionally significant quantity of thiamine, riboflavin and nicotinic acid that are lost during processing (Nweke and others

2002). The starch content increases with the growth of the storage roots and reaches a maximum between the 8th and 12th month after planting. Thereafter, the starch decreases and the fiber content increases (Tewe and Lutaladio 2004). Cassava starch contains 17 percent amylose and 83 percent amylopectin, while major cereal starches have a typical ratio of 28:72, respectively (Swinkels 1985). Cassava roots also contain sucrose, maltose, glucose and fructose in limited levels. The raw starch of the cassava root has a digestibility of 48.3 percent while cooked starch has a digestibility of 77.9 percent (Tewe and Lutaladio 2004).

Cassava root is a poor source of protein. However the quality of cassava root protein is fairly good as far as the proportion of essential amino acid as a percentage of total nitrogen is concerned. The protein of cassava tuber is rich in arginine but low in essential amino acids including methionine, lysine, tryptophan, phenyalanine, tyrosine, cysteine and cystine (Tewe and Lutaladio 2004; Blagbrough and others 2010; Falade and Akingbala 2011). Only about 60 percent of the total nitrogen is derived from amino acids and about one percent is in the form of nitrates, nitrites and hydrocyanic acid. The remaining 38-40 percent of the total nitrogen remains unidentified. Peeling results in the loss of part of the valuable protein content of the root because the peel contains more protein than is found in the root flesh (Tewe and Lutaladio 2004).

1.3.1. Protein deficiency

There is a long-standing debate on the nutritional value of cassava because it contains only 1 or 2 percent protein and is low in minerals and some essential vitamins. Stephenson and others (2010) found that cassava intake is inversely

correlated with protein intake and diet quality in children from Nigeria and Kenya. Those individuals who consume cassava as a staple do not compensate for the very low protein: energy (P:E) ratio of cassava by including in their diet amounts of protein-rich foods such as legumes and fish. Stephenson and others (2010) commented that the increase of the P:E ratio (4-8 fold) using genetic modification will help to reduce the deficiency of protein intake of great part of the population who mainly consume cassava.

1.3.2. Cyanogenic content and toxicity

The magnitude of cyanogen varies greatly among different plant species. Cassava contains cyanogenic glucosides in the form of linamarin (95%) and, to a lesser extent, lotaustralin (5%) (Nweke and others 2002; Blagbrough and others 2010; Montagnac and others 2009). The amount of cyanogenic glucosides varies with the part of the plant, its age, variety, and environmental conditions as soil moisture and temperature (Okigbo 1980; Oluwole and others 2007).

Traditional methods of cassava processing (sun drying, heap fermentation, production of farinha in Brazil and gari in West Africa), and cooking usually remove most of the free cyanide, thus reducing toxicity (Nweke and others 2002). Giraud and others (1992) demonstrated the ability of certain strains of lactic acid bacteria (LAB) to break down cassava linamarin that would lead to the production of a standardized and non-toxic foodstuff. Other studies also confirmed that detoxification of cassava gave the best results when a starter culture consisting of *Lactobacillus plantarum* was used (Holzapfel 2002). The possibility of chronic

toxicity is associated with the habitual consumption of large quantities of cassava products or with consumption of insufficiently processed cassava when the diet is deficient in protein (Nweke and others 2002; Blagbrough and others 2010).

Damaging cassava roots brings linamarin, synthesized from the amino acid valine, into contact with the cyanogenic enzyme linamarase to produce acetone cyanohydrins that decompose either by hydroxynitrile lyase (HNL) or spontaneously to release acetone and HCN (Figure 2) (Montagnac and others 2009; Blagbrough and others 2010).

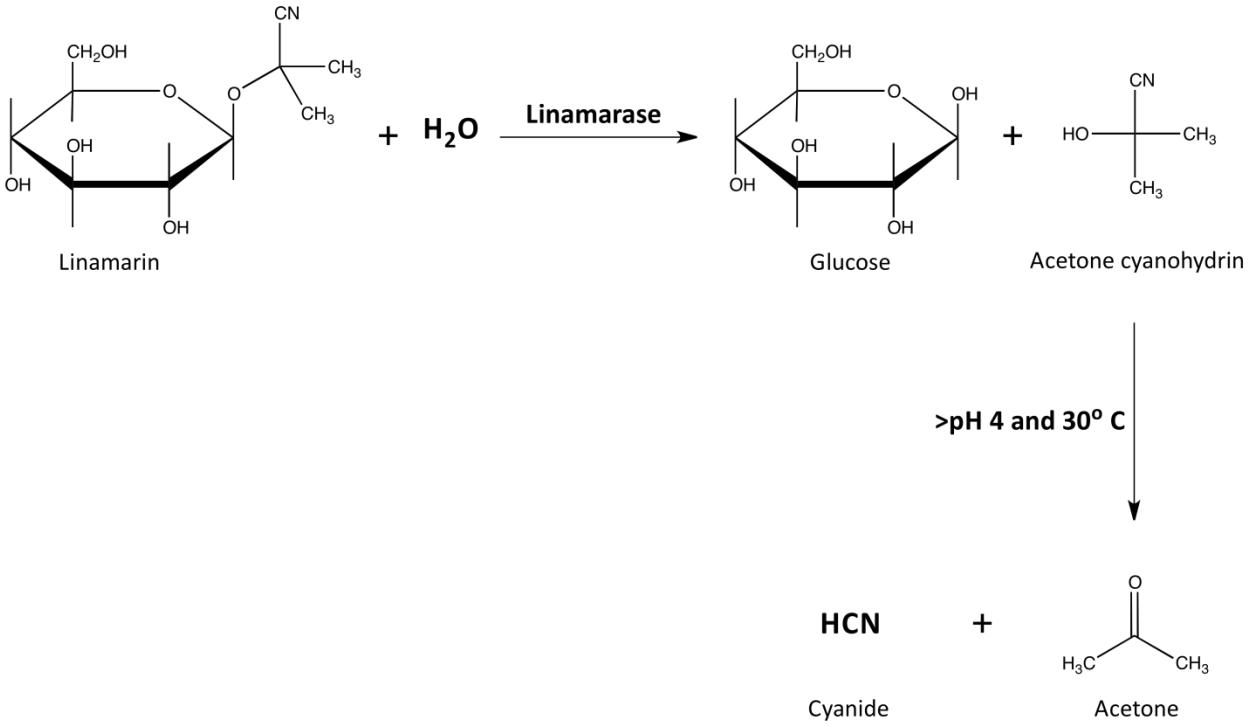

Figure 2. Cyanogenesis process in cassava

The presence of cyanide in cassava has caused a global scare as to the safety of cassava and its products for human and animal consumption. The concentration of the glycosides varies considerably among varieties and also with climatic and cultural conditions. The normal range of cyanoglucosides content in fresh roots is from 15-400 ppm calculated as mg HCN/kg fresh weight but occasionally varieties with very low HCN content of 10 mg/kg or very high HCN content of 2 000 mg/kg have been reported. Cassava is often classified as "bitter" or "sweet" according to the amount of cyanide present. Therefore, there are cultivar dependent differences in root cyanogen levels. Total root linamarin levels range between 100 and 500 mg linamarin/kg fresh weight for low- and high-cyanogenic cultivars, respectively. However, no cassava cultivars lack cyanogenic glycosides. Several studies have shown that bitterness or sweetness could not be highly correlated with the level of cyanogenic glucosides (White and others 1998; Tewe and Lutaladio 2004; Oluwole and others 2007).

In long-term trials, the carry over effect of cyanide, particularly for gestating animals, can be deleterious as placental thiocyanate transfer occurs in gestating pigs consuming cassava-based feeds with a HCN level of 500 ppm. However through proper processing, cyanide levels of less than 50 ppm can be obtained particularly in sundried samples (Tewe and Lutaladio 2004). Studies on thiocyanate values in human serum and urine samples indicate that conversion of cyanide to thiocyanate is a significant pathway in the metabolism of HCN (Vetter 2000).

Currently, safety limits for cyanide in cassava is 10 ppm (or 10 mg/kg dry weight). However, levels below 100 ppm are considered safe in cassava chips and pellets imported

into the European Union (EU) from Indonesia and Thailand for feeding of different classes of livestock. Hydrocyanic acid levels above 250 ppm will rarely be encountered in practical cassava-based rations (Tewe and Lutaladio 2004). Intoxication because of high consumption level of unprocessed cassava has resulted in cases of goiter and cretinism in iodine deficient regions, tropical ataxic neuropathy, and konzo, a permanent paralysis of the legs (McMahon and others 1995; Blagbrough and others 2010).

1.4. Production

Cassava is cultivated in many tropical countries situated in the equatorial belt, between 30° north and 30° south of the equator, which attests to its adaptability to a wide range of ecosystems (Prakash 2006). The current global cassava production is around 250 million tons, of which Africa accounts for half (51%), while Asia and Latin America produce 35% and 14%, respectively (Figure 3) (FAO 2009). Around 60% of global production is concentrated in five countries Nigeria, Brazil, Thailand, Indonesia and the Congo Democratic Republic (Prakash 2006). Nigeria is the world's leading producer. Strong domestic investment in the sector assisted by good weather is likely to propel Ghana's cassava output to new heights, nearing 15 million tons in 2011. Food security drives and favorable growing conditions could also yield strong gains in Angola, Mozambique and the United Republic of Tanzania.

In Asia, cassava production increased by 6 percent to 83 million tons in 2011. The industrial utilization of cassava in the form of alcohol and ethanol has been the main driver of the sizeable expansion in the crop's cultivation throughout the region, amounting to

almost 60 percent in the past decade. Many sectors, principally in Southeast Asia have benefited from the allocation of additional land for cassava and from subsidies and mandatory ethanol-gasoline blending requirements (FAO 2011).

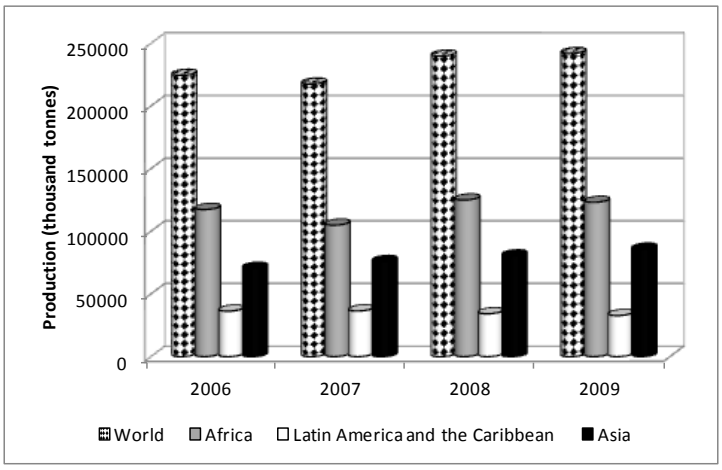

Figure 3. World Cassava Production 2006-2009

Source: FAO (2009)

1.5. Consumption

Most cassava utilization is in the form of food – fresh roots (Figure 4) and processed products such as flour and fermented meal preparations – and is largely concentrated in Africa. Both cassava roots (an important source of carbohydrates) and leaves (source of proteins and minerals) are suitable for consumption (Prakash 2006). The consumption of cassava (mostly in the form of fresh roots and basic processed products) continues on an upward trend in Sub-Saharan Africa. Per capita food availability could rise by 4 kg to around 113 kg per year. Measures to promote domestic cassava flour over imported cereals, either for direct consumption or through blending, remain active throughout the world and constitute an important determinant in boosting cassava food consumption. For

instance, Brazil mandates the inclusion of 10 percent cassava flour in wheat flour and it is estimated that 50 percent of the country's cassava crop is utilized in such blending. Though several major producing countries in West Africa, especially Nigeria, have also promoted this initiative, many have fallen short of enforcement, owing to the limited availability of cassava flour (FAO 2011).

Figure 4. Cassava storage roots

1.6. Commercialization

World trade in cassava products, excluding trade among EU countries stands at about 12% of global production. The bulk of world trade in cassava is in the form of pellets and chips for feed (60%) and the balance mostly in starch and flour for food processing and industrial use. The fresh root is commercialized in a very small amount due to the product bulkiness and perishable nature (Prakash 2006).

In Africa, the majority of cassava produced (88%) is used for human food, with over 50% used in the form of processed products. Animal feed and use for starch are only minor uses of the crop. In the Americas, animal feed is far more important, accounting for approximately one-third of consumption, and human food represents only 42% of

production. Starch also represents an important use of cassava in South America. The situation in Asia is greatly influenced by the export of cassava chips by Thailand to the European community for use as animal feed (Hillocks and others 2002).

Continued supply problems in Thailand, the world's largest exporter, which gave rise to record quotations (estimated price Jan-Oct 2011: US$ 80/ton of Thai cassava root) combined with the improved competitiveness of maize, have contributed to a subdued global market for cassava products in 2011. International cassava trade is being increasingly driven by industrial demand of the product and quality requirements in terms of high starch content (30 percent or more) that are guiding procurement decisions. With problems in sourcing competitive and quality raw material from Thailand, international buyers have begun to source cassava products from elsewhere in the region, especially Cambodia and Vietnam. Global imports of cassava chips and pellets continue to be driven by the need to fulfill capacity in the rising alcohol sector (including ethanol), mostly in China (FAO 2011).

1.7. Approaches on genetic transformation of cassava

After years of development, the cassava plant regeneration system has been fully developed based on somatic embryogenesis, shoot organogenesis from cotyledons and somatic embryos and friable embryogenic calli providing the necessary elements for the genetic transformation of cassava. This technology has gradually matured and substantial progress has been made in validating gene function and transgenic breeding. The

commonly used methods for the genetic transformation of cassava include *Agrobacterium*-mediated gene delivery and particle bombardment (Liu and others 2011).

With the development of cassava biotechnology, the use of functional genomics and genetic engineering to solve the problems associated with the germplasm enhancement of cassava can play a major role in promoting cassava cultivation and industrial application all over the world. To date, improved traits of cassava achieved by transgenic technology include virus resistance, improved nutritional quality, reduced cyanide content and improved biomass (Liu and others 2011).

1.8. Enhanced nutritional quality

As mentioned above, cassava storage root is very low in protein content. Increasing the protein content can improve the dietary and nutritional balance of people who rely mainly on cassava. The BioCassava Plus project team has developed a number of transgenic cassava lines with value-added traits such as improved protein content, approximately fourfold increase, and improved vitamin A, iron, and zinc contents (Abhary and others 2011; Liu and others 2011).

Abhary and others (2011) genetically modified cassava using the patatin promoter to direct transgenic expression of zeolin in the tuberous roots. Zeolin is a fusion product between phaseolin, the major storage protein in common beans (*Phaseolus vulgaris*), and a truncated γ-zein protein from maize (*Zea mays*). The next phase of the BioCassava Plus program focuses on bringing products to the farmer and consumer and includes stacking

traits in farmer-preferred cultivars, testing those products in the field, ensuring their safety and stability, and educating consumers (Sayre and others 2011).

1.9. Cassava starch properties

Cassava starch, also called tapioca starch, is a major starch source that can be easily extracted and isolated. Starch granules in storage tissues can vary in shape, size and composition. Cassava starch has a particular property related to its high single stage swelling and peak viscosity, but the swollen granules lack ability to retain the swollen structure and collapses instantaneously. Thus, there is a weak nature of the inter and intra molecular hydrogen bonding of the starch granules. In this respect, cassava starch differs from starches isolated from sources such as wheat, maize, potato, etc. (John and Raja 1999). Nwokocha and others (2009) determined cassava starch was not better in withstanding processing conditions compared to cocoyam due to cassava starch higher peak viscosity and low setback temperature. In consequence, cassava thickening characteristics are poorer than cocoyam starch.

Regarding the polymers present in most starches, a linear molecule termed amylose and a highly branched polymer amylopectin, potato (21%) and tapioca starch (17%) have much lower amylose content as compared to 28% for maize and wheat starch. The linear starch chains (amylose) have an increased tendency to line up into bundles or micelles (retrogradation). Tapioca starch contains amylose molecules which have a substantially higher degree of polymerization (DP) than the amylose molecules of corn and wheat starch. Tapioca amylose has a DP-range average of 3000 similar to potato starch (Swinkels 1985).

As reported by Abraham (1993), cassava starch has been prized for its clarity of paste, neutral flavor, light color and reasonably good adhesive strength; although is less stringy and less cohesive as cited by Swinkels (1985). Besides the starch derivatives and food products, cassava starch commercial and industrial applications include the manufacture of adhesives and alcohol and sizing of paper and textiles.

1.10. Cassava processing

In many parts of the world, cassava is consumed fresh, directly after boiling, along with spices and salt, or mixed with several other vegetables. Several food commodities are processed from or substituted with different levels of cassava. Some of the objectives of cassava processing are to produce a more stable product, reduce the content of toxic compounds to innocuous levels, and diversify the food and other uses of cassava among others. These products can be either unfermented or fermented foods or drinks (Table 1) and they can be boiled, steamed, fried, roasted, baked, dried, fermented. Cassava juice or pulp can be consumed so it can be in a liquid or solid form. Some of these products are limited to specific countries while others are available in several regions of the world. Sweet varieties of cassava are consumed raw after removing the skin and rind. Cassava can also be cooked and consumed in slices or mashed. In Brazil, cassava may be boiled in sugar syrup to produce a sweet food or with other vegetables to make a soup called sancocho or cocido (Falade and Akingbala 2011).

Table 1. Some traditional unfermented and fermented cassava products

Unfermented Cassava Products	Fermented Cassava Products
Unfermented fufu	Cassava beer
Cassava reep/tucupay	Kasili
Landang or cassava rice	Farina
Macaroni	Oyek
Cassava pudding	Gatot
Sago wafers	Chick-wangue
Wafer	Tape-flour tape
Cassava rava and pregelatinized cassava starch	Cassava bread
	Cassava flour
Wayana cassava cakes	Cassava starch
Fried cassava chips	Fufu
Tapioca	Lafun
Cassava chips and pellets	Akyeke (or attieke)
Cassava flour	Agbelima
Cassava starch	Gari

Source: Falade and Akingbala (2011)

2. Fermented foods

Fermentation can be defined as the biochemical modification of primary food products brought about by the action of microorganisms and their enzymes. Fermentation is intentionally carried out to enhance the taste, aroma, shelf-life, texture, nutritional value, and other properties of food (Motarjemi and others 1996).

Fermented foods are food substrates that are invaded by edible microorganisms whose enzymes, particularly amylases, proteases and lipases hydrolyze the polysaccharides, proteins and lipids to non-toxic products with characteristic sensory properties appealing to the consumers (Steinkraus 1997).

Fermented foods intended for infants or young children are mainly lactic-acid-fermented cereals (e.g. maize, sorghum, millet), root crops (e.g. cassava), milk, and, to a lesser extent, fish, meat, or vegetables. The fermentation process can be accelerated by the addition of a starter culture of lactic acid bacteria (LAB), either by adding already fermented material (back-slopping) or a carrier material to which LAB attach (Motarjemi and others 1996).

2.1. Benefits

There is considerable evidence that lactic acid fermentation inhibits the survival and multiplication of a number of bacterial pathogens. Lactic acid fermentation has been associated with the reduction of certain naturally occurring toxins in plant foods. For example, cyanide levels in cassava are reduced in several traditional fermented cassava products, although this reduction may be principally the consequence of endogenous enzymes rather than microbial activity (Steinkraus 1983).

Because of limited shelf life, cassava is often processed by boiling, roasting, drying, or fermentation (Gouado and others 2008). The most popular processing method, especially for the varieties which are high in the cyanogenic glucosides, is by fermentation. Fufu is one of the major foods forms of cassava fermentation. Fufu is prepared by fermenting peeled cassava cubes, sieving and drying to obtain the dried product (fufu flour). Fufu is reconstituted by stirring in boiling water to form a dough and eaten with sauces or soup (Achi and Akomas 2006).

According to Brauman and others (1996), fermentation reduces potentially toxic endogenous cyanogens present in variable concentrations (300 to 500 ppm), and improves palatability for further processing. During the grating of the root, endogenous linamarase in the root is released, contacting the linamarin which is broken down. However, the endogenous linamarase is not sufficient to break down all the glucoside in the root and traces are usually carried into the gari, another fermented cassava product (Kostinek and others 2005). These traces have been implicated in causing diseases such as goiter and tropical ataxic neuropathy when cassava foods are consumed for prolonged periods in conditions of low protein diet. It is now well accepted that the flavor of fufu is produced by the fermentative activities of lactic acid bacteria and yeasts (Oyewole and Odunfa 1990; Sobowale and Oyewole 2008). During the consequent fermentation, roots are softened, endogenous cyanogenic glycosides (linamarin and lotaustralin) are degraded, and characteristic flavors develop (Brauman and others 1996). Lactic acid bacteria are assumed to subsist on the small amounts of free sugars present in cassava roots. It is presumed that they will grow faster and produce more of the components which flavor the foods more rapidly, when the quantity of sugars in the cassava mash is increased through their own amylolytic activities (Okafor and others 1998). Okafor and others (1998) determined that fermentation is more rapid and linamarin (cyanide) removal more effective when liquid is not squeezed out of the mash.

Fermentation offers a number of advantages including food preservation, improved food safety, enhanced flavor and acceptability, increased variety in the diet, improved nutritional value, improved functional properties and reduction in anti-nutritional

compounds (Kuye and Sanni 1999). Influence of fermentation on the protein content was evaluated in fufu, which is a creamy white fermented paste (Fagbemi and Ijah 2006). In addition, biochemical changes in other cassava products (flour and gari) subjected to *Saccharomyces cerevisae* were also studied (Oboh and Akindahunsi 2003). This study concluded that baker's yeast, a cheap, non-pathogenic and saprophytic fungus efficiently increased the protein content of cassava products.

Some phenolic compounds such as tannins, affect nutritive value of food products by forming a complex with protein, inhibiting digestion and absorption (Oboh and Akindahunsi 2003). Phenolic compounds have been also reported to have negative biological effects such as the inhibition of iron absorption (Rawel and others 2000). In addition, tannins also impart a dull color to processed products, which affects market value (Oboh and Akindahunsi 2003). Oboh and Akindahunsi (2004) found that the tannin contents of baker's yeast-fermented cassava product gari were very low when compared with the tannin content of other cassava products. The tannin content of the fermented cassava product flour was significantly ($p>0.05$) higher than that of gari, indicating that the process of garification could also decrease the tannin content of cassava products. Products could also be considered to be safe with regard to tannin poisoning since the levels reported are far below the critical value of 0.7-0.9%. Considering also the results by Rawel and others (2001) that showed the influence of the reaction of plant phenolics with proteins on their physicochemical properties and in-vitro enzymatic degradation, a better understanding is needed of the interrelated parameters that affect the nutritional value.

2.2. Microorganisms associated with fermentation of cassava

Facultative heterofermentative *Lactobacillus plantarum* and obligate heterofermentative *Lb. brevis* and *Leuconostoc mesenteroides* are an example of the lactic acid bacteria found in cassava fermented products. Lactic acid bacteria have a high impact on the sensory qualities and preservation of cassava. Important sensory properties of fermented cassava are flavor and the visco-elastic properties of the cooked paste (Amoa-Awua and others 1996).

Among other species that have been identified to be responsible for the fermentation of cassava products such as fufu are *Bacillus subtilis, Klebsiella. spp, Candida tropicalis, Candida krusei, Lactobacillus plantarum* and *Leuconostoc* (Falade and Akingbala 2011).

Lactobacillus plantarum was predominant among the lactic acid bacteria, producing ethanol and high concentrations of lactate that acidified the retting cassava juice during fufu processing. Traditional fermentation of cassava involved the use of a mixed microflora, which develops spontaneously, resulting in simultaneous biochemical changes. In some cases, previous batches are saved for further use as an inoculum and reduce fermentation times. As a result, flavor, aroma, and texture of the products vary with season, location, and producer. Until recently, there has been a dearth of information about the microorganisms that can be used as starter cultures for the production of fermented cassava products. There is a need to optimize the fermentation process during the production of indigenous foods. Attempts to improve, upgrade or industrialize cassava fermentations, particularly in Africa would involve the isolation, identification, and production of microbial cultures in order to have an available culture bank for additional fermentations. Such a practice will

enhance the industrial production of traditional fermented foods while supporting the nutritional intake of the population and reduce the expenses on imported foods (Ray and Ward 2006; Falade and Akingbala 2011).

2.3. Lactic acid bacteria and *Lactobacillus plantarum*

Lactic acid bacteria (LAB) are Gram-positive, non spore forming with rod and coccoid morphology, fermentative bacteria that grow anaerobically and are traditionally applied in the conservation of a variety of fermented foods. LAB are microaerophilic, catalase, and oxidase negative, and the pH of cultures is below 4.0 during stationary growth phase (de Vries and others 2006; Todorov and Franco 2010). In addition, LAB compete with other microbes by secreting antagonistic compounds and modifying the microenvironment by their metabolism. The antimicrobial compounds produced by LAB are natural preservatives that can be used as preparations for increasing the shelf-life and safety of minimally processed foods (Niku-Paavola and others 1999).

The largest group of LAB belongs to the genus *Lactobacillus* that comprises more than 50 different species. In many cases, these lactobacilli are also used as starter cultures in industrial and artisanal food fermentation since they contribute to the conservation, flavor, and texture of the fermented food product. While the fermentative conversion of sugars present in the raw materials into lactic acid is their main function, production of anti-microbial peptides, exopolysaccharides and a variety of other metabolites are important properties (de Vries and others 2006; Todorov and Franco 2010).

LAB are grouped into seven taxonomic genera*: Lactobacillus* and *Carnobacterium* (rods) and *Lactococcus*, *Enterococcus, Streptococcus, Leuconostoc, and Pediococcus* (cocci). Depending on its ability to metabolize different carbohydrates, the genus *Lactobacillu*s is divided into 3 groups: Group A (only strict homofermentative species); group B (facultative heterofermentative species); and group C (only strict heterofermentative species). *L. plantarum* belongs to group B that includes facultative heterofermentative species producing lactic acid by the fermentation of glucose. In addition, species of this group also produce acetic acid, ethanol and formic acid (Todorov and Franco 2010).

Lactobacillus plantarum is a heterofermentative, facultative, anaerobic microorganism found in a variety of foods (Table 2). Normally, *L. plantarum* is present on the surface of different plants in low concentrations together with other microorganisms (de Vries and others 2006; Todorov and Franco 2010). It is also found in the human gastrointestinal tract and can be involved in spoilage of certain foods such as meat, wine or orange juice. *Lactobacillus plantarum* has the capacity for the utilization of many different sugars, uptake of peptides, and formation of most amino acids. Moreover, *Lactobacillus plantarum* has been tested for health effects showing reduction in carriage of faecal enterobacteriacea, reduction of certain risk factors for coronary artery diseases, and a dose dependent reduction in the symptoms of irritable bowel syndrome (IBS) (de Vries and others 2006).

The presence of *L. plantarum* in certain foods is important for the sensory characteristics of the final products and this may explain the application of this species as a starter culture for production of fermented vegetables. *L. plantarum* has been reported as the prevalent microorganism associated with the spontaneous fermentation of cassava

starch and the production of organic acids and aromatic compounds (Todorov and Franco 2010).

Table 2. Food products containing *Lactobacillus plantarum*

Product category	Foods
Plants	Olives Cocoa beans Cassava Sauerkraut Togwa Wine
Milk	Stilton cheese Traditional feta cheese Ricotta forte cheese
Meat	Fermented dry sausage Fermented Italian sausage

Source: de Vries and others (2006)

Georgieva and others (2009) also demonstrated the capacity of *L. plantarum* strains isolated from cheese to survive over extended shelf-times at refrigerated temperatures, their viability and growth in the presence of preservatives combined with variable acidifying and coagulation ability and enzyme activity that make the species appropriate for food applications.

2.4. Fermented cassava foods

Fermented cassava foods can be acid or air fermented based on their mode of fermentation:

a) Acid fermented products:

- Fermentation of grated products such as gari, attieke and agbelima.
- Fermentation of soaked products such as alebo, lafun and Nigerian fufu.

b) Air fermented roots:

- Mold growth is encouraged due to slow drying times.
- Mold growth promoted by heaping the roots and covering them with leaves

The processing steps after fermentation allow the production of other products. For example, fermented grated roots can be steamed to produce attieke, toasted to produce gari, or no further processing to obtain agbelima (Westby 1994).

2.5. Fufu

Fufu is an acid-fermented cassava product that is produced through submerged fermentation of peeled cassava roots in water, and traditionally consumed in Nigeria and other West African countries. After steeping, the softened pulp is screened, sedimented, dewatered in cloth bags, cooked and pounded into fufu (Figure 5) (Falade and Akingbala 2011). Fufu is a variant of dried fermented cassava product consumed as cooked dough at homes and eateries with various soups across West African countries where they are identified with different tribal or ethnic names (Shittu and Adedokun 2010).

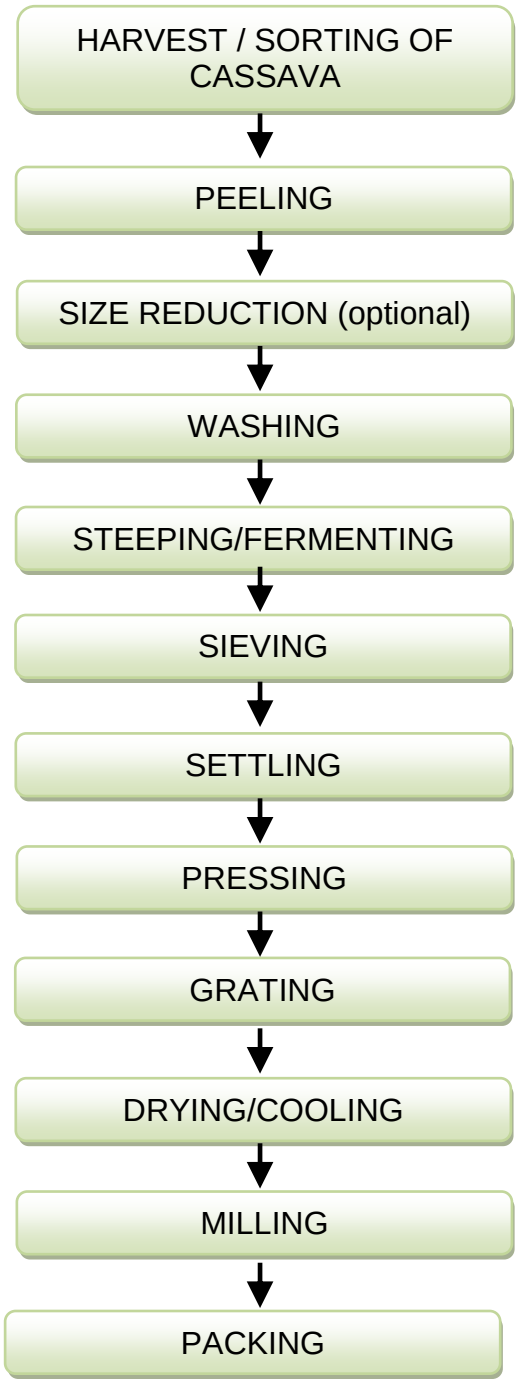

Figure 5. Processing of Fufu flour

Source: IITA (2005)

Fufu is considered by consumers to be of good quality when it has a smooth texture, a characteristic sour aroma and is creamy-white, grey or yellow in color as cited by Tomlins and others (2007). The quality of fufu, however, varies with processors and seasons and this variability has been attributed to various local practices by the processors (Sanni and others 1998b). Fufu is traditionally sold as a wet paste with moisture about 50% that makes it a highly perishable product with a short shelf-life (Tomlins and others 2007).

A practical approach to improving the shelf life and marketability of fufu is drying (Sanni and Akingbala 2000). Reconstitution of fufu flours in hot water has become popular in Nigeria and overseas, thus contributing to improving the livelihoods of rural small-scale cassava processors (Tomlins and others 2007). The drying of fufu at high temperature (60°C) reduced the repulsive odor of fufu but the product was sticky and soft due to acid hydrolysis of starch. In consequence, the quality of the dried fufu was unacceptable when compared with wet fufu (Akingbala and others 1991). Sanni and Akingbala (2000) reported the highest sensory scores for overall acceptability of dried fufu obtained with a rotatory or cabinet dryer. Moreover, the authors stated that those drying methods would allow production on a large scale.

According to Ray and Sivakumar (2009), the microorganisms associated with fufu are *Lactobacillus, Leuconostoc and Streptococcus* [all lactic acid bacteria (LAB)] with *Bacillus subtilis, Enterococcus, Klebsiella and Candida krusei*. During the fermentation, several chemical compounds are formed. These include steam volatile acids (formic, acetic and propionic); non-volatile acids (lactic and succinic); volatile neutral compounds (ethanol,

acetone and isopropanol); non-volatile neutral compounds (glycerol and 2,3-butanediol); butyric, butanediol, acetoin and some gases (Kuye and Sanni 1999).

3. Post harvest physiological deterioration (PPD)

The major constraint for the use of cassava is that farmers, processors and consumers suffer substantial losses during storage of the root tubers because their easy deterioration after harvest. The deteriorative nature is classified into physiological (or primary) and microbial (or secondary) deterioration (Tanaka and others 1983).

Physiological deterioration or p**rimary deterioration** is a complex physiological and developmental process, which starts 24 h to 48 h after harvest (at 20–30 °C) and 65-80% air relative humidity. Iyer and others (2010) determined that the onset of this deterioration occurred very early after wounding during harvest. **Secondary deterioration** or second stage (after 5 to 7 days) involves a microbial decay and is due to microorganisms such as bacteria and fungi (Huang and others 2001). The short shelf-life severely limits the marketing options because it increases the likelihood of losses, marketing costs, and limits access to urban markets to those close to the production sites. The biochemical processes and histological changes of the first stage are known as post-harvest physiological deterioration (PPD) or vascular streaking, which is a blue black discoloration of the xylem parenchyma, followed by general discoloration of the storage parenchyma (Uritani 1999;Buschmann and others 2000a; Van Oirschot and others 2000; Huang and others 2001; Sanchez and others 2006). Keeping roots at 10 °C and 80% air relative humidity delays the onset of PPD by 2 weeks (Sanchez and others 2006). Uritani

(1999) showed that when roots were cut into a block, PPD appeared very soon and at uniform pattern in the outer part of the secondary xylem parenchyma, called B-part on the cut surfaces, as shown (Figure 6).

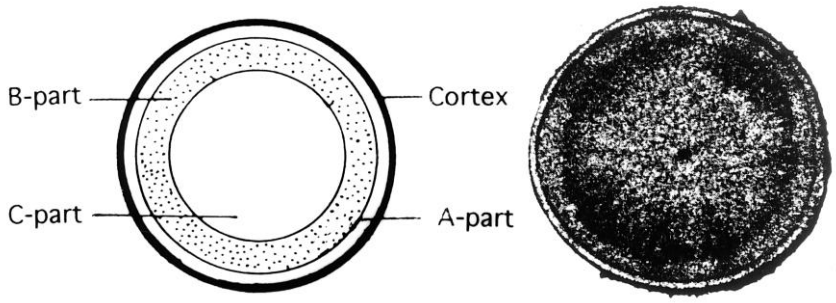

Figure 6. Physiological deterioration of cassava root block, in diameter of 5 to 6 cm

Left: The scheme of the block showing physiological deterioration.
Right: cassava blocks left in the laboratory condition (at 26 - 30 °C and 78 - 92% RH) for 3 days. Physiological deterioration occurred in the B-part corresponding to the outer part of the secondary xylem parenchyma. A-part and C-part are the outer and inner parts of B-part, respectively.

A rapid accumulation of fluorescent compounds observed microscopically in the parenchyma have been described. The fluorescent compounds are hydroxycoumarins, scopolin, scopoletin and esculin. Other compounds that have been identified from cassava roots and that may play a role in PPD are leucoanthocyanins, cyanidin and delphinidin, flavan-3-ols, (+)-catechin and (+)-gallocatechin, and 22 diterpenoid compounds (Buschmann and others 2000b).

This results in brownish discoloration (so called vascular streaking) in the intervening parts between the outermost and inner parts of parenchymatous tissue, thereby rendering the root unpalatable and unmarketable (Uritani and others 1983; Blagbrough and others

2010; Iyer and others 2010). Tanaka and others (1983) assumed that the brownish discoloration that is vascular streaking is due to the oxidation of produced phenolic components such as (+)-catechin by phenol oxidase (frequently implicated in the enzymatic discoloration of tissues) and peroxidase (implicated in the oxidative damage of vegetables during storage). In addition, the authors affirm that the cellular membranes in particular cells of the intervening parts are disorganized or damaged indirectly by some environmental factors. Then, the oxidizing enzymes are allowed to catalyze the oxidation of the phenolic compounds to quinones, which may be polymerized or combined with amino acids and proteins, leading to brown colored insoluble substances. The interactions between enzymes such as peroxidase and compounds such as the coumarin, scopoletin, are largely confined to the vascular tissues where the visible symptoms of deterioration are observed (Blagbrough and others 2010).

With the technological developments in cassava breeding, it is possible to address PPD through genetic improvements using various cassava germplasm or transgenic technologies, thus promoting cassava utilization. Although cassava transgenic technology has been applied in different aspects, no study on the modification of the PPD process has yet been reported (Liu and others 2011).

Morante and others (2010) suggested that tolerance to PPD can be found in different sources and they seem to be acting through different biochemical/genetic mechanisms that require a systematic evaluation for additional sources of tolerance.

4. Peroxidase (POD)

4.1. Definition

Peroxidase (POD) is one of the most important enzymes responsible for polyphenol degradation. This oxidative enzyme which is found in cells of plants, animals, and microbes, is a heme protein with the ferric protoheme group, the prosthetic group, with molecular weights ranging from 30,000 to 150,000 Da (López-Nicolás and García-Carmona 2009). Plant peroxidases are mostly soluble, with lesser proportions membrane associated and covalently bound forms (Damodaran and others 2008) where the most extensively studied is the horseradish peroxidase (Prestamo and Manzano 1993).

4.2. Action mechanism

Peroxidase reacts nonselectively via free-radical mechanisms, using hydrogen peroxide as the electron acceptor. The following equation describes most of the reactions catalyzed by peroxidase (López-Nicolás and García-Carmona 2009):

$$H_20_2 + 2AH_2 \longrightarrow 2H_20 + 2AH$$

Where:

AH_2 and AH represent a reducing substrate and its radical product, respectively.

Polyphenols, indoles, amines and sulfonates have been reported as substrates of peroxidase.

4.3. Peroxidase and its reactions

Plumbley and others (1981) found that discolored cassava roots contained peroxidase activity in the soluble, covalently-bound, and ionically-bound fractions. Extracts from non-discolored tissue lacked activity in the latter fraction and reduced activity in the other two fractions.

POD is able to catalyze at least four types of reactions: peroxidatic, oxidatic, catalytic, and hydroxylation.

a) Peroxidatic reactions.- These occur when phenolic compounds or amines are the hydrogen donors. These reactions require hydrogen peroxide (H_2O_2). With phenolic compounds, the reaction products are either quinones or other polymerized products. The substrates may be guaiacol, resorcinol, aniline, etc. (Whitaker 1972; Matheis and Whitaker 1984a).

b) Oxidatic reactions.- These occur when certain hydrogen donors that require O_2. Ascorbic acid is a substrate example (Whitaker 1972; Matheis and Whitaker 1984a).

c) Catalytic reaction.- It is a very slow reaction where POD converts H_2O_2 to water and oxygen in the absence of a hydrogen donor (Whitaker 1972; Matheis and Whitaker 1984a).

d) Hydroxylation.- In the presence of certain hydrogen donors such as dihydroxyfumaric acid, POD can hydroxylate various aromatic compounds such as tyrosine and phenylalanine (Whitaker 1972; Matheis and Whitaker 1984a).

POD generated *o*-quinones can undergo a variety of reactions in foods, especially with compounds that have amino and sulfhydryl groups. Proteins also contain phenolic tyrosine groups that can serve as substrate for POD in the absence of low molecular weight phenolic substrate proteins (Matheis and Whitaker 1984b). To this regard, Minamihata and others (2011) demonstrated the peroxidase-mediated oxidative tyrosine coupling reaction for site specific cross-linking. In addition, treatment of proteins with H_2O_2 and peroxidase was shown to cause oxidation of tyrosine residues, and form dityrosine and tertyrosine in proteins (Matheis and Whitaker 1984b). In another study, Faergemand and others (1998) found that microbial peroxidase was able to induce formation of polymers in whey proteins but its mode of action differed from the other oxidoreductases evaluated, laccase and monoamine oxidase.

4.4. Applications

Some of the applications of peroxidase include among others the paper pulp industry, synthesis of polymers, production of biofuel, elimination of phenolic contaminants, decolorization and deodorization of various compounds (López-Nicolás and García-Carmona 2009). Peroxidase is inactivated by free radicals during the peroxidative removal of phenol from aqueous solutions. This is known as the suicide-peroxide inactivation of peroxidase that has to be fixed to obtain as a result an efficient treatment of the phenolics. In addition, peroxidase and especially horseradish peroxidase has been used to polymerize phenolic and aromatic amine compounds (Hamid and Rehman 2009).

4.5. Roles of POD and effects on food processing

Physiological roles of peroxidases include formation and degradation of lignin, oxidation of the plant regulator indole acetic acid associated with ripening, and removing cellular H_2O_2 (Damodaran and others 2008). Oxidation and polymerization of phenolic compounds as a result of enzymatic activity of peroxidases may result in the formation of brown pigments. This can take place during the growth of fruits such as dates or during mechanical damage in processing (DeMan 1999). However, this enzymatic browning is still open to question. Peroxidase is used as a blanching indicator due to its heat stability. The heme prosthetic group, glycosylation, four disulfide linkages, and the presence of 2 moles of Ca^{2+} are responsible for this heat stability characteristic. In this regard, the heme prosthetic group can be released during regeneration of peroxidase activity that occurs at pH 5.5-8.0. This group may be involved in catalysis of oxidative reactions that have been implicated in off-flavor in canned fruits and vegetables (Burnette 1977; Damodaran and others 2008). Among other reactions where peroxidase is involved and may impact the quality of foods are the formation of phenoxy radicals that indirectly oxidize lipids and cause the direct oxidations of capsaicin (pungent compound of peppers) (Damodaran and others 2008).

4.6. Methods for determination of POD activity

Quantitative enzyme assays can measure either the total amount of a particular enzyme in units of moles or, more commonly, the catalytic activity associated with a particular enzyme (Wrolstad and others 2005).

The peroxidase activity measurement involves the presence of various hydrogen donors, such as guaiacol, pyrogallol, and mesidine among others. The commonly used assay has guaiacol as substrate. This assay consists on the addition of guaiacol and hydrogen peroxide to a buffer with a pH 6 or 7. Peroxidase is then added to start the reaction. The increase of absorbance is measured at 470 nm as a function of time. The major product of the reaction is tetraguaiacol (Whitaker 1972).

4.7. POD in cassava

Cassava storage roots suffer from a rapid deterioration known as a post-harvest physiological deterioration that can render them unpalatable and unmarketable within 24–72 h after harvest (Sayre and others 2011). The onset of PPD is associated with accumulation of secondary metabolites from the phenylpropanoid pathway, and increases in many enzyme activities, including peroxidase among others. PPD is characterized by a brownish discoloration also called vascular streaking (Tanaka and others 1983) or blue-black discoloration as mentioned by Reilly and others (2007). This vascular streaking is assumed to result from peroxidase mediated oxidation of scopoletin (Tanaka and others 1983; Wheatley and Schwabe 1985). Biochemical localization data indicate that required components of the reaction, namely scopoletin, H_2O_2, and peroxidase activities, are initially localized to the vicinity of the xylem vessels where vascular streaking symptoms occur. Moreover, peroxidase isoforms with activity towards scopoletin are present in the root (Reilly and others 2003).

5. Phenolic compounds

5.1. Definition

Phenolic compounds as food components represent, with more than 6000 identified substances, the largest group of secondary metabolites in plant foods. They are generally characterized by the presence of an aromatic ring bearing one or more hydroxyl substituents. They are usually found in plants bound to sugars as glycosides (Kroll and others 2003).

Plant phenols are present in many foodstuffs as color imparting ingredients. Interest in these compounds is related to their dual role as substrates for oxidative browning reactions and as antioxidants, underlying their impact on sensory and nutritional qualities of fruits vegetables, their role in plant growth and metabolism and, more recently, their demonstrated physiological activity in humans (Kroll and others 2003).

5.2. Structure and distribution of phenols in plants

The major groups of phenolic compounds are distinguished by a number of constitutive carbon atoms in conjunction with the basic phenolic skeleton. The food relevant groupings include the hydroxybenzoic acid derivatives (HBAs) with a general structure C_6-C_1 (Figure 7). The second group is that of those phenolic compounds having the general formula C_6-C_3 (Figure 8), representing a series of trans-phenyl-3-propenoic acids differing in their ring substitution. The third and the largest group are represented by the flavonoids, with more than 4000 compounds characterized. They are also the most widespread and diverse, built upon a C_6-C_3-C_6 skeleton (Figure 9) in which the three-

carbon bridge between the phenyl groups is commonly cyclized with oxygen (Kroll and others 2003).

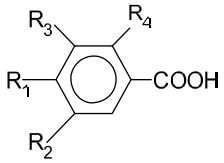

Figure 8. The structure of hydroxybenzoic acid with a general structure C_6-C_1

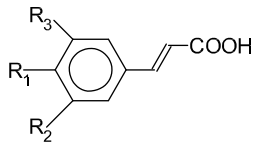

Figure 7. The structure of hydroxycinnamic acid

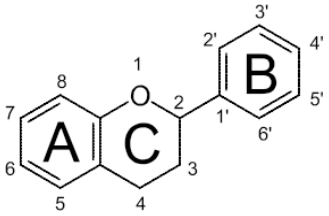

Figure 9. The structure of the flavonoids with a C_6-C_3-C_6 flavone skeleton

5.3. Phenolics in cassava

Hydroxycoumarins occur widely as secondary metabolites in the plant kingdom. They are constitutive in some species, but scopoletin in sunflower and other plants accumulates following mechanical wounding, insect feeding damage, and fungal or bacterial infection. Hydroxycoumarins are involved in the process of defense of the plants against phytopathogens, response to abiotic stresses, regulation of oxidative stress, and probably

hormonal regulation. Scopoletin as a phytoalexin was induced by various fungi in the leaves of *Hevea brasiliensis* (Euphorbiaceae). In cassava roots 1-2 days after harvesting there is an accumulation of hydroxycoumarins: scopoletin, scopolin (its glucoside) and esculetin, esculin (its glucoside) (Figure 10) and due to the 150-200 fold increase of scopoletin concentration, it is assumed that scopoletin is involved in the post-harvest physiological deterioration (PPD) of cassava roots (Blagbrough and others 2010). Hydroxycoumarins are derived from L-phenylalanine by the action of phenylalanine ammonia lyase (PAL), and via E-cinnamic acid (Figure 10) in the phenylpropanoid pathway. In cassava roots, specific phenolic compounds identified are 3-flava-3-ols (gallocatechin, catechin (Figure 11), and catechin gallate), hydroxycoumarins (Figure 12), and the flavones 3-glycosides known as rutin and kaempferol 3-rutinoside (Montagnac and others 2009). The phenolic compounds associated with the development of physiological deterioration have been identified and include scopoletin, scopolin, esculin, proanthocyanidins, (+)-catechin, and (+)-gallocatechin (Iyer and others 2010).

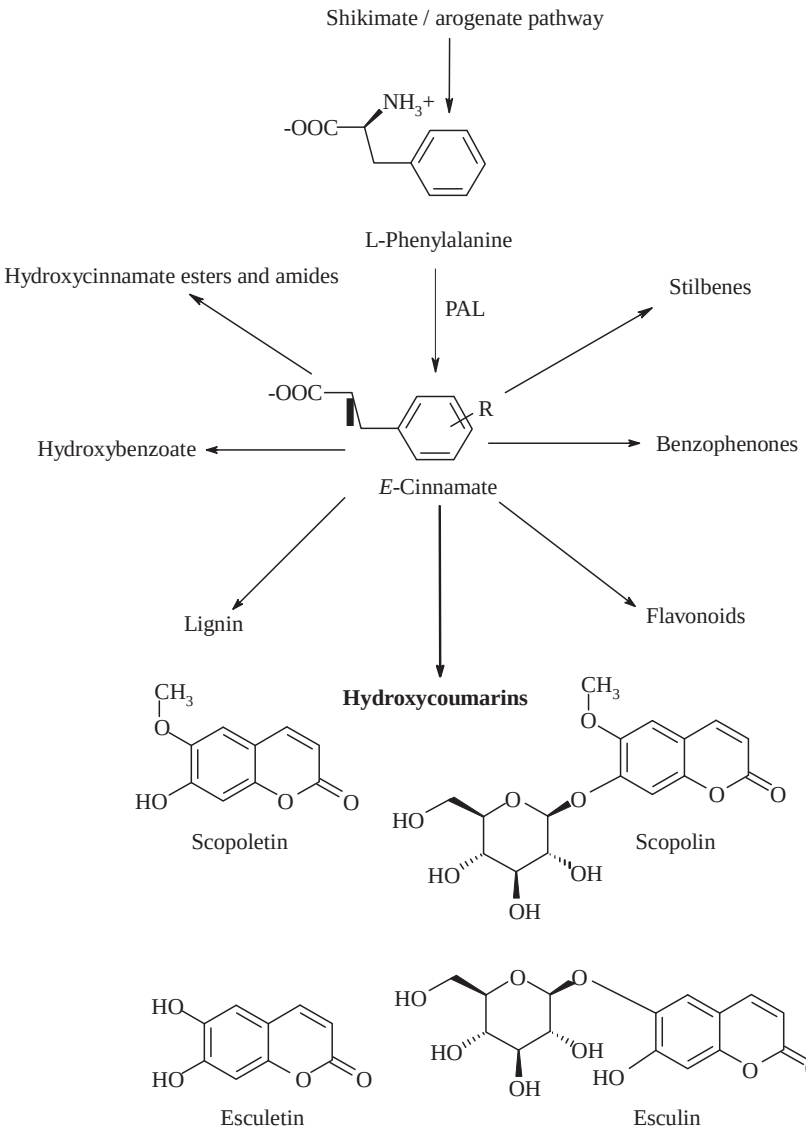

Figure 10. Biosynthetic pathways of phenylpropanoids and hydroxycoumarins

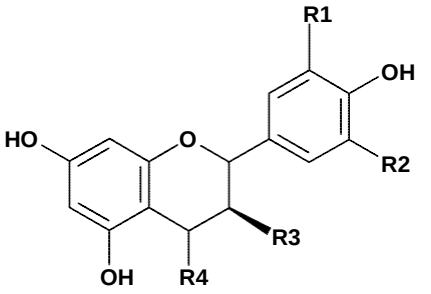

Figure 11. Catechin

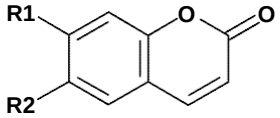

Figure 12. Hydroxycoumarin

5.4. Phenolics in sweet potato

Sweet potatoes (*Ipomoea batatas* L.) are rich in dietary fiber, minerals, vitamins, and antioxidants, such as phenolic acids, anthocyanins, tocopherol and β-carotene (Woolfe 1992). As cited by Teow and others (2007), carotenoids and phenolic compounds, besides acting as antioxidants, also provide sweet potatoes with their distinctive flesh colors (cream, deep yellow, orange and purple). According to Teow and others (2007), the phenolic contents of nineteen sweet potato clones with varying flesh colors ranged from 0.14 to 0.51 mg of chlorogenic acid equivalent/g fresh weight. On the other hand, Khurnpoon and Rungnoi (2012), reported a total phenolic content range from 126.7 – 500.76 μg GAE/g dry matter in white fleshed cultivar and from 96.17 – 477.01 μg GAE/g dry matter in yellow fleshed sweet potatoes.

Some polyphenols such as chlorogenic acid, isochlorogenic acid and related compounds were produced in response of wounding in sweet potato (Uritani 1999). Uritani (1998) determined coumarin production in sweet potato, mainly umbelliferone and scopoletin, as a result of infection by some fungi as *C. fimbriata.* In other study, Thompson (1981) showed the presence of chlorogenic acid and 4-O-caffeoylquinic acid in sweet potato cultivars. Similar results also were found by Truong and others (2007) who determined caffeic acid, chlorogenic acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 3,4-di-O-caffeoylquinic acid in sweet potato cultivars in the United States with chlorogenic acid as the highest in root tissues. In eight sweet potato root varieties, either 5- caffeoylquinic acid or 3,5-di caffeoylquinic acid were most abundant as reported by Jung and others (2011).

5.5. Scopoletin

Scopoletin was identified as 7-hydroxy-6-methoxy coumarin (Figure 10). Scopoletin and its glycoside are ubiquitous in the plant kingdom, occurring in low concentrations in the vascular systems of healthy plants. Scopoletin accumulates around necrotic lesions of virus-infected plants and is subsequently transported to the vascular bundles (Peterson and others 2003).

According to Rickard (1981), biochemical analysis showed many changes in the phenolic constituents of cassava roots occurred following injury but the increased fluorescence was mainly due to the production of scopoletin and scopolin. Wheatley and Schwabe (1985) found that scopoletin plays a very crucial role in the development of physiological deterioration in cassava roots after harvest. While studying the constituents in deteriorated cassava, Bayoumi and others (2010) determined that scopoletin and its glucoside scopolin accumulate in cassava roots during PPD.

6. Proteins and Chimeric proteins

6.1. Zein

Cereal grain evolved to store large amounts of proteins in tightly organized aggregates. Zeins are the major maize proteins (prolamins), accounting for more than 50% of total endosperm proteins. In maize, γ-Zein is the major storage protein synthesized by the rough endoplasmic reticulum (ER) and stored as an insoluble mass in specialized organelles called protein bodies (PB) (Forato and others 2003; Alvarez and others 2010; Mainieri and others 2004). PBs are composed of the products of gene families (Pompa and Vitale 2006).

According to de Virgilio and others (2008) PBs are stable structures naturally formed by certain seed storage proteins within the endoplasmic reticulum (ER). The only known biological function of zeins is as a nitrogen source for embryos during germination. They are important protein sources for human and livestock diets and are used as a biodegradable polymer in industrial applications (Forato and others 2003).
Zeins are classified according to their solubility and sequence homology into α zeins (alcohol soluble and represent more than 70% of total zeins), β (10-15%), γ (5-10%) and δ zeins (traces) (Forato and others 2003). As determined by Wang and others (2003) using MALDI-MS, α-Zein was dominant in the commercial zein with two small peaks at m/z 14 466 and 20 386 . SDS-PAGE, which usually divides α-zein into two groups, based on their migration (Z19 and Z22), showed two bands at 22k Da and 24k Da.

6.2. Phaseolin

Phaseolus vulgaris beans represent an important source of dietary protein for humans in various regions of the world. Phaseolin (globulin, 7S fraction, vicilin family) the major storage protein of *P. vulgaris,* accounts for more than 50% of the total seed proteins. Phaseolin is an oligomeric protein, consisting of three polypeptide subunits α-, β- and γ-phaseolin with molecular weight distribution from 43 to 53 kDa (Romero and others 1975; Yin and others 2011).

The nutritive value of phaseolin is limited by a low sulfur amino acid (AA) content and a high resistance of the protein to enzymatic hydrolysis. Heat treatment is known to improve drastically phaseolin hydrolysis. However, this is not always sufficient to obtain a

satisfactory nutritional value (Marquez and Lajolo 1981; Montoya and others 2008; Yin and others 2011).

6.3. Sporamin

Sporamin represents about 60 to 80% of the total soluble protein of sweet potato (*Ipomoea batatas*) roots. Sporamin is a mixture of related proteins with 20 kDa molecular weight and it probably represents the major component of "ipomoein" that is the sweet potato globulin fraction (Hattori and others 1990). According to Matsuoka and Nakamura (1992) sporamin is a non-glycosylated monomeric protein. Maeshima and others (1985) showed that sporamin accounted for more than 80% of the soluble proteins in sweet potato and that sporamin is not a single species of protein. There are two subfamilies, sporamin A and sporamin B. The ratio of sporamin A to sporamin B content was 2:1 based on electrophoresis analysis and the intensity of the bands. Sporamin A and B have lysine and methionine, respectively as the limiting amino acids. Sun and others (2009) determined that sporamin B had 180 amino acid residues and the molecular weight was about 19242 Da while Maeshima and others (1985) cited by Shewry (2003) reported a mass of 31000 for sporamin A and 22000 for sporamin B.

The limiting amino acid in sporamin A is lysine while methionine, a sulfur amino acid, is the limiting amino acid in sporamin B (Woolfe 1992). Sporamin plays a defensive role against pests in the vegetative tissues of the plants (Shewry 2003). Thus, expressing sporamin in cyanogen-free cassava can help in insect resistance because of the absence of cyanogens and increase the nutritional value of the cassava storage roots (Abhary 2011).

6.4. Zeolin

Seed storage proteins of legumes and cereals are also major food proteins for humans, and, nutritionally, they largely complement each other: Storage proteins from legumes are poor in sulfur amino acids and those of cereal are poor in Lys and Trp (Mainieri and others 2004).

An alternative strategy to enhance protein accumulation in cassava roots was to express chimeric storage proteins that were designed to form protein bodies, which would accumulate in the endoplasmic reticulum. The initial chimeric storage protein tested was zeolin expressed under control of the patatin promoter. Zeolin is a fusion product between phaseolin, the major storage protein in common beans (*Phaseolus vulgaris*), and a truncated gamma-zein protein from maize (*Zea mays*), which directs the fused polypeptide to form stable protein bodies (PBs) within the endoplasmic reticulum (ER) (Mainieri and others 2004; Abhary and others 2011; Sayre and others 2011). Zeolin protein bodies isolated from transgenic cassava storage roots of plant line p600-25 range in size from 3 to 7 mm in diameter (Abhary and others 2011). Because the storage proteins of cereals and legumes nutritionally complement each other, zeolin can be used as a starting point to produce nutritionally balanced and highly stable chimeric storage proteins (Archinti and others 2004). The proline-rich repeat ($PPPVHL_8$) at the N-terminal of the γ-zein protein was shown in previous reports to have an affinity towards the binding luminal protein chaperone (BiP) chaperone in the ER. When this portion of γ-zein is fused to a soluble protein, such as phaseolin, the recombinant product showed the capability to form insoluble PBs (Abhary 2011).

Zeolin contains 89 amino acids of mature γ-zein, starting from the fifth residue after the γ-zein signal peptide. The total number of amino acids is 525, including the 24 residues of the N-terminal signal peptide of phaseolin, which is removed cotranslationally. The zein sequence includes the repeated and Pro rich domains. The zeolin construct was placed under the transcriptional control of the cauliflower mosaic virus 35S promoter (constitutive promoter), used to produce transgenic tobacco (*Nicotiana tabacum*) by Agrobacterium transformation and its behavior was studied in young leaves. Zeolin maintains the main characteristics of wild-type γ-zein: it is insoluble unless its disulfide bonds are reduced and forms ER-located protein bodies (Archinti and others 2004; Mainieri and others 2004; Bellucci and others 2007).

6.5. Sporazein

As cited by Leyva-Guerrero and others (2012), a chimeric protein consisting of sporamin and a truncated maize zein sequence was developed and received the name sporazein.

As reported by Abhary (2011), a combination of coding sequences derived from β- and γ-zeins was organized into a flexible system to be used as a fusion template in order to achieve superior quality, quantity and stability of desired proteins in plants. The required sequences were engineered into an easily cloned technology for expression and accumulation as protein bodies in transgenic host cells. A vector that contained the proline-rich repeat of γ-zein fused to a soluble protein, such as sporamin, and a hydrophobic stretch plus cysteine amino acids from β- zein sequence on the 3`end of the vector was

made and sporazein was obtained. Sporazein is a nutritionally balanced protein of 49.6 kDa in which the zein components drive accumulation of the product to form protein bodies within the endoplasmic reticulum (Fregene and others 2010).

7. Modification of protein by enzymes in the presence of phenolic compounds

Several factors influence the oxidatively modified protein: (i) the rates of formation of various kinds of reactive oxygen species (ROS); (ii) the levels of antioxidant defenses that protect against ROS-mediated protein damage; (iii) the sensitivity of proteins to oxidative attack; and (iv) the repair or elimination of damaged proteins. The abilty of ROS to modify proteins may be prevented by the by the action of various enzymic and nonenzymic antioxidants that can neutralize their prooxidant capacities but there are other factors that can make the damage to occur (Stadtman 2006). Oxidative modification of proteins involves disorders in the primary, secondary and tertiary structures that results in aggregation or fragmentation of the protein molecule based on the amino acid composition (Dubinina and others 2002).

Protein degradation can be influenced by its potential interaction with major (carbohydrates, fats and degradation products) and minor nutrients such as secondary plant metabolites (phenolic compounds) (Rawel and others 2007). Proteins and phenolics interactions can be of five potential types: Hydrogen bonding, Π-bonding, hydrophobic linkage, ionic linkage; and covalent linkage (Felton and others 1992; Kroll and others 2003). In the presence of low molecular weight phenolic compounds, PPO

(polyphenoloxidase) and POD (peroxidase) generated *o*-quinones can react with amino, sulfhydryl , thioether, phenolic, indole and imidazole groups of proteins. In some cases, these reactions eventually lead to protein cross-linking. In the absence of low molecular weight phenolic compounds, tyrosyl groups of proteins can serve as substrates for both PPO and POD, again leading to protein cross-linking (Matheis and Whitaker 1984b).

Kroll and others (2003) cited that the quinone, as a reactive electrophillic intermediate, can readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan moieties in a protein chain. Then a second addition may occur, which leads to formation of cross-linked protein polymers.

Enzyme catalyzed reactions that are known to cross link proteins are those catalyzed by transglutaminase, lipoxygenase, polyphenol oxidase, lysil oxidase and peroxidase (Matheis and Whitaker 1984b). Enzyme-catalyzed browning reactions of amino acids and proteins with oxidized phenolic compounds may cause deterioration and a decrease of nutritional value of food during storage and/or processing. Peroxidases are able to oxidize a wide variety of phenolic compounds (Oudgenoeg and others 2001).

Modification of proteins by PPO and POD may lead to impairment of nutritional properties, since the enzyme-generated quinones react with the side chains of essential amino acids. Cross linking of proteins may also reduce susceptibility to enzyme hydrolysis. Undesirable colors may also be formed when phenolic compounds are present (Matheis and Whitaker 1984a).

Addition of low-molecular weight hydrogen donors (phenolic or diamide compounds) to the protein, H_2O_2, and peroxidase system also caused cross linking of proteins via an

oxidative deamination reaction. It has also been reported that the addition of peroxidase, peroxidase and H_2O_2 or peroxidase, H_2O_2, and catechol improved the dough-forming properties and the baking performance of wheat flours (Matheis and Whitaker 1984b).

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CHAPTER III

EFFECTS OF OXIDATIVE ENZYMES AND PHENOLIC COMPOUNDS ON SWEET POTATO PROTEIN IN A MODEL SYSTEM

ABSTRACT

Sporamin, the major storage protein in sweet potato, is a candidate for transgenic incorporation into cassava roots. Effects of an oxidative model system on sporamin, as occurs during post-harvest physiological deterioration in cassava after harvest, were evaluated. Thus, a partial purified sweet potato protein extract was obtained through acetone precipitation.

Oxidative model systems included a pure phenolic compound (scopoletin) and a crude cassava phenolic extract in addition to horseradish peroxidase and hydrogen peroxide. Sweet potato major protein, sporamin, in its native form and as part of different model systems was incubated at room temperature and aliquots collected at 0, 1, 6 and 24 h. Proteins were visualized by SDS-PAGE and analyzed by matrix-assisted laser desorption/ionization time-of-flight-time-of-flight (MALDI-TOF/TOF) and LC-MS/MS. Oxidation of the methionine residue was observed at 0 and 24 h incubation. Sporamin did not show a major degradation during 24 h of incubation in the oxidative phenolic systems. Polymerized protein did appear when pure scopoletin was present in the oxidative model system. However, the band formed in the stacking gel was very small. In addition, model

systems obtained at 24 h of subjected to pepsin digestion for 1, 6 and 24 h, exhibited resistance to pepsin at 1 h. This study showed that inclusion of sporamin in cassava may address the objective of increasing nutritional value.

INTRODUCTION

Major drawbacks of the cassava crop include (1) low protein content in the tubers, (2) rapid post-harvest tuber perishability and (3) a high content of the two cyanogenic glucosides linamarin and lotaustralin in all tissues. Depending on cultivars, the protein content in cassava storage roots ranges from less than 1% to 5% (dry weight). The nutritional value of this small amount of protein is further reduced by the particularly low levels of the essential amino acids (EAA) lysine and leucine and of the sulfur-containing EAA (SAAs) methionine and cysteine. Thus, fortification of cassava is primarily the inclusion of essential amino acids and secondarily of trace elements (Sautter and others 2006; Stupak and others 2006; Jørgensen and others 2007).

The recombinant storage protein "Zeolin", made up of maize γ-zein and bean phaseolin has been successfully incorporated into cassava (Stupak and others 2006). This chimeric protein is a major improvement of this crop which is characterized by low endogenous storage proteins (Stupak and others 2006). Researchers at the Danforth Center (St. Louis, MO, US) have used this single chimeric gene (Zeolin) to induce the formation of protein bodies in parenchyma cells. Cassava roots with more than 3.5% fresh

weight or 10.6% dry weight total protein content have been attained (Abhary and others 2008; Abhary and others 2011). According to de Virgilio and others (2008), prolamins such as maize γ-zein form stable protein bodies when expressed in vegetative tissues of transgenic plants, indicating that protein body formation does not require cereal seed-specific molecules besides the prolamins. Furthermore, the N-terminal domains of γ-zein can confer the ability to form protein bodies when fused to the bean vacuolar storage phaseolin in the chimeric protein zeolin. Wild-type γ-zein is insoluble unless its disulfide bridges are reduced. Zeolin has similar solubility properties. Zeolin characteristics in transgenic cassava have not yet been studied in a processing and post harvest context to gain insights in their modification or contribution to product properties. Some studies referred to the transformation of alfalfa with zeolin, and its accumulation has been studied. It has been previously shown that in transgenic tobacco leaf, zeolin is retained in the endoplasmic reticulum where its accumulation in protein bodies can reach 3.5% of total leaf protein content (Archinti and others 2004; Mainieri and others 2004; Belluci and others 2007a, b).

A more recent modification is sporazein fortified cassava. Sporazein is derived from the major storage protein in sweet potato (sporamin) coupled with peptides from corn with large amounts of sulfur-containing amino acids. The sporazein name is a combination of sporamin and zein (Abhary M. e-mail message, 2010). Sporamin A, a vacuolar storage protein of the tuberous root of sweet potato (*Ipomoea batatas*) has also been incorporated into cassava. Sporamin accounts for 60 to 80% of the total soluble protein in the sweet potato tuber (Hattori and others 1990; Huang and others 2007). Sporamins are encoded by

a multigene family, grouped based on nucleotide homology into two subfamilies, namely sporamins A and B. From studies of transgenic tobacco cells, transformed with a sporamin gene, it was revealed that post-translational processing of a sporamin precursor into two smaller molecular weight forms occurs (Yeh and others 1997). The activity of sporamin as a trypsin inhibitor may account for its ability to confer resistance to the lepidopteran pest, *Spodoptera litura*, when expressed in transgenic tobacco (Shewry 2003). Moreover, recent studies on tobacco plants transformed with the sporamin gene have shown that it is an effective anti-insect agent. Based on these observations, sporamin may provide a viable alternative in designing transgenic crops to control insect pests (Yeh and others 1997). Phenolic compounds are known to have effects in plant protein utilization (Kroll and others 2003).

Montagnac and others (2009) mentioned that specific phenolic compounds identified in cassava roots are 3 flavan-3-ols (gallocatechin, catechin, and catechin gallate), hydroxycoumarins (scopoletin), and the flavone 3-glycosides rutin and kaempferol 3-rutinoside. Phenolic compounds accumulate in cassava roots as they deteriorate. Van Oirschot and others (2000) pointed out that the onset of post-harvest physiological deterioration (PPD) coincides with increases in the levels of phenolic compounds including scopoletin.

Scopoletin is a phenolic coumarin and an important member of the group of phytoalexins isolated from many plants (Gnonlonfin and others 2012). Biosynthetic pathways to hydroxycoumarins during PPD in cassava roots using stable isotope labeling showed that the major pathway to scopoletin and its glucoside scopolin was through *p*-

coumaric, caffeic and ferulic acids (Bayoumi and others 2008b) but the pathway is not yet fully understood. Scopoletin has been shown to have an important role in traditional medicine in Africa such as the management of convulsions. Also, scopoletin in the presence of horseradish peroxidase (HRP) enhances eosinophil activation that is one of the key cells in the pathogenesis of asthma (Gnonlonfin and others 2012). As reported by Panda and Kar (2006) scopoletin also inhibited hepatic lipid peroxidation *in vitro* and increased the activity of antioxidants enzymes, superoxide dismutase and catalase.

Enzyme-catalyzed browning reactions of amino acids and proteins with oxidized plant phenols may cause deterioration of food during storage and processing leading to a loss in nutritional quality, which is especially serious in underprivileged countries. This loss of nutritional quality is attributed to the destruction of essential amino acids and a decrease in digestibility and inhibition of proteolytic and glycolytic enzymes. The quinones, primary products of enzymatic oxidation, can also readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan residues in proteins causing changes in physical, structural and biological properties (Friedman 1996; Rawel and others 2001; Kroll and others 2003). Moreover, oxidation decreases consumer acceptability of foods due to the low molecular weight off-flavors developed (Choe and Min 2009).

Thus, the interest in plant phenols is connected to their dual role as substrates for oxidative browning reactions and as antioxidants, underlining their impact on sensory and nutritional qualities of fruits and vegetables. Phenolic compounds can interact with proteins through covalent and non-covalent binding under specific conditions that include the production of a reactive and redox active quinones or a semiquinone radical that are

electrophilic species capable of undergoing nucleophilic addition at reactive side chains of the proteins (Rawel and others 2005; Rawel and Rohn 2010). This irreversible complexation of plant phenols with dietary proteins may result in polyphenol-rich foods being nutritionally poor, causing protein deficiency (Rawel and others 2001). Prigent and others (2007) showed the modification of proteins by non-enzymatically and enzymatically covalent induced interactions. In the case of covalent interactions with the phenolic compound, chlorogenic acid, modifications of functional properties such as decrease of solubility of proteins was observed.

Reilly and others (2003) indicated that the flavan-3-ol compounds (+) gallocatechin, and (+) catechin, and the coumarin compounds scopoletin and esculetin were capable of acting as free-radical scavengers. Scopolin did not show free radical scavenging activity. In addition, they determined that the cassava storage root contains a range of easily oxidized compounds that could participate as electron donors in enzymatic or non enzymatic oxidation reactions during the post-harvest period.

Increases in activity of both peroxidase and catalase, two key enzymes involved in turnover of H_2O_2, occur during PPD (Beeching and others 1998). In addition, the peroxidase substrate, scopoletin, shows a transient accumulation in cassava roots and has been proposed as a key component of the deterioration response (Reilly and others 2003).

Reilly and others (2003) mentioned that since oxidation of scopoletin by peroxidases is known to give rise to a bluish product of unknown structure, PPD may result from peroxidase mediated oxidation of scopoletin. They showed that within the root parenchyma all required components of the reaction, namely scopoletin, H_2O_2, and

peroxidase activities, are initially localized to the vicinity of the xylem where vascular streaking symptoms occur. In non-pruned plants or more susceptible cultivars, where catalase levels are relatively higher, a significant proportion of H_2O_2 scavenging could occur via peroxidase-mediated reactions requiring the participation of cellular components, including scopoletin, as electron donor.

In response to wounding or infection, polyphenols were produced in the tissue adjacent to the wounded surface or infected region. These were (+)-catechin and derivatives in cassava (Uritani 1999). Therefore, effects of enzymatic activity (mainly peroxidase) and phenolic compounds (including scopoletin) on sporamin, major protein in sweet potato, as a potential plant source for fortification of cassava were evaluated in designed model systems in this study.

Oudgenoeg and others (2001) concluded that cross-linking of the tyrosine-containing peptide Gly-Tyr-Gly (GYG) and ferulic acid can be achieved by kinetic control of the reaction catalyzed by HRP. Therefore, tyrosine containing sweet potato protein may cross link with the phenolic scopoletin that increases during PPD.

Our objectives were achieved by partial purification of sporamin from sweet potato. A model system containing the compounds involved in PPD (oxidative enzymes and phenolics) and the protein of interest was used to evaluate interactive effects. Protein characterization was done to determine the influence of the different PPD components using novel MALDI-TOF/TOF and LC-MS/MS methods to qualify PPD-modified peptides of sweet potato protein as if its gene were part of cassava.

MATERIALS AND METHODS

MATERIALS

Cassava and sweet potato roots were purchased from the local market (Safeway, Pullman, WA). Folin-Ciocalteu reagent, gallic acid, α-lactalbumin (Type III, calcium depleted, from Bovine milk), scopoletin (≥99%), peroxidase (Type VI from horseradish) and pepsin from porcine stomach mucosa (Pepsin A, lyophilized powder) (activity 3100U/mg solid) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium carbonate, monobasic and dibasic potassium phosphate reagents, phosphoric acid, HPLC grade methanol, and hydrogen peroxide (3% and 30%) were from J.T. Baker Inc. (Phillipsburg, NJ). The CB-X total protein assay kit was purchased from G-Biosciences (St. Louis, MO). Trypsin (sequence grade) was obtained from Promega (Madison, WI). Microplates were 96-well BD Falcon™ 353915 (Fisher Scientific, Pittsburgh, PA). Acetone (HPLC grade) and ethanol (200 proof) were purchased from Washington State University Central Stores. Spectra/Por 4 Regenerated cellulose membrane (cutoff 12-14 kDa) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA).

METHODS

Extraction of phenolic compounds

Cassava roots were induced to post-harvest physiological deterioration as described by Buschmann and others (2000) and Bayoumi and others (2008a). Briefly, cassava roots (550 g) were peeled, cut into approximately 1 cm^3 cubes and allowed to deteriorate for 4

days under controlled conditions (23 °C, 75% R.H.). Daily, 1 g of cassava cubes was blended in a homogenizer (Ultra Turrax T25 basic) with 10 mL of absolute ethanol and allowed to extract at room temperature (20 °C) for 1 hour. Extraction was done in triplicate and each of the replications was decanted into 150 mL round bottom flask using a small funnel and Whatman #1 filter paper. The ethanol extracts were dried to 3 mL in a rotary evaporator (Brinkmann Model: B-169 Vacuum aspirator) at 35-40 °C, filtered through 0.45 µm Durapore® membrane filters (Type HV) (Millipore Corporation, Burlington, MA) into glass vial with Teflon lids (VWR International, LLC., Brisbane, CA) and held at -20 °C for phenolic analysis and further use in the sweet potato protein model systems.

Total Phenolic Content

Total phenolic content was determined using the procedure described by Singleton and Rossi (1965) and modified by Chaovanalikit and Wrolstad (2004). A 0.5 mL sample of the ethanol extract or a series of gallic acid standards (0, 50, 100, 150, 200 ppm) were mixed with 0.5 mL of 2N Folin-Ciocalteu reagent and 7.5 mL of deionized water. The mixture was held at room temperature (20 °C) from 30 sec to 8 min before adding 1.5 mL of 20% sodium carbonate (w/v), and kept at the same temperature for 2 h before measuring the absorbance at 765 nm. Results were expressed on a fresh weight basis as mg gallic acid equivalents GAE/g.

Phenolic Compounds Determination by HPLC

HPLC separation and quantitation were modified from methods described by

Macheix and others (1990). Phenolic extracts (10 µL injections) were analyzed for scopoletin with an Agilent 1100 series HPLC system with auto sampler and a UV detector (Agilent, Technologies, Avondale, PA) equipped with a 5 µM C-18 Zorbax-SB column and guard column (250×4.6 mm). The mobile phase consisted of a solvent gradient of 90:10 (0.5% phosphoric acid: 100% methanol) that increased linearly to 30:70 over 40 min, maintained at 30:70 for 5 min, cleaned with 100% methanol for 13 min, and then re-equilibrated to 90:10 for 2 min at a flow rate of 1.0 ml/min. Scopoletin was detected at 350 nm. Quantification was performed by comparing sample retention times and using response factors generated from scopoletin as external standard. Scopoletin, in the range of 52 to 260 µg/mL, was prepared in HPLC grade methanol in a mix containing other coumarins. The HPLC analysis was performed in triplicate (Iyer and others 2010).

Crude enzyme preparation

A modified method as described by Liu and others (2009) and Luo and Huang (2012) was used for cassava peroxidases preparation. Briefly, freeze dried cassava flours (5 g) were extracted with cold 0.05 M potassium phosphate buffer and stirred at 4 °C for 2 min with an Omni mixer homogenizer (Omni International, Waterbury, CT) and allowed to sit for 30 min at 4 °C. Extract was centrifuged (15344 xg) at 4 °C for 15 min to eliminate debris and starch. The crude extract was collected in amber bottles for immediate peroxidase enzyme activity determination.

Peroxidase activity

Fifty μL of enzyme cassava extract were added to 3 ml of assay medium containing 0.1 M potassium phosphate buffer (pH 6), 10 mM guaiacol and 0.1M H_2O_2. Changes in absorbance at 470 nm were measured every 15 s for 3 min. One unit of enzyme activity is defined as the amount of tetraguaiacol formed per min using the extinction coefficient of tetraguaiacol of 26.6/mM · cm. Enzyme activity was expressed as units/g fresh weight. min (Angelini and others 1990; Ozturk and Demir 2002; Luo and Huang 2012).

Sweet potato protein extraction (SPP)

Sweet potato protein extraction and partial purification was carried out as described by Akazawa and others (1957). Briefly, 60 g of sweet potato tissue were mixed with 1000 mL of cold acetone (HPLC grade) at -70 °C. Mix was blended for 3 min in a laboratory blender, model 51Bl30 (Waring Commercial, Torrington, Connecticut) at room temperature and vacuum filtered using Whatman filter paper N° 1. The acetone powder recovered was washed with 700 mL of cold acetone at -70 °C and dried in a desiccator at 4°C for 2 h. Acetone powder was extracted with 150 mL of cold 0.1 M potassium phosphate buffer pH 7.2 containing 0.5% ascorbic acid and stirred at 4 °C for 20 min. Solution was centrifuged at 15344 xg for 30 min at 0 °C. The solution was dialyzed overnight at 4 °C against the phosphate buffer (not containing ascorbic acid) with a one change of buffer solution. The dialyzed solution was centrifuged at 8500 xg for 20 min at 0 °C. The supernatant was recovered and stored at -20 °C for further analysis.

Soluble protein measurement

Sweet potato protein extract was analyzed by the Bradford Assay using the CBX Kit (G-Biosciences, St. Louis, MO) according to manufacturer's protocol. A calibration curve was constructed with bovine serum albumin included in the CBX kit as a standard.

Sweet potato protein model systems

In the protein model systems, a protein source (sweet potato protein extract) with a soluble protein content of 2 mg/mL, a pure enzyme (POD) and the substrate (cassava phenolic extract (1262.2 μg/g) or scopoletin) were combined in model systems as shown in Tables 1 and 2. The systems were allowed to react for 0, 1, 6 and 24 h at room temperature with continuous vortex agitation. An aliquot of 200 μL was taken at each specified time and the reaction was quenched with a catalytic amount of catalase (150 μg/ml, 3.3 μL). Effect of combinations of variables (protein, phenolics, enzyme and hydrogen peroxide) was evaluated. Protein changes in each model system were evaluated by MALDI-TOF/TOF and LC-MS/MS analyses. Sweet potato protein extract was run as a control. Simultaneously, α-lactalbumin, which has been proved to show oxidative polymerization resulting in a dimer formation (Heijnis and others 2010), was run for testing efficacy of the method.

Gel electrophoresis (SDS-PAGE)

The sweet potato protein molecular weight distribution and effect of oxidative enzyme activity and phenolics on protein was determined by sodium dodecyl sulfate electrophoresis-polyacrylamide gel (SDS- PAGE) using a Mini Protean electrophoresis cell

(Bio Rad, Hercules, CA). Precast polyacrylamide gels 4–20% Mini-PROTEAN® TGX™ were used (Catalog N° 456-1094) (Bio Rad, Hercules, CA). SDS–PAGE was performed with 20 µL sample load with 20 µg or 40 µg protein. Molecular weight markers used were broad range (Catalog N° 161-0317) (Bio Rad, Hercules, CA) including myosin (200 kDa), β – galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovoalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). Electrophoresis was carried out at 20 °C using 200 V constant for 37 min. The proteins were stained with Bio-Safe Coomassie Blue G-250 stain (Catalog N° 161-0786) (Bio Rad, Hercules, CA). Gel images were captured with a Coolpix S60 camera (Nikon Corp., Japan).

In Gel digestion

Protein bands of interest, corresponding to the samples incubated for 0 and 24 h, were excised from the gel. Proteins were reduced and alkylated prior to trypsinolysis as described by Shevchenko and others (1996).

In Solution digestion

Selected solutions of model systems incubated at 0 and 24 h were digested with sequencing grade modified trypsin (Promega, Madison, WI) as described by Kinter and Sherman (2000).

Qualitative analysis of PPD-modified peptides of sweet potato by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF/TOF)

MALDI-TOF mass spectrometry was used to characterize the changes caused by the interaction of oxidized phenolics with sweet potato proteins.

Sample preparation

The matrix material, alpha-cyano-4-hydroxy-trans-cinnamic acid (alpha-CHCA), used for peptides obtained after in gel digestion was dissolved at 5 mg/mL in 50:50 water:acetonitrile with 0.1% trifluoroacetic acid (TFA). To obtain a uniform sample on the MALDI plate, matrix solution was first spotted onto the MALDI plate and allowed to dry. The samples were diluted 1 to 1 with the matrix solution and spotted onto the previously spotted matrix. Diluted peptide samples were concentrated and desalted using NuTip C8 from Glygen Corp. The sample was acidified using 1% TFA solution, aspirated up and down at least 10 times using the acetonitrile wetted tip and the tip rinsed using 0.1% TFA to remove any remaining salt. The peptides were eluted from the tip using 2 μL of the matrix solution and spotted onto the top of the previously dried matrix spots.

Whole proteins solution samples, with and without digestion, were prepared in a similar way using sinapinic acid as matrix, dissolved at 10 mg/mL in 50:50 water:acetonitrile with 0.1% TFA and processed the same way as peptides. Samples were spotted onto a dried spot of sinapinic acid matrix after use of the C8 tip to desalt and concentrate the sample.

MALDI-TOF/TOF

MALDI analysis was performed using an Applied Biosystems MDS Sciex 4800 TOF/TOF Analyzer (Framingham, Massachusetts, USA). The mass spectra of peptides or whole proteins from trypsin digest were run three times using the MS Reflector Positive method and the Mid Mass Linear Positive or High Mass Linear positive methods, respectively, supplied by the manufacturer. The peptides or protein samples were deposited on the MALDI spot, air-dried and ionized by a 200 Hz Nd-YAG frequency tripled laser of 355 nm, averaging 400 laser shots. Peptides spectra were acquired for the mass range from 500 to 5000 Da while protein spectra mass range was from 16000 to 50000 Da.

Analysis of PPD-modified peptides of sweet potato protein by Liquid Chromatography Mass Spectrometry (LC-MS/MS)

LC-MS/MS spectra were obtained using an Esquire HCT electrospray ion trap (Bruker Daltonics, Billerica, MA) and LC Packings Ultimate Nano high-performance liquid chromatography system Bruker as described by Noh and others(2008). The trypsin-digest of gel pieces was separated by reverse phase HPLC and then ionized by electrospray.

MALDI-TOF/TOF and LC-MS/MS Data Analysis

The resulting MS/MS spectra from either MALDI or LC-MS/MS were submitted to MASCOT software v.2.1 (Matrix Science, London, England) and analyzed by peptide mass fingerprints (PMF) for identification of the protein. Data submitted to MASCOT had alkylation of cysteine (C) "carbamidomethyl" with iodoacetamide as a fixed modification.

Variable modifications included deamidation, methionine oxidation to sulfoxide and oxidation of methionine to sulfone as supplied by Matrix Science. In addition, a variable modification "scopoletin" was created for tyrosine (Y) through a linkage with scopoletin. Zero missed trypsin site was the default search for MALDI while one miss-cut was for LC-MS/MS. In addition semytrypsin was used in LC-MS/MS to identify variable N terminal start sites.

Digestibility of proteins

In order to study the digestibility of native sweet potato protein extract and as part of an oxidative model system, a time course of *in vitro* pepsin digestion was done and adapted from previous studies by Macierzanka and others (2009) and Monogioudi and others (2011).

The sweet potato extract protein concentration in all model systems (Table 3) was 1.33 mg/mL. All model systems were adjusted to pH 2.5 with 1M HCl before digestion. The concentration of pepsin was 50 μg/mL. The experimental conditions used to study proteolysis by pepsin corresponded to typical body temperature and low pH in *in vitro* digestion. Pepsin was used instead of trypsin due to the trypsin inhibitor characteristic of the sweet potato major protein as cited by Shewry (2003). Pepsin digestion was carried out with the previously incubated (24 h) model systems at 37 °C under continuous moderate agitation (125 rpm). Aliquots of the digests were collected at 1, 6 and 24 h and the proteolysis was immediately stopped by raising the pH to 7 with 5M NaOH prior to SDS-PAGE run as previously described.

Data Analysis

Cassava total phenolics, scopoletin concentration and peroxidase activity data were analyzed for significant differences using a one-way analysis of variance (ANOVA). Tukey's HSD multiple comparisons of means were used to analyze both cassava flours and fermented cassava flours (wet fufu) data with XLSTAT (Version 7.5.3, XLSTAT Addinsoft, France) at the $p \leq 0.05$ confidence level. All the tests were performed in triplicate.

RESULTS AND DISCUSSION

Phenolics and enzyme activity in cassava roots

Cassava roots undergo a process known as postharvest physiological deterioration (PPD) that presumably involves several enzymes such as peroxidase and a phenolic compound such as scopoletin. Based on that information, sweet potato protein extract was placed in an oxidation system in order to mimic post harvest physiological deterioration and evaluate any protein modification that may be detrimental during its use for enrichment of cassava roots.

Fresh cassava roots were first evaluated for total phenolics and scopoletin content as observed in Figure 1. Regarding the total phenolic content of cassava roots over a period of 4 days, the greatest concentration (0.24 mg GAE/g fresh weight) was found the first day. This content decreased at the 2nd and 3rd days with an increase at 4th day (0.17 mg GAE/g fresh weight). No significant differences were found between total phenolic

content at 3rd and 4th days ($p>0.05$). In relation to the scopoletin content, a different trend was observed over the same period of time. Thus, a decrease was observed at the 2nd day with a regular increase up to 1011.58 µg/g at the 4th day. No significant differences were found in scopoletin concentration ($p>0.05$). Buschmann and others (2000) evaluated the hydroxycoumarins present in cassava directly after harvest and determined a small amount of scopoletin at first that they interpreted as a direct response to wounding during harvest. Scopoletin increased after 3 and 4 days of storage. Even though the scopoletin concentration increase showed a trend similar to what Buschmann and others (2000) reported, factors such as cultivar, harvesting time and method of analysis used among others, may have influenced the differences found in scopoletin concentrations. On the other hand, our results disagreed with Uritani and others (1984), who found that coumarin compounds such as scopoletin, scopolin and esculin were nearly absent in the fresh cassava tissue. They also determined that bluish fluorescent phenolic components were produced in response to injury in cassava root tissue.

Peroxidase activity was also measured during the 4 day PPD induction. As shown in Figure 2, low peroxidase (POD) activity was found at 1 and 2 days of evaluation. A noticeable increase was observed at 3 and 4 days. Significant differences in peroxidase activity were observed between the 1st and 4th days with the latter showing the greatest activity. When compared the POD activity with the scopoletin concentration determined over the same period of time, an evident increase of the POD activity was detected with a simultaneous slight increase of the scopoletin concentration at the 4th day (Figure 2).

Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions on the different model systems in order to visualize sweet potato protein (SPP) polymerization. Due to the partial purification of the sweet potato material, other proteins were detected besides the major protein sporamin (Figures 3a, b and 4a, b).

The SDS-PAGE results from 0 h incubation are shown in Figures 3a and 4a, with the inclusion of a pure phenolic compound (scopoletin) and a crude phenolic cassava extract as substrates, respectively. Under the conditions used, none of the SPP incubated with horseradish peroxidase (HRP)/hydrogen peroxide (H_2O_2) was apparently cross linked (Lane 4). In addition, the SPP did not appear to be affected by the scopoletin/HRP/H_2O_2 treatment (Lane 3). The H_2O_2 alone had no detectable effects on the SPP (Lane 7). This last result is in contrast to Matheis and Whitaker (1984) who stated that H_2O_2 produced a slight increase of the dimer content of lysozyme. As reported by Matheis and Whitaker (1984) and Heijnis and others (2010), a low H_2O_2 concentration was used in order to minimize possible oxidation of protein-bound sulfur-containing amino acids. An overdose of H_2O_2 results in the inactivation of HRP (Heijnis 2010). On the contrary, Faergemand and others (1998) reported that higher concentrations of H_2O_2 were able to induce oxidative polymerization of β-lactoglobulin while not affecting α-lactalbumin. Most of the available data on the chemical modification or proteins by peroxidase/H_2O_2 or by peroxidase alone indicate that tyrosine is the amino acid residue in proteins most sensitive to modification (Matheis and Whitaker 1984).

As observed in lanes 3 and 5 (Figure 3b), containing scopoletin complete oxidative model systems showed polymerized protein in the stacking gel. Presumably, the major sporamin sweet potato protein was cross-linked. Even though reducing conditions were used, scopoletin influenced cross linking of SPP. Differences on the intensities of the high molecular weight bands in lanes 3 and 5 (Figure 3b) was due to the greater amount of protein loaded in the well in lane 5 (40 μg protein). No previous reports have been found related to scopoletin participation in cross-linking of proteins in an oxidative system. While studying a two-step approach for DNA immobilization, Gajovic-Eichelmann and others (2003) used a novel electro-deposited polymer based on scopoletin. Results of the electropolymerization showed that polymerization of scopoletin was not hindered by the protein streptavidin. Ward and others (2001) found that during the enzymatic polymerization of ferulic acid, dehydrodimers and trimers decreased when increasing the concentration of H_2O_2 and time of incubation. This indicated that dimers and trimers were further oxidized resulting in the formation of higher oligomers. In our study, no apparent protein dimers or trimers were observed in SDS-PAGE. Stahmann and others (1977) suggested that the presence of new bands due to the reaction with peroxidase was due to changes in the charge of the protein rather than a change in size. Additional investigation is necessary to understand the factors leading to peroxidase polymerization of scopoletin and/or sporamin.

On the other hand, no high molecular weight bands were observed in lanes 3 and 5 (Figure 4b) when a crude phenolic cassava extract was used instead of pure scopoletin as peroxidase substrate. This may be due to the lower concentration of scopoletin found in the

crude phenolic extract (Figure 1) compared to the amount of scopoletin in the model systems. The final concentration of scopoletin in the model system was 1.6 mM while the final concentration of scopoletin in the crude phenolic extract was 0.16 mM. In addition, the presence of other phenolic compounds such as rutin and kaempferol-3-*O*-rutinoside as identified by Gomez-Vasquez and others (2004) in cassava, may have influenced the formation of a high molecular weight compound and/or degradation of the sporamin.

As reported by Faergemand and others (1998) and as confirmed in our preliminary experiments (data not shown), low or high concentrations of peroxidase did not produce the formation of polymers. On the other hand, an effect was observed in α-lactalbumin, a protein used to test the efficiency of the system. The α-lactalbumin dimer band decreased in intensity when the concentration of peroxidase was reduced from 14 to 0.14 μM.

In general, according to the SDS-PAGE analyses, the intensities of the sporamin bands remained nearly unchanged at all incubation times (1 and 6 h data not shown) and novel bands were not formed, indicating that there was no major degradation of protein material. Incubation times greater than 24 h could cause protein changes. Balange and Benjakul (2009), while studying the effects of oxidized phenolic compounds on the gel property of mackerel found that proteins may cross-link in different forms based on degree and site of interactions induced by the oxidized phenolic compounds. Rohn and others (2002) also concluded that the reaction of phenolics with proteins/enzymes is influenced by the formation of semiquinone intermediates and pH. They observed that the reactivity of the phenolics increased with increasing pH and consequently a decrease of the enzyme activity occurred.

As reported by Heijnis (2010), α-lactalbumin formed dimers under specific concentrations of HRP and H_2O_2 as observed in Figures 3b-4b (lane 9). Reducing agents decrease the relative amounts of dimers (Karasawa and others 2010; Wang and others 2010). Thus, existence of dimeric species found here under reducing conditions suggests that formation of the dimer is not exclusively mediated by disulfide bonds. Dimer formation can be also mediated by redox-dependent and -independent mechanisms. In the case of the sporamin model systems, no dimers were observed when non reducing conditions were used (data not shown). As shown in lane 8 (Figures 3b-4b), SPP prevented the formation of α-lactalbumin dimer. Huang and others (2007) demonstrated that sporamin B in sweet potato may have significant antioxidant activities. Sporamin B may contribute significantly to change of the redox state and as a potent antioxidant against both hydroxyl and peroxyl radicals. As cited by Shewry (2003), sporamin has antioxidant activity that is associated with intermolecular thiol/disulfide exchange. In addition, sporamin is also able to scavenge both 1-1-diphenyl-2-picrylhydrazyl radicals and hydroxyls radical. Previous study also showed the ability of scopoletin to scavenge superoxide anion that may prevent diseases related to oxidative damage (Shaw and others 2003).

MALDI-TOF/TOF and LC-MS/MS

MALDI analysis of the whole protein samples (without trypsin digestion) and in the presence or not of oxidative conditions showed an apparent protein dimer formation in the spectra of the sporamin as shown in Figures 5 a, b, c at 24 incubation. MALDI analysis of the

whole protein in solution digestion samples did not work very well possibly due to sporamin being a trypsin inhibitor; therefore, trypsin was not available for protein cleavage. It is assumed that extreme reduction and alkylation conditions would inhibit the trypsin inhibitor activity of sporamin.

The excised sporamin bands were reduced, alkylated and digested with trypsin, and resulting peptides were extracted and analyzed by peptide mass fingerprints (PMF) using MALDI-TOF/TOF to evaluate detailed changes of the peptide patterns and molecular weights in derivatized monomer molecules of sporamin. MALDI-TOF-MS was used as the preferable method because of its robustness in complex samples and the prevalence of singly charged peaks. LC-MS/MS was used as a complementary approach, particularly for smaller peptides. According to Wang and others (2003), even though MALDI has a high accuracy, resolution (peak separation) and versatility, its resolution is lower than that of LC-MS/MS.

In gel digestion with trypsin of the SPP band from SDS-PAGE showed the presence of sporamin A and B. The genetic variant of the proteins are consistent with the gene sequences provided by the Basic Local Alignment Search Tool (BLAST) offered by the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to the MASCOT search program. For example, the upper band (Figure 6) was identified as sporamin A with three different lengths at the N-terminal based on the LC-MS/MS analysis and MASCOT evaluation using semitrypsin as the cleavage enzyme: SSETPVLDINGDEVR, SETPVLDINGDEVR and EPASSETPVLDINGDEVR. The average masses for those peptides of Sporamin A, calculated with General Protein Mass

Analysis software (GPMAW 6.0) supplied by Chem SW, Inc. (2003), were 19905.35 Da, 19818.28 Da and 20202.35 Da, respectively. The differences between those N-terminals and the N-terminal of the nucleotide sequence (Figure 7a) provided by NCBI may be due to processing conditions and/or oxidation of the sweet potato material during extraction. EPASSETPVLDINGDEVR is the longest N-terminal trypsin peptide found in sporamin A and may account for the higher mass of the whole sporamin protein sample. The C-terminal was found to be LALSNSPFVFVIKPTDV with no processing or modification observed when comparing it with the sequence provided by NCBI (Figure 7a).

The lower band was identified as sporamin B (Figure 6) and two different N-terminals, identical to the ones found for Sporamin A, were found based on the LC-MS/MS analysis and MASCOT evaluation: SSETPVLDINGDEVR and SETPVLDINGDEVR with average masses of 19974.22 Da and 19887.15Da, respectively. The C-terminal peptide determined was LALSNTPFVFVIKPTDM. The C-terminal in sporamin B was not modified as a similar C-terminal was observed in the sequence provided by NCBI for sporamin B (Figure 7b).

Also, in gel digestion of the high molecular band (L) (Lanes 3, 5-Figure 3b) analyzed by LC-MS/MS and data evaluated with MASCOT determined that this band presented sporamin A with peptides in the N and C terminal similar to those found in the sequence provided by NCB. Furthermore, a unique fragment (44-59) only present in sporamin B was observed (Figure 9b).

Sporamin bands from lanes 1, 3 and 4 from 0 h and 24 h incubation time (Figures 3 a, b) were digested and analyzed by MALDI. At 0 h (lane 1), about 74% of the protein sequence of sporamin A (Figure 8a) and 58% of sporamin B (Figure 8b) were matched by

identifying 20 and 13 peptides, respectively. At 24 h (lane 1), about 56% of the protein sequence of sporamin A (Figure 9a) and 59% of sporamin B (Figure 9b) were covered by identifying 12 and 10 peptides, respectively. The nominal mass (M_r) found for sporamin A was 20987 Da and 20764 Da for sporamin B. Irrespective of the incubation time, oxidation of the methionine (C-terminal) in sporamin B was observed. Thus, the observed mass of the peptide sequence for the protein segment 170-186 was approximately 1909 Da compared to the non oxidized form peak observed at *m/z* 1893 (data not shown).

The sporamin protein band corresponding to a complete oxidative system that included scopoletin (lane 3, Figures 3a, b) was also analyzed by MALDI. Apparent oxidation of methionine was observed at 0 h and 24 h. At 0h, methionine sulfoxide was detected in the N terminal peptide (sporamin A, fragment 24-43) as determined by an increase in mass fron 1956 to 1972 Da. Methionine oxidation was also observed in fragment 164-170 in sporamin A (Figure 8a) with an oxidized form at peak *m/z* 931.44. Sporamin B showed an oxidized methionine form at peak *m/z* 1908.98 (fragment 170-186) (Figure 8b) with no observable non oxidized form. At 24 h, a more noticeable oxidation of methionine was observed in fragment 164-170 (Figure 9a) of sporamin A compared to 0 h incubation. Regarding sporamin B, oxidation of the C-terminal also was detected (fragment 170-186) (Figure 9b). In addition, segment 60-89 of sporamin B at 24 h incubation time showed oxidation of the methionine (3302.46 Da).

The sporamin band obtained as a result of the oxidative system without the presence of scopoletin was also evaluated (lane 4, Figures 3a, b). Methionine oxidation corresponding to N and C terminals was found for both 0 and 24 h incubation for fragments

24-43 (Figures 8a, 9a) and 170-186 (Figures 8b, 9b), respectively. Oxidation also was observed at 24 h in fragment 60-89 (Figure 9b) of sporamin B. The oxidized form showed an observable mass of 3302.45 Da compared to 3285.55 Da for the non oxidized form.

Sporamin bands from lanes 3 and 4 at 24 h of incubation were compared. Oxidized forms of methionine (N-terminal) were observed in both lanes with an observable oxidized mass of approximately 1972 Da, while the non oxidized mass was approximately 1956 Da (Figures 10a, b). Regarding the C-terminal, no major oxidation was observed in the sporamin band from lane 4 (Figure 11a), while a major oxidation was observed in the band from lane 3 where scopoletin was present (Figure 11b). We suspected that the mass increase of 16 Da represents the oxidation of the methionine residue to methionine sulfoxide. In addition, there are several factors that can account for differences between the spectra. These include other types of modifications, differences in digestion cleavage patterns between bands, differing levels of protein purity, and differences in peak signal-to-noise between spectra (Person and others 2003).

To confirm the assignments based on MALDI mass measurements, MS/MS fragmentation of the in gel digested protein samples was performed. The in-gel digest of the selected sporamin protein bands was subject to microspray LC-MS/MS analysis using an ESI ion trap mass spectrometer. The protein coverage for sporamin A or sporamin B was not as good as the coverage determined with MALDI. However, the N-terminal was detected with LC-MS/MS. LC-MS/MS did not allow a complete ionization of the peptides of the sporamin protein under the conditions applied. A more purified protein or the presence of a single sporamin either A or B will allow a better application of this technique.

All amino acids are susceptible to oxidation but their susceptibility varies greatly. Oxidation of residues within proteins may be mediated by different systems including oxidases, hydrogen peroxide and metals among others. Of the amino acids, methionine is characterized by its high vulnerability to oxidation resulting in methionine sulfoxide (Levine and others 1996). Also, tyrosine, the only phenol among the essential amino acids, is a target of oxidation mainly from OH radicals (Eickhoff and others 2001; Berges and others 2011; Minamihata and others 2011). Based on the nucleotide sequences provided by NCBI, sporamin A and B have 5 and 2 methionine residues, respectively. Seven tyrosine residues are present in sporamin A or B. In this study, oxidation was mainly observed, as mentioned before, on those peptides fragments where methionine was present. Because of methionine oxidation was also detected at 0 h of incubation of the oxidized model system, quantification is required to determine the degree of oxidation at 24 h. In addition, oxidation of the methionine was also found in the sporamin band without the addition of oxidizing elements, thus further analysis is necessary.

Han and others (2000) reported that oxidation of tyrosine by peroxidase requires H_2O_2. Thus, they pointed out that production of dityrosine demonstrated the accumulation of H_2O_2 in the reaction mixture. As they stated, during peroxidase-mediated chorion hardening, the enzyme after being oxidized by H_2O_2, oxidizes tyrosine residues on protein to tyrosine radicals that interact to form dityrosine, thereby resulting in chorion protein cross-linking that contributes to the formation of a rigid and insoluble chorion in mosquitos.

Digestibility of sweet potato proteins

Digestibility of sweet potato proteins in an oxidative system was studied by SDS-PAGE as shown in Figure 12. Native SPP extract as well as SPP in an oxidative system showed resistance to proteolysis by pepsin.

The presence of a pure phenolic compound (scopoletin) (lanes 2-3) or not (lane 4) (Figures 12a-c) or the presence of a crude phenolic extract (lanes 6-7) (Figures 12a-c) did not influence the resistance to pepsin digestion of the sweet potato major protein (sporamin). Similar protein patterns were observed at the different digestion times, suggesting that there was no influence of time on the resistance of the protein to the cleavage. This method did not allow monitoring masses of the digested proteins and only an estimated value was obtained by comparison with the standards used (lane 10) (Figures 12a-c).

SDS-PAGE showed high molecular weight protein of the soluble protein fraction of sweet potato (large aggregates) (lanes 3 and 5) (Figure 3b), which appeared in an oxidized model system when pure scopoletin was included. This polymerized protein was apparently readily digested at 1 h as no high molecular protein band is observed in lane 3 (Figures 12a-c) after digestion. Further, the time course of the *in vitro* digestion of this fraction demonstrated that the major protein in sweet potato, sporamin, was not digested at 1, 6 or 24 h while lactalbumin was digested rapidly by pepsin at 1 h. In a similar study, Maloney (2011) concluded that protein resistance to digestion was due to the compact protein structure that hinders the ability of the digestive enzymes to reach cleavage sites. As mentioned by Maloney (2011), another possibility is the unique amino acid sequence of

the protein that causes digestive enzymes to not have cleavage sites. Sun and others (2012) also determined no visible change of the sweet potato protein after digestion with pepsin or pancreatin after 30 min. They also observed a slight increase of the sweet potato protein digestion with pepsin after heating. Different heating methods had different effects on the digestibility of SPP. Autoclaving, boiling at 100 °C for 1 h or microwaving allowed a complete digestion of the SPP.

In previous studies, Hou and others (2005) found that digestion with chymotrypsin and pepsin did not eliminate the antioxidative activity of sporamin and an increase of the scavenging activity of the trypsin inhibitors against DPPH was observed.

CONCLUSIONS

Several factors may affect the polymerization of sporamin in an oxidative model system. Scopoletin at the level of 1.6 mM, exhibited a slight cross-linking effect with sporamin A and B proteins from sweet potato in SDS-PAGE. However, a crude cassava phenolic extract containing scopoletin at smaller concentrations did not have any apparent effect on sporamin polymerization. A greater concentration of pure scopoletin or cassava crude phenolic extract may have a greater cross-linking effect on sporamin.

Comparison of molecular weights from MALDI spectra with those predicted from gene sequencing for the major changes in the sporamin oxidative system does not show major protein degradation. LC-MS/MS found the N-terminals in all samples evaluated but a

good ionization of the peptides was not obtained. In consequence, we were not able to confirm the results provided by MALDI. Qualitatively, oxidized forms of methionine were found in the sporamin with and without the components of an oxidative model system. Further work needs to be done in order to quantify the level of oxidation of the protein.

This study results have an impact on the understanding how sporamin, the major protein of sweet potato, may be affected by factors responsible for postharvest physiological deterioration in cassava (PPD). Therefore, sporamin damage can occur when included in cassava and exposed to scopoletin production during postharvest physiological deterioration. In contrast, sporamin is only slightly affected by PPD considering the small intensity high molecular weight band formed. No visible cross-linking was observed in this study. Inclusion of sporamin protein in cassava may address the objective of improving nutritional properties in cassava.

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Table 1. Model systems for the evaluation of PPD oxidation factors (enzyme and pure phenolic) on the sweet potato proteins

Compounds	Final concentration						
	1	**2**	**3-5**	**4-6**	**7**	**8**	**9**
Protein	**SPP[1] 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP- αLAC 0.85-4.17 mg/mL**	**αLAC[2] 8.33 mg/mL**
Phenolic (Scopoletin)	-	1.6 mM	1.6 mM	-	-	-	-
Peroxidase	-	-	14µM	14µM	-	14µM	14µM
H_2O_2	-	-	1mM	1mM	1mM	1mM	1mM

[1]SPP: Sweet potato protein extract
[2]αLAC : α-Lactalbumin

Table 2. Model systems for the evaluation of PPD oxidation factors (enzyme and crude cassava phenolic extract) on the sweet potato proteins

Compounds	Final Concentration						
	1	**2**	**3-5**	**4-6**	**7**	**8**	**9**
Protein	**SPP[1] 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP- αLAC 0.85-4.17 mg/mL**	**αLAC[2] 8.33 mg/mL**
Crude phenolic extract	-	29 µg GAE[3]/µL	29 µg GAE/µL	-	-	-	-
Peroxidase	-	-	14µM	14µM	-	14µM	14µM
H_2O_2	-	-	1mM	1mM	1mM	1mM	1mM

[1]SPP: Sweet potato protein extract
[2]αLAC : α-Lactalbumin
[3]GAE: Gallic acid equivalent

Table 3. Model systems of sweet potato protein extract and PPD oxidation factors (enzyme and phenolics) at 24 h of incubation for the evaluation of pepsin digestion for 1, 6 and 24 h at 37 °C

Compounds	**Final concentration**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Protein	**SPP[1] 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**SPP 1.7 mg/mL**	**αLAC[2] 8.33 mg/mL**
Phenolic	-	1.6 mM (Scopoletin)	1.6 mM (Scopoletin)	-	-	29 µg GAE/µL (Crude phenolic extract)	29 µg GAE/µL (Crude phenolic extract)	-	-
Peroxidase	-	-	14µM	14µM	-	-	14µM	14µM	14µM
H_2O_2	-	-	1mM	1mM	-	-	1mM	1mM	1mM

[1]SPP: Sweet potato protein extract
[2]αLAC : α-Lactalbumin
[3]GAE: Gallic acid equivalent

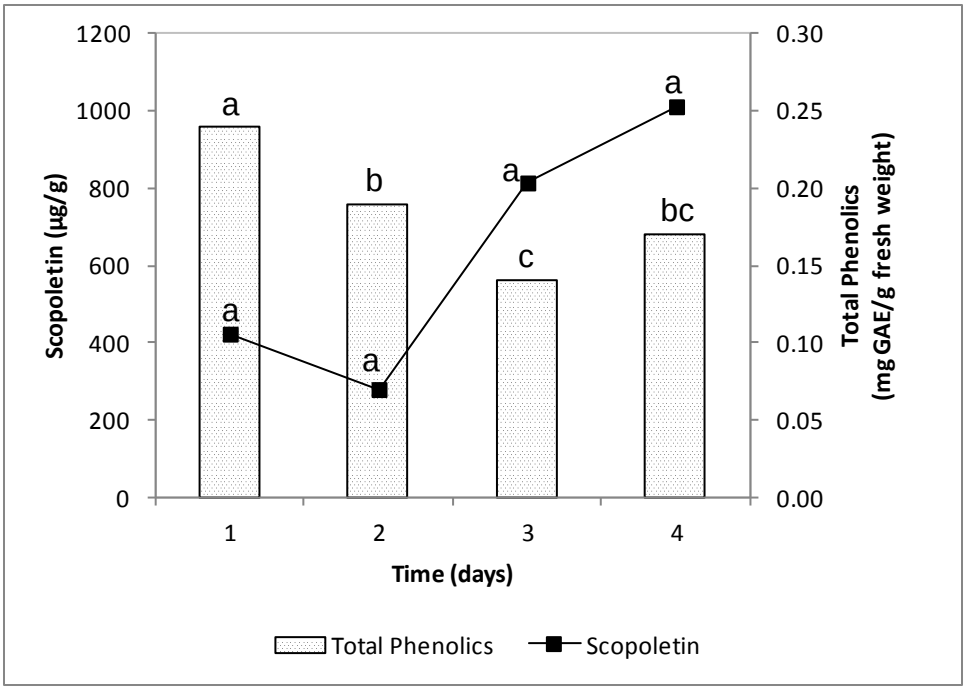

Figure 1. Scopoletin and total phenolic concentration of cubes from wild type cassava roots over a storage time of 4 days at 23 °C. Different letters represent significant differences among time points for each parameter ($p \leq 0.05$).

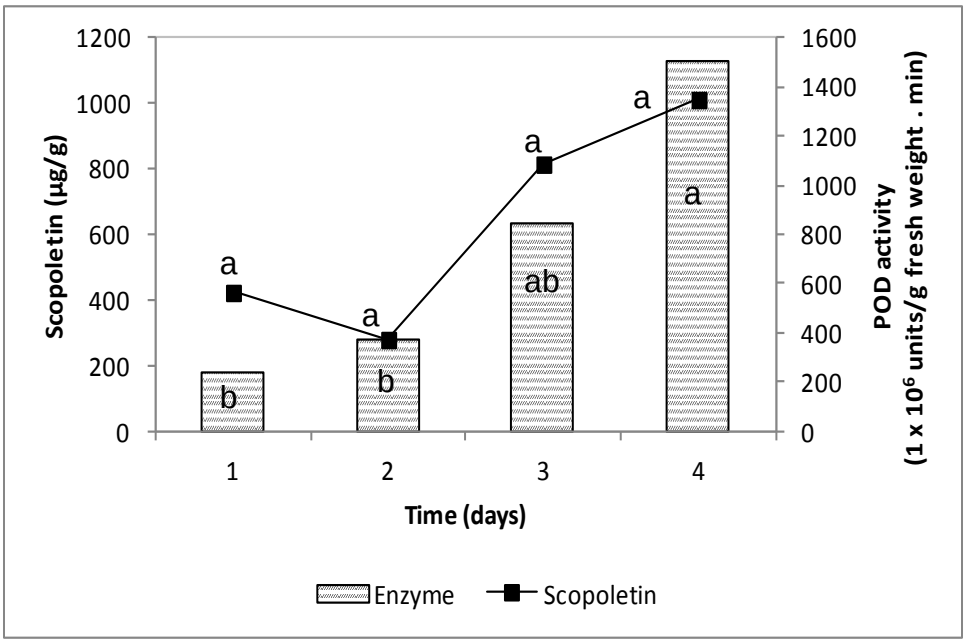

Figure 2. Scopoletin and peroxidase activity of wild type cassava roots over a storage time of 4 days at 23 °C. Different letters represent significant differences among time points for each parameter ($p \leq 0.05$).

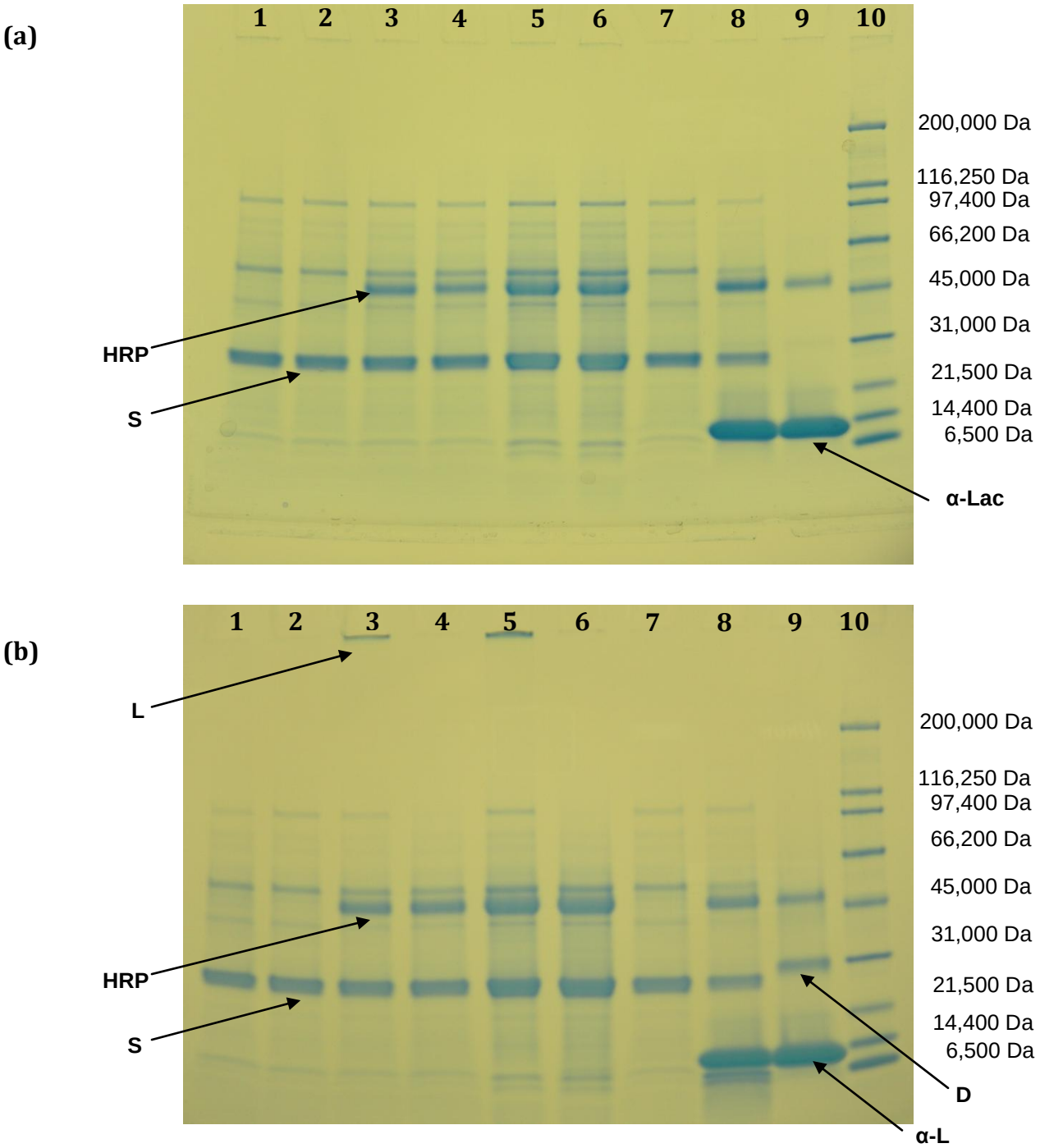

Figure 3. SDS-PAGE pattern with reducing conditions at 0 h **(a)** and 24 h **(b)** incubation using 4-20% gradient gel loaded with: **(1)**Sweet potato protein extract, **(2)**Sweet potato protein extract +Scopoletin, (**3&5)**Sweet potato protein extract + Scopoletin + Peroxidase + H_2O_2, **(4&6)**Sweet potato protein extract + Peroxidase + H_2O_2, **(7)** Sweet potato protein extract + H_2O_2, **(8)** Lactalbumin + Sweet potato protein extract + Peroxidase + H_2O_2, **(9)**Lactalbumin + Peroxidase + H_2O_2, **(10)**Standard solution. **α-L:** α-Lactalbumin; **S:** Sporamin; **HRP:** Horseradish peroxidase; **D:** dimer; **L:** large aggregates.

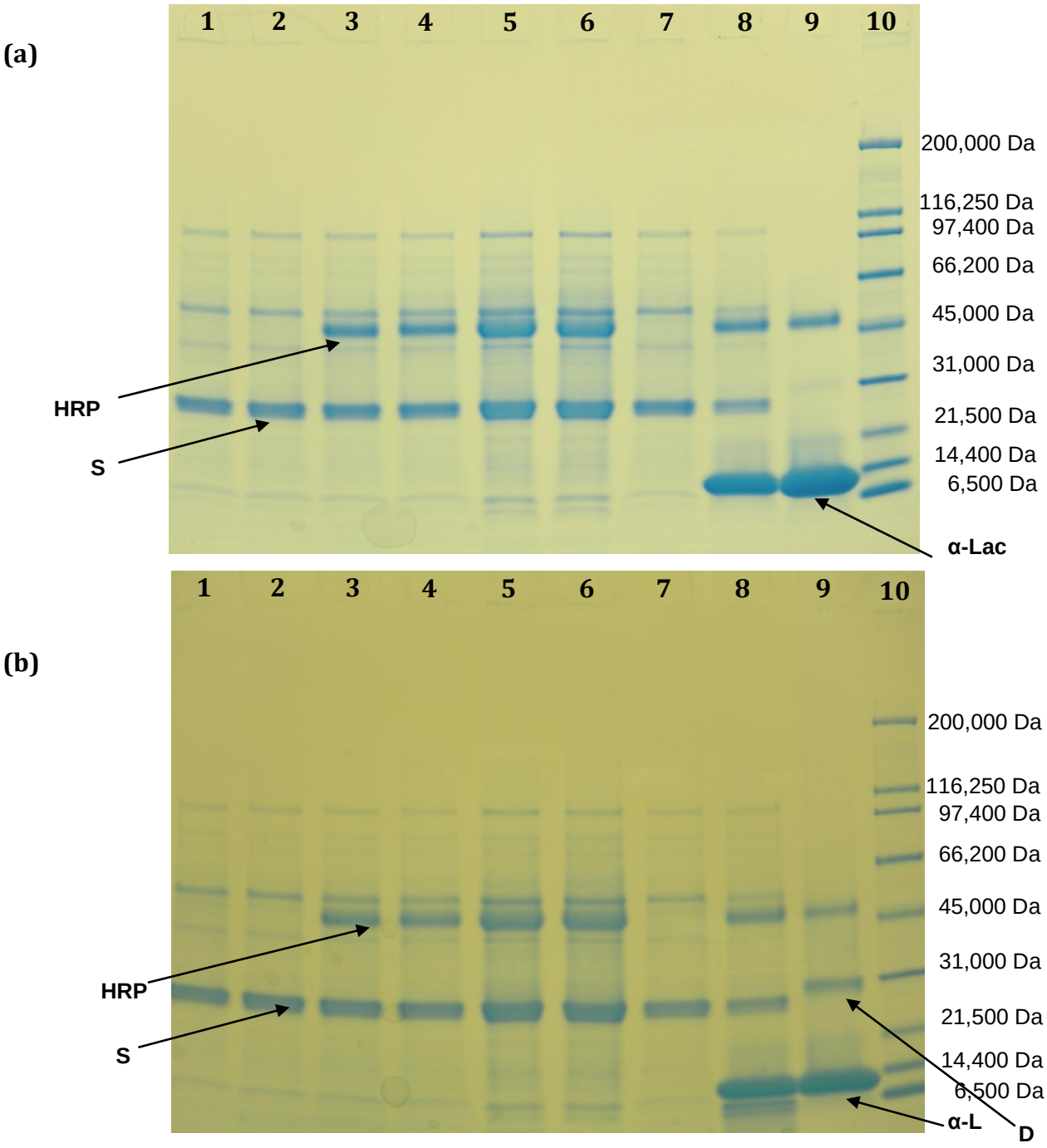

Figure 4. SDS-PAGE pattern with reducing conditions at 0 h **(a)** and 24 h **(b)** incubation using 4-20% gradient gel loaded with: **(1)**Sweet potato protein extract, **(2)**Sweet potato protein extract +crude phenolic extract, (**3&5)**Sweet potato protein extract + crude phenolic extract + Peroxidase + H_2O_2, **(4&6)**Sweet potato protein extract + Peroxidase + H_2O_2, **(7)** Sweet potato protein extract + H_2O_2, **(8)** Lactalbumin + Sweet potato protein extract + Peroxidase + H_2O_2, **(9)**Lactalbumin + Peroxidase + H_2O_2, **(10)**Standard solution. **α-L:** α-Lactalbumin; **S:** Sporamin; **HRP:** Horseradish peroxidase; **D:** dimer.

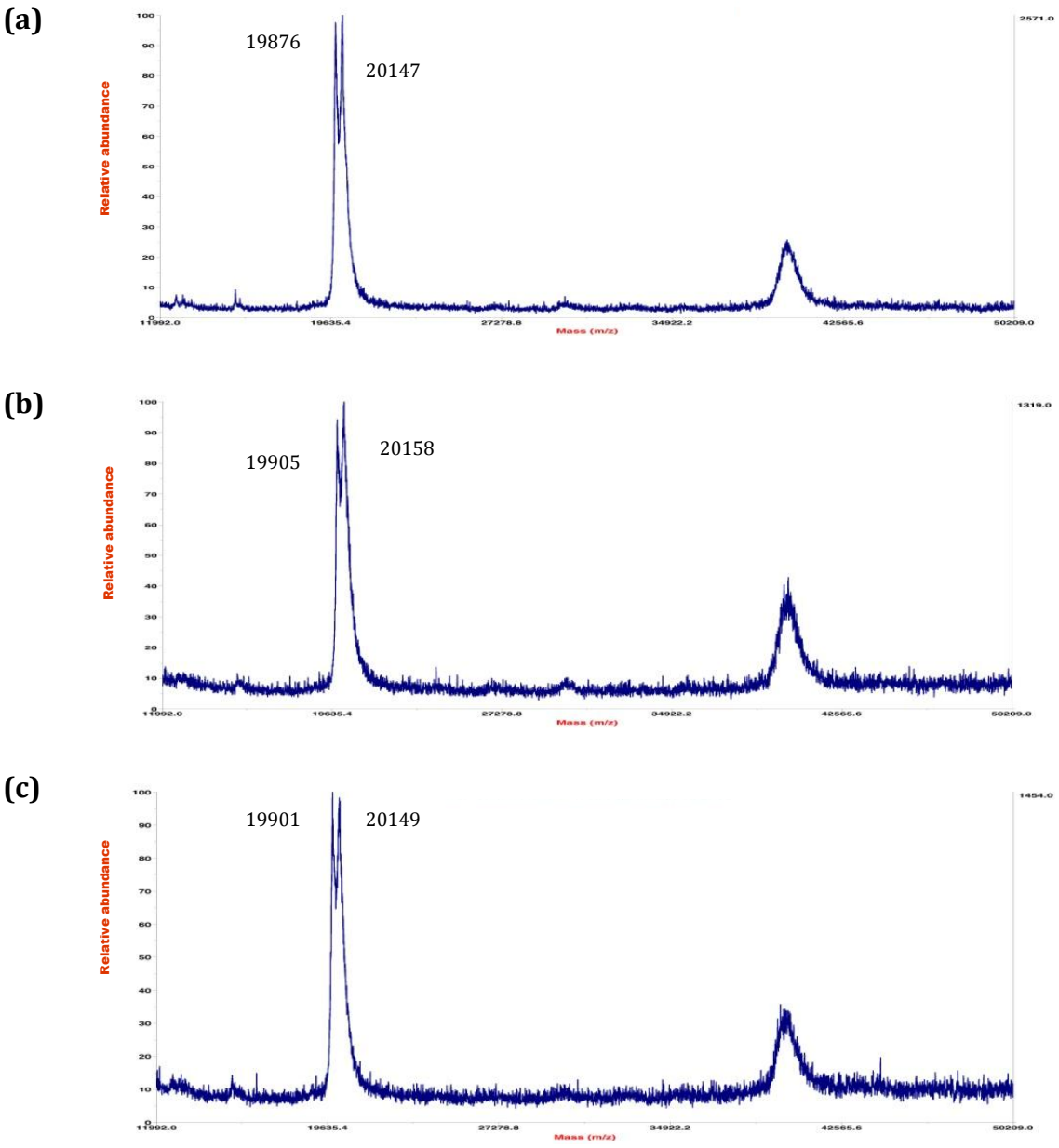

Figure 5. MALDI of sweet potato protein solution and derivatives at 24 h incubation: (a) Sweet potato protein extract (control), (b) Sweet potato protein extract + Scopoletin + Peroxidase + H_2O_2, (c) Sweet potato protein extract + Peroxidase + H_2O_2.

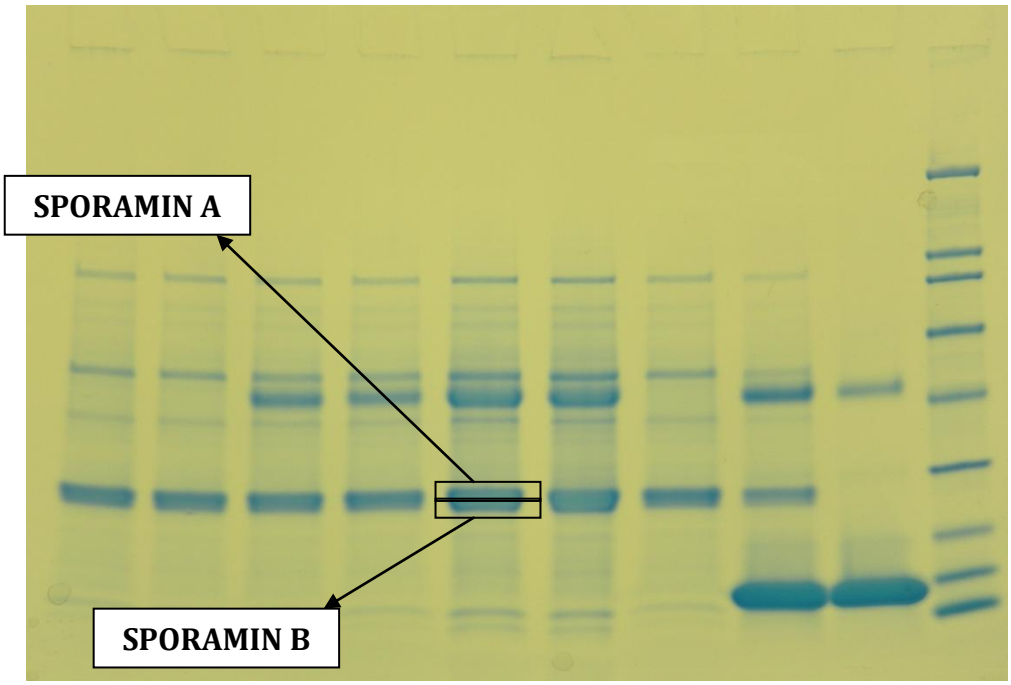

Figure 6. Sporamin A and B in sweet potato protein major bands in SDS-PAGE examined by LC-MS/MS.

(a)

EPTTHEPA**SSETPVLDINGDEVR**AGGNYYMVSAIWGAGGGGLRLAHLDMMSKCASDVIVSPNDL
DNGDPITITPATADPESTVVMASTYQTFRFNIATNKLCVNNVNWGIQHDSASGQYFLKAGEFVSD
NSNQFKIEVVDANLNFYKLTYCQFGSDKCYNVGRFHDPLMRTTR**LALSNSPFVFVIKPTDV**

(b)

LRETA**SSETPVLDINGDEVR**AGENYYIVSAIWGAGGGGLRLVRLDSSSNECASDVIVSRSDFDNGD
PITITPADPESTVVMPSTFQTFRFNIATNKLCVNNVNWGIKHDSESGQYFVKAGEFVSDNSNQFKI
EVVNDNLNAYKISYCQFGTEKCFNVGRYYDPLTRATR**LALSNTPFVFVIKPTDM**

Figure 7. Amino acid sequences of (a) Sporamin A and (b) Sporamin B obtained with the Basic Local Alignment Search Tool (BLAST) provided by the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Lighter and darker shadings indicate the N and C terminals of the sequences, respectively.

a) Sporamin A

```
  1 EPTTHEPASS ETPVLDINGD EVRAGGNYYM VSAIWGAGGG GLRLAHLDMM
 51 SKCASDVIVS PNDLDNGDPI TITPATADPE STVVMASTYQ TFRFNIATNK
101 LCVNNVNWGI QHDSASGQYF LKAGEFVSDN SNQFKIEVVD ANLNFYKLTY
151 CQFGSDKCYN VGRFHDPLMR TTRLALSNSP FVFVIKPTDV
```

b) Sporamin B

```
  1 LRETASSETP VLDINGDEVR AGENYYIVSA IWGAGGGGLR LVRLDSSSNE
 51 CASDVIVSRS DFDNGDPITI TPADPESTVV MPSTFQTFRF NIATNKLCVN
101 NVNWGIKHDS ESGQYFVKAG EFVSDNSNQF KIEVVNDNLN AYKISYCQFG
151 TEKCFNVGRY YDPLTRATRL ALSNTPFVFV IKPTDM
```

Figure 8. Coverage of sporamin A and B at 0 h incubation indicated by the darker shading of the sequences.

a) Sporamin A

```
  1 EPTTHEPASS ETPVLDINGD EVRAGGNYYM VSAIWGAGGG GLRLAHLDMM
 51 SKCASDVIVS PNDLDNGDPI TITPATADPE STVVMASTYQ TFRFNIATNK
101 LCVNNVNWGI QHDSASGQYF LKAGEFVSDN SNQFKIEVVD ANLNFYKLTY
151 CQFGSDKCYN VGRFHDPLMR TTRLALSNSP FVFVIKPTDV
```

b) Sporamin B

```
  1 LRETASSETP VLDINGDEVR AGENYYIVSA IWGAGGGGLR LVRLDSSSNE
 51 CASDVIVSRS DFDNGDPITI TPADPESTVV MPSTFQTFRF NIATNKLCVN
101 NVNWGIKHDS ESGQYFVKAG EFVSDNSNQF KIEVVNDNLN AYKISYCQFG
151 TEKCFNVGRY YDPLTRATRL ALSNTPFVFV IKPTDM
```

Figure 9. Coverage of sporamin A and B at 24 h incubation indicated by the darker shading of the sequences.

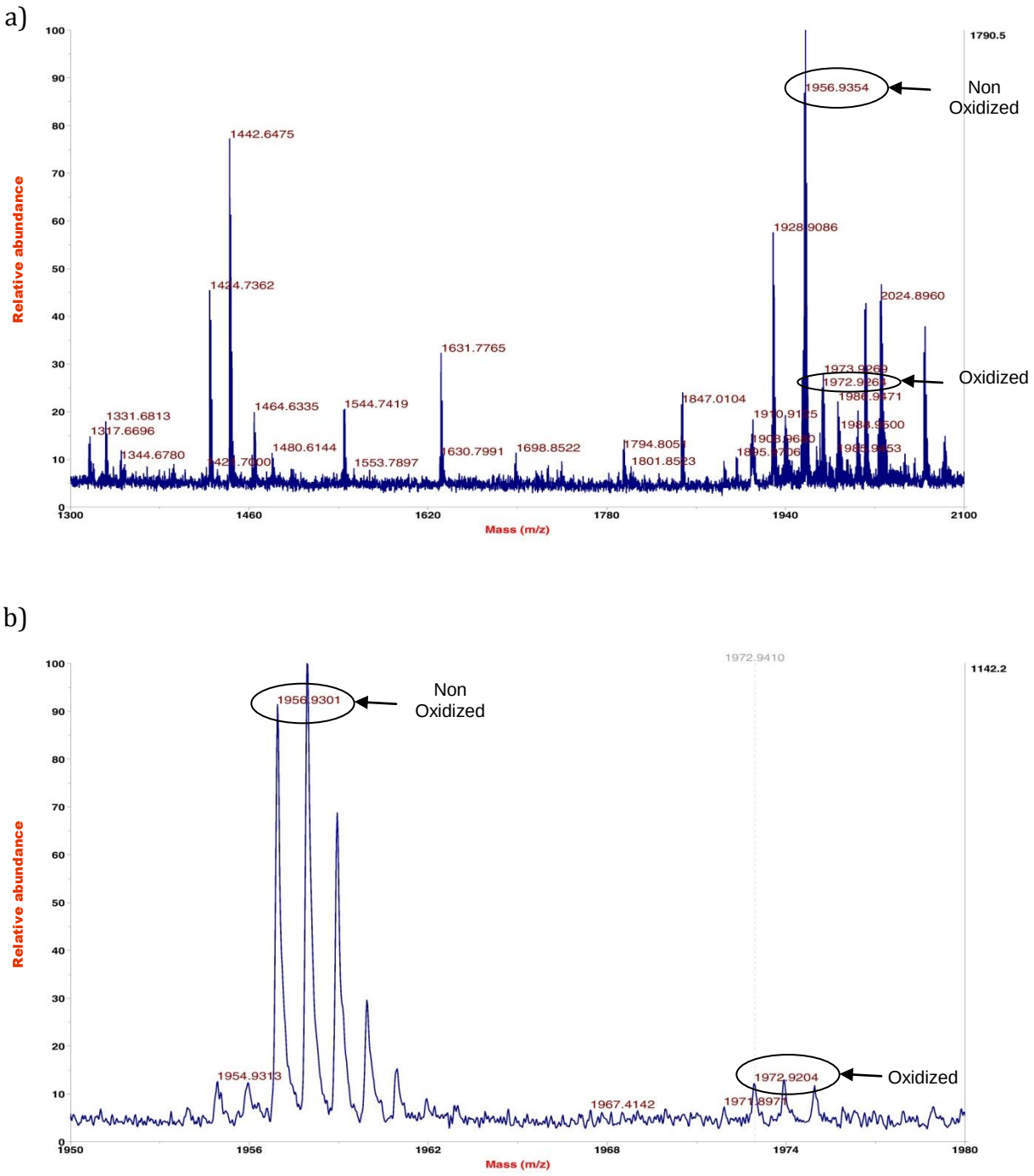

Figure 10. Reflector positive mode- MALDI-TOF spectra of the sporamin band in an oxidative model system (a) with scopoletin, and (b) without scopoletin showing the oxidized and non oxidized N-terminal peptides at 24 h incubation.

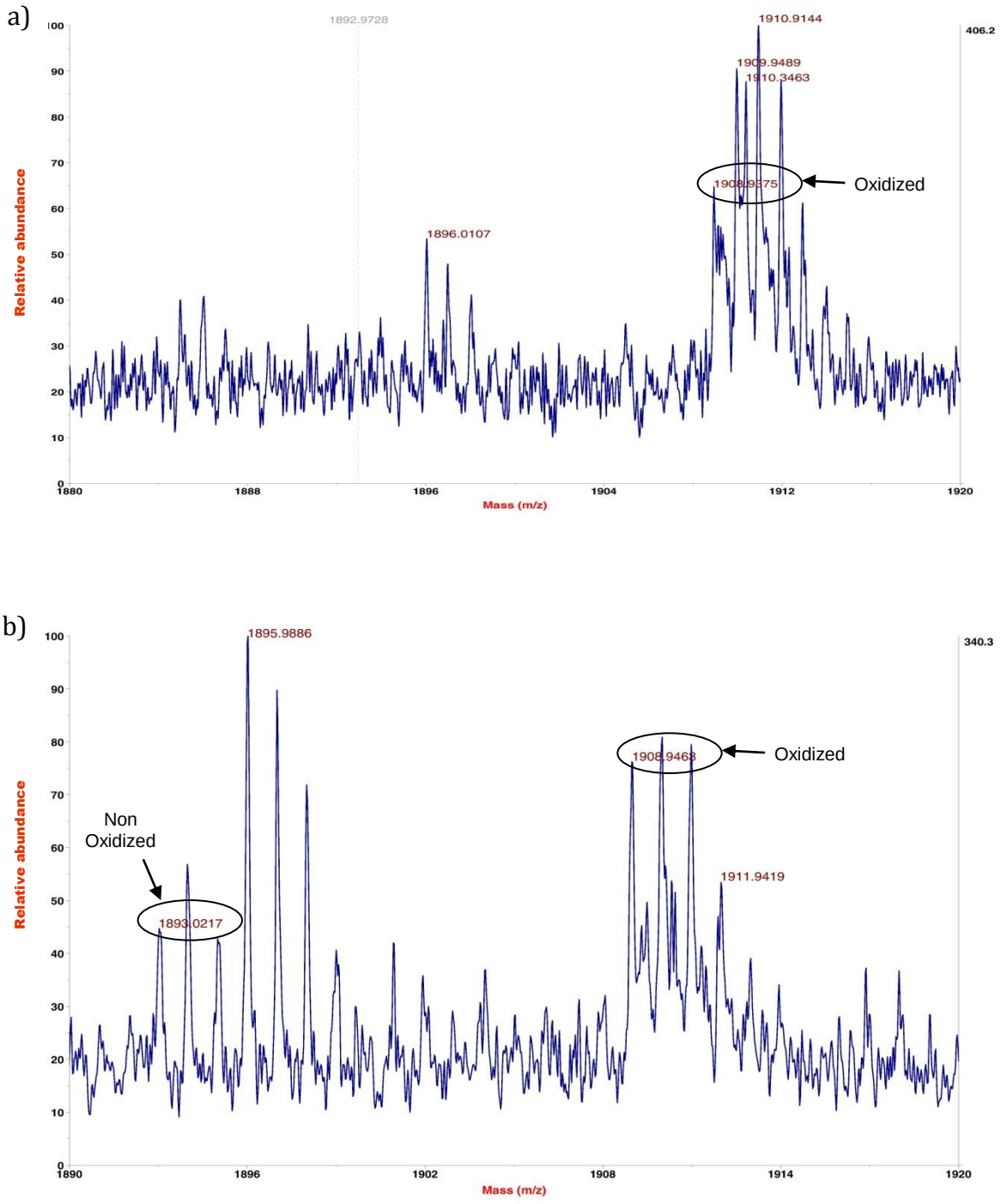

Figure 11. Reflector positive mode- MALDI-TOF spectra of the sporamin band in an oxidative model system (a) with scopoletin, and (b) without scopoletin showing the oxidized and non oxidized C-terminal peptides at 24 h incubation.

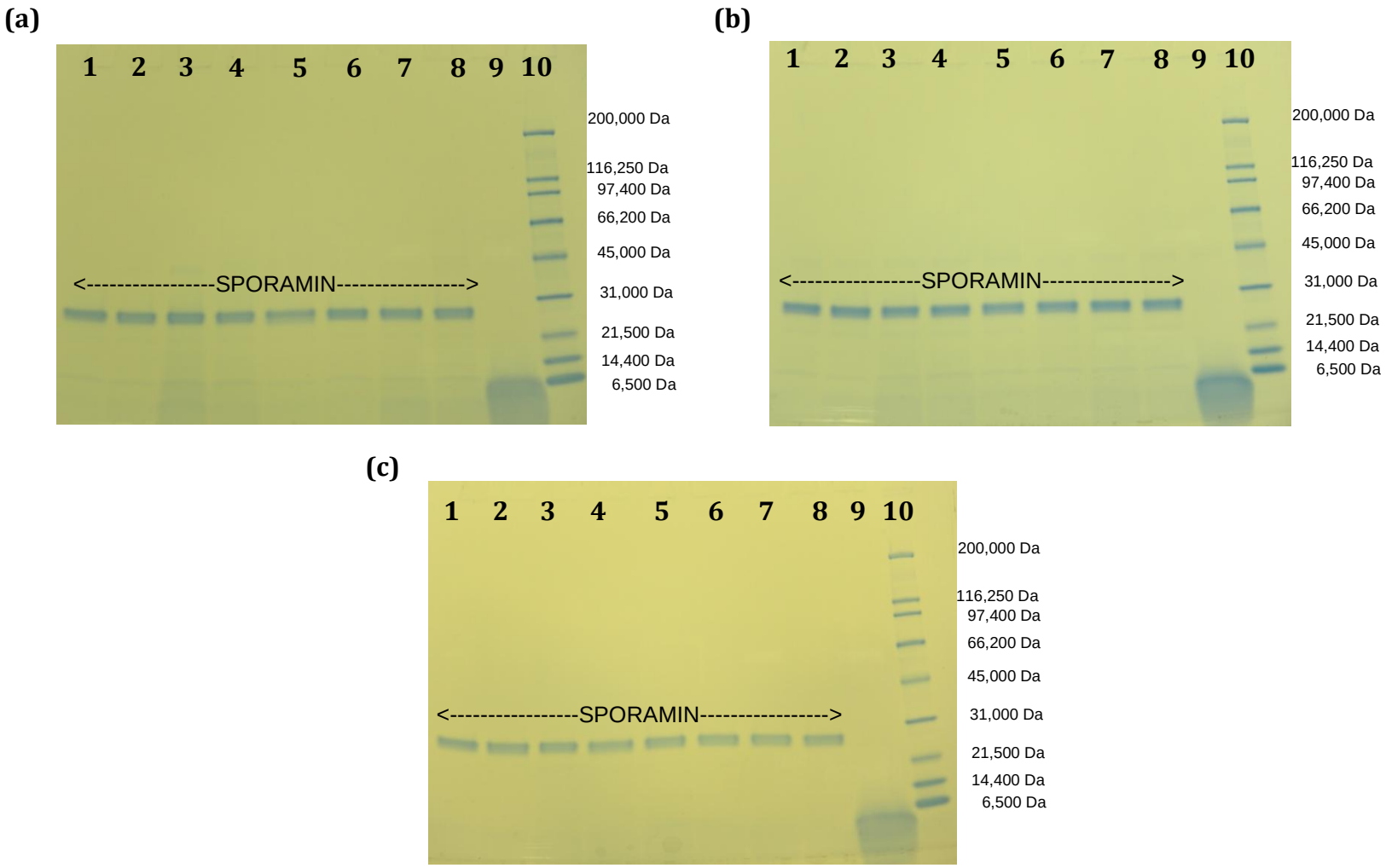

Figure 12. SDS-PAGE pattern with reducing conditions at **(a)**1 h, **(b)**6 h and **(c)**24 h of pepsin digestion using 4-20% gradient gel loaded with: **(1)**Sweet potato protein extract, **(2)**Sweet potato protein extract +Scopoletin, (**3)**Sweet potato protein extract + Scopoletin + Peroxidase + H_2O_2, **(4)**Sweet potato protein extract + Peroxidase + H_2O_2, **(5)**Sweet potato protein extract, **(6)**Sweet potato protein extract + crude phenolic extract, **(7)**Sweet potato protein extract + crude phenolic extract+ Peroxidase + H_2O_2, **(8)**Sweet potato protein extract + Peroxidase + H_2O_2, **(9)**Lactalbumin + Peroxidase + H_2O_2, **(10)**Standard solution.

CHAPTER IV

PHYSICO-CHEMICAL ANALYSIS OF FERMENTED PROTEIN FORTIFIED CASSAVA FLOUR (WET FUFU)

ABSTRACT

Suitability of *Lactobacillus plantarum* strain 6710 was investigated for use as starter for wet fufu, a common cassava-based staple in West Africa, using protein (zeolin, sporazein, sporazein plus pro-vitamin A) and pro-vitamin A fortified cassava flours and low protein wild type cassava flours in lab-scale fermentation trials at 32 °C for 4 days.

All cassava flours were allowed to ferment with or without the addition of the starter culture. Initial lactic acid bacteria (LAB) count in non inoculated (NF) and inoculated (LF) wild type cassava flours were 5.19 log CFU/g and 8.19 log CFU/g, respectively. LAB count of non inoculated flour increased 4 log units in 24 h, while inoculated sample increased 1 log unit. After 3 days, the LAB count in the wild type inoculated flour was similar to non inoculated sample, 8.74 and 8.69 log CFU/g, respectively. A similar trend was observed for the fortified cassava flours with the added starter, with numbers reaching a maximum at 24 h then decreasing.

The added strain rapidly increased titratable acidity (TA) when using wild type cassava e.g. 0.24 to 0.96% lactic acid at 24 h to 1.18% at 96 h. TA also increased with sporazein, sporazein plus pro-vitamin A and pro-vitamin A samples to 1.67, 1.78 and 1.73%

at 96 h, respectively. The starter culture resulted in a rapid reduction in pH to a mean of 3.73 at 24 hours compared to 4.47 for non inoculated or naturally fermented samples (controls).

Volatile compounds such as 2-propanone, 2-butanone and 1-hexanol were detected in all cassava samples at all fermentation times. Acetic acid was found in all samples but the wild type and fortified zeolin, sporazein plus pro-vitamin A and pro-vitamin A samples at 0 h fermentation time. A similar aroma profile was found in all samples suggesting that protein fortification does not have a detrimental effect on the aroma of fufu. This study showed that use of *L. plantarum* is feasible for production of wet fufu from protein fortified cassava making possible increased consumption of a nutritious product.

Keywords: Cassava, fufu, protein, *Latobacillus plantarum*.

Practical Application: Consumption of fermented cassava products constitutes an important part of a daily diet in Sub-Saharan Africa where cassava is a staple food. Cassava is mainly starch and its protein content is very low in the roots. Preparation of fufu, a common cassava-based fermented product, with protein fortified cassava material will provide a more nutritious product and help reduce malnutrition. The effects of using protein fortified materials on fermentation and chemical characteristics of fufu and how these affected the lactic acid bacteria growth were elucidated.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the staple root crop of over 800 million people (Nassar and others 2007) in the humid tropics and ranks sixth in terms of overall global crop production (Han and others 2001). The significant growth in the urban population in developing countries is bringing with it associated changes in consumer demand, especially for food and feed products, opening up new opportunities and challenges for the production of cassava such as flour and starch for food and non-food uses.

Cassava is the major source of calories for many in underdeveloped countries. However, a cassava-based diet does not provide complete nutrition. Cassava is cultivated mainly for its edible storage root, which contains 85% starch and only 1-2% protein (Sheffield and others 2006).

As mentioned by Abhary and others (2011) few reports exist of successful transgenic modification of starchy storage organs to accumulate storage proteins. Abhary and collaborators genetically modified cassava, resulting in high protein content material.

A major limitation of cassava is the potential toxicity of cassava roots or products. A common biochemical feature of cassava plant is the accumulation of cyanogens in vegetative tissues, especially the edible leaves and tubers. According to Nweke and Bokanga (1994), cassava produces the cyanogenic glycosides linamarin and lotaustralin. In Africa, improperly processed cassava is a major problem. It is associated with a number of cyanide-related health disorders, particularly among people who are already malnourished

(Maziya-Dixon and others 2007). Numerous cassava processing methods have been devised, including sun drying, soaking and fermentation followed by drying or roasting, to reduce the toxicity (cyanogenic compounds) while at the same time converting the highly perishable fresh roots of cassava into stable products (Vasconcelos and others 1990). According to Westby (1994) and Brauman and others (1996), the fermentation of cassava roots, called retting, allows the reduction of potentially toxic endogenous cyanogens, which are present in variable concentrations (300 to 500 ppm), and improves palatability for further processing. Among fermented products, fufu is a favorite cassava-based entrée popular in many parts of West Africa (Uyoh and others 2009).

Fufu (foofoo or foufou) is a staple in many West African countries, especially Cameroon, Ghana and Nigeria. Fufu is a fermented wet paste product, usually consumed in the form of cooked or pounded hand-molded balls, with vegetables, spicy sauces or soup (Fungwe 1998; Obadina and others 2009). For production of fufu, the cassava roots are peeled, washed, cut into pieces and submerged in water at room temperature for 5 days (Ray and Sivakumar 2009), but it has been found that local processors ferment cassava for different lengths of time (Oyewole and Ogundele 2001). During this period, the cassava roots ferment and soften, releasing hydrogen cyanide into the soak water, reducing pH level and imparting the characteristics flavor of the retted cassava meal. The mash is sieved through small baskets to remove the ligneous central strands. The solid residue is pressed to drain off the water and formed into small balls. The fufu is sold to consumers in wet form in small units packaged in plastic or polypropylene bags or in ready to eat cooked form (Ray and Sivakumar 2009).

Species of lactic acid bacteria (LAB) such as *Lactobacillus, Leuconostoc* and *Streptococcus* are the predominant micro-organisms in fufu along with *Bacillus subtilis, Enterococcus, Klebsiella and Candida krusei* (Ray and Sivakumar 2009).

According to Sobowale and Oyewole (2008) most of the LAB isolated were strains of *Lactobacillus plantarum.* In Africa, most food fermentations are done on a household level, and are still conducted as spontaneous processes. Initiation of spontaneous fermentation takes a relatively long time, with a high risk of failure that can result in spoilage and/or survival of pathogens. Thus, the use of starter cultures is recommended, as it would lead to a rapid acidification of the product inhibiting the growth of spoilage and pathogenic bacteria and to a product with consistent quality.

During the fermentation of foods, several chemical compounds are formed. These include steam volatile acids (formic, acetic and propionic); non-volatile acids (lactic and succinic); volatile neutral compounds (ethanol, acetone and isopropanol); non-volatile neutral compounds (glycerol and 2,3-butanediol); butyric, butanediol, acetoin and some gases (Kuye and Sanni 1999). According to Uyoh and others (2009), fufu may be unacceptable for many people because of the flavor of the product. *Lactobacillus plantarum* has been found to have the highest acid producing ability in cassava tubers (Oyewole 1990; Kostinek and others 2008), therefore, a strain of this species, selected on the basis of technological properties in previous investigations (Huch and others 2008), was used in an attempt to reduce fermentation time that may cause the production of unacceptable aroma compounds.

The role of *L. plantarum* in cassava fermentation for fufu processing underlies our study to elucidate the contribution of a strain of the selected *L. plantarum* to the production of high quality protein fortified fermented cassava (wet fufu). Proteolytic activity of the *L. plantarum* strain used is unknown. While traditional fufu processing involves the utilization of cassava roots, this study proposes the use of cassava flour as starting material.

The objectives of this study were to determine the influence of a selected lactic acid bacteria starter culture on the course of fermentation on protein and pro-vitamin A fortified cassava flours. We evaluated microbiological and physico-chemical characteristics including the aromatic compounds of a fermented cassava product (wet fufu) relative to different fermentation times. This could help producers to evaluate the viability of providing high protein fermented cassava products.

MATERIALS AND METHODS

Cassava flours

Four types of protein fortified cassava flours (zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO)) and wild type cassava flour were provided by the International Laboratory for Tropical Agricultural Biotechnology (ILTAB) (St. Louis, MO).

Microbiological Assessment

Culture

L. plantarum BFE 6710 strain grown as a stab culture was provided by the Max Rubner-Institut (Karlsruhe, Germany) (Edward and others 2011) and routinely grown in Lactobacilli de Man, Rogosa and Sharpe (MRS) broth (Difco™, Becton Dickinson and Company, Sparks, MD) at 32 °C for 24 h under aerobic conditions.

Culture propagation and storage

After growth, culture was placed in a cryo tube with 50% glycerol and stored at -72 °C for further use (stock culture). Working cultures were obtained from stock culture. The cells were streaked on MRS agar and incubated at 32 °C for 48 h such that the surface of the bacterial colony was slightly above the surface of the agar and transferred into MRS broth. Cultures were propagated twice before use. The preculture was centrifuged at 8000 xg at 4 ºC for 10 min. The pellet was washed twice with buffered peptone water (BBL™, Becton Dickinson and Company, Sparks, MD) and centrifuged. The final cell pellet was resuspended into 9 mL of buffered peptone water with an initial concentration of 7×10^{10} CFU/mL.

Growth of starter culture in cassava flour

All wild type and fortified cassava flours were preserved in sealed plastic containers at 4 °C. Ninety g of cassava flour were transferred to sterilized plastic containers (under UV light for 15 min). Initial inoculation of all flours by *L. plantarum* was done by

transferring a cell pellet suspension with a concentration of 7 x 10^{10} CFU/mL into cassava flour. Sterile water was added to adjust the total moisture content of the cassava sample to the average moisture of the cassava root (68%). A second set of non inoculated flours was also prepared. Due to the limited amount of available cassava flour material, this experiment was done only once. Non inoculated (NF) and inoculated flours (LF) were covered and kept at 32 °C for 96 h fermentation.

Monitoring lactic acid bacteria (LAB) growth

Cassava sample was removed at 0, 24, 48, 72 and 96 h of fermentation and assessed as follows: One 5 g sample of mash from each container was placed in a stomacher bag (Seward 80 mL, Worthing, UK). Forty-five mL of buffered peptone water was added and the sample was homogenized for 2 min using a stomacher (Seward Stomacher® 80 Biomaster). One hundred µL of appropriate ten-fold serial dilutions of the sample in 0.2% peptone water (Bacto™, Becton Dickinson and Company, Sparks, MD) were spread-plated in duplicate on MRS agar for cultivation of lactic acid bacteria (LAB) with incubation at 32 °C for 48 h for enumeration.

Chemical analyses

Fermented cassava (wet fufu) was removed at different fermentation times and kept at -70 °C for 7 days until analysis.

Moisture.- Moisture of cassava flours was determined by the air oven method 925.10 (AOAC 2000b). Sample (2 g) was dried at 130±3 °C for 1 h.

Ash.- Ash of cassava flours was determined by the direct method 923.03 (AOAC 2000a). Sample (3-4 g) was weighed into an ashing dish (crucible) and ignited in the muffle furnace at 550ºC until light gray ash resulted (approximately 8 h).

Soluble Protein.- Unfermented and fermented (wet fufu) cassava flours were extracted with 100 mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM EDTA, 0.2% Triton X-100 with 4% 2-mercaptoethanol. The homogenate was vortexed for 5 min at 2500 rpm and centrifuged at 1500 x g for 10 min at 4°C. Supernatant was analyzed by the Bradford method (CBX kit) (G-Biosciences, St. Louis, MO) according to manufacturer's protocol. A calibration curve was constructed with bovine serum albumin included in the CBX kit as a standard.

Nitrogen.- Total nitrogen of cassava flours was determined by Approved Method 46-30.01 (AACC 2010) with a LECO Instrument FP-528 nitrogen analyzer (Leco, St. Joseph, MI) equipped with a thermoconductivity detector. Briefly, 0.1 g of sample was placed in an aluminum foil and folded as a capsule and analyzed by the instrument.

Total starch content.- Total starch of cassava flours was determined by the total starch assay procedure (amyloglucosidase/α-amylase) K-TSTA 04/2009 (Starch assay kit,

Megazyme International Ireland Ltd., Wicklow, Ireland). Methods (e) and (a) were combined in that sequence.

pH

pH was determined by a modified AOAC pH of flour method 943.02, sec. 32.1.20 (AOAC 2000d). A 3 g portion of flour or fermented cassava flour (wet fufu) was homogenized with 30 mL of Milli Q water. Homogenized samples were dispersed for 30 min by shaking. An electronic pH-meter AB15 Plus accumet® Basic (Fisher Scientific) was used with a pH electrode accu TupH.

Titratable Acidity

The titratable acidity (TA) was determined by a modified AOAC glass electrode method 942.15, sec. 37.1.37B (AOAC 2000c). A 3 g portion of flour or fermented cassava flour (wet fufu) was homogenized with 30 mL of Milli Q water. Homogenized samples were dispersed for 30 min by shaking. Sample was titrated to pH 8 with 0.1 M NaOH using a titration unit TitroLine easy Schott Instruments GmbH (Mainz, Deutschland, Germany). The titer volume was multiplied by 0.09 to give %TA as lactic acid.

Physical analyses

Fermented cassava (wet fufu) was removed at different fermentation times and kept at -70 °C for 7 days until evaluation.

Particle Size Distribution of Cassava Flour (PSDC)

Particle size distribution of cassava flour was determined using a sonic sifter (model L3, ATM Corp., Milwaukee, WI). A modified method as specified by Nair and others (2011) was followed with some modifications. Briefly, cassava flour (2 g) was sifted through a series of pre-weighed stainless steel screens with 125, 149, 177, 250 and 420 µm openings by vibration in the sift/pulse mode with a sift amplitude of 10 and two cycles of shaking/sieving time of 6 min each. After 12 min of sieving, the weight of flour on each sieve was determined. PSDC was expressed as percentage of flour on each sieve divided by total flour weight.

Cassava starch granular morphology characterization by microscopy

Cassava flours and freeze dried fermented cassava (wet fufu) were placed on microscope slides. The samples were visually inspected for granule swelling and damage with a Nikon Eclipse E600 microscope (Nikon Inc. Instrument Inc., Melville, NY). Images were taken using a 50 X/0.90 oil-immersion lens (Plan; Nikon) and captured with the QCapture software (release 2.66.4, Surrey, BC, Canada). Starch granules were visualized microscopically under both normal and polarized light.

Pasting properties

Cassava flours starch pasting properties were measured in with the Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd., Warriewood, Australia) interfaced with a personal computer equipped with Thermocline software for Windows (release 2.1,

Newport Scientific Pty. Ltd., Warriewood, Australia) according to AACC Approved Method 76-21.01 (AACC 2000). The sample was slurried by mixing 3.5 g (14% moisture basis) with 25 mL of water into the RVA canister, stirred manually by rotating the plastic paddle for 10 s to disperse the sample uniformly and to remove lumps, and inserted into the tower. The heating and cooling cycles were programmed in the following manner: The samples were held at 50 °C for 1 min, heated 50 to 95 °C in 4.42 min, held at 95 °C for 2.7 min, cooled to 50 °C in 3.82 min, and held at 50 °C for the remainder of the run. The total run time was 13 min. Parameters estimated were peak viscosity, setback viscosity, final viscosity, pasting temperature and time to reach peak viscosity (Petruccelli and others 1993; Maziya-Dixon and others 2007).

Color evaluation

A Minolta colorimeter (Minolta Spectrophotometer CM-2002; Minolta Camera Co., Ltd., Osaka, Japan) was used to measure the color of cassava flours. Values of L*, a*, and b* were recorded, where L* is lightness or darkness, whose value varies from 100 for perfect white to zero for black; +a* is redness (0-60) and –a* is greenness (0 to -60); +b* is yellowness (0-60) and –b* is blueness (0 to -60) (Carreno and others 1995; Francis 1998). The colorimeter was calibrated using an internal white calibration plate. The cassava flour was placed into plastic Petri dishes, slightly shaken to form a layer of 5 mm thickness, covered with the Petri dish lid and the color of the surface of the flour read on the meter. The average L, a* and b* values were obtained from two randomly selected points on the surface of the flour. The color measurement was performed in

triplicate. The whiteness index, WI, the hue angle, H* (the angle for a point calculated from a* and b* coordinates in the color space by which red, yellow, green and blue are identified) and chroma, C* (quantitative component of the color that distinguishes between vivid and dull colors) were determined according to the following equations (Aloys and Zhou 2006; Matos and Rosell 2012).

$$WI=100 - [(100-L^*)^2 + (a^*)^2 + (b^*)^2]^{1/2} \quad (1)$$

$$H= \tan^{-1} (b^*/a^*) \quad (2)$$

$$C= (a^2+b^2)^{1/2} \quad (3)$$

The net color ($\triangle E$) difference was evaluated with the following equation, using the parameters L*, a* and b* and comparing the different fortified cassava flours with the wild type cassava flour.

$$(\triangle E)^* = [(L_o^*-L^*)^2 + (a_o^*-a^*))^2 + (b_o^*-b^*))^2]^{1/2} \quad (4)$$

Volatile analysis

The method of Iyer and others (2010) with some modifications was used for the identification of volatile compounds in fermented cassava flour (wet fufu) at all fermentation times. Volatiles were collected using headspace-solid phase microextraction (HS-SPME). Optimization of the method was done in order to determine the amount of fermented cassava flour (wet fufu) needed for the analysis based on our experience in GC-analysis.

Briefly, samples at each fermentation time were stored at -70 °C until analysis. A slurry made up of cassava (0.5 g), sodium chloride (0.98 g) and distilled water (3.0 mL) was prepared in a 10 mL headspace amber rounded bottom vial (Cat. No. SU860100) (Sigma – Aldrich, Milwaukee, WI) with a magnetic stirring bar. Sample vials were immediately sealed with a screw cap with PTFE/silicone septum (Cat. No. SU860101) (Sigma – Aldrich, Milwaukee, WI). Automation of the procedure was achieved using a CTC CombiPal autosampler (Zwingen, Switzerland), which was programmed using a CycleComposer software version A.01.04 (Agilent Technologies Inc.) and equipped with a sample tray, a temperature controlled agitator tray and a fiber-conditioning device. A SPME stableflex fiber coated with 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) (57293-U) (Supelco, Bellefonte, PA) was conditioned for 1 h at 270 °C in the fiber conditioner of the COMBI PAL system before use. Sample was stirred at 250 rpm, the fiber was exposed to the headspace of the slurry and the volatile compounds were collected for 60 min based on previous optimization studies (Iyer and others 2010).

The volatiles adsorbed onto the SPME fiber were thermally desorbed into the injection port of an Agilent Technologies 6890 series gas chromatograph (Agilent, Avondale PA) equipped with a 6890N GC split/splitless injector and data collection provided by Chemstation software version E.02.00.493 and a HP-5MS column (5% Phenyl Methyl Siloxane) (30 m x 0.248 mm x 0.25µm film thickness) (J&W Scientific, Folsom, CA). Helium was used as the carrier gas. The injector and detector temperatures were 200 °C and 250°C, respectively. The column temperature was initially maintained at 33 °C for 5 min before increasing to 50 °C at a rate of 2 °C/min, and then to 225 °C at a rate of

5 °C/min. The sample was desorbed for 5 min and set in the splitless mode. Fermented cassava flour (wet fufu) volatile compounds were identified by using a mass spectrophotometer MS 5975C (inert XL MSD) and the MS spectra was compared against a NIST library. The quantitative data were determined by running known standards and developing response factors based on water matrices. The final values were reported as µg/mL.

Data Analysis

Cassava flours physico-chemical data were analyzed for significant differences using a one-way analysis of variance (ANOVA). Fermented cassava flours (wet fufu) physico-chemical data were analyzed using a three-way analysis of variances (ANOVA). Tukey's HSD multiple comparisons of means were used to analyze both cassava flours and fermented cassava flours (wet fufu) data with XLSTAT (Version 7.5.3, XLSTAT Addinsoft, France) at the $p \leq 0.05$ confidence level. The ANOVA performed on fermented cassava flours (wet fufu) used sample, fermentation and time as fixed effects. All the tests were performed at least in duplicate. Principal component analysis was applied to identify the volatile compounds associated with each one of the fermented cassava flours at all fermentation times.

RESULTS AND DISCUSSION

Microbial count

The microbial count assessed on MRS media showed that there was a background microbial population of presumptive, indigenous LAB in the unfermented (0 h) NFWT, NF SPRO and NF PRO samples with 1.55 x 10^5, 6.47 x 10^4 and 9.63 x 10^5 CFU/g, respectively. Background microbial population also was found by Huch and others (2008) who determined LAB counts of 2 x 10^2 CFU/g to 6 x 10^3 CFU/g in the non inoculated unfermented cassava mash. Brauman and others (1996) determined that LAB accounted for more than 99% of the total microflora during cassava root fermentation.

Variations of cell numbers are evident in non inoculated (Figure 1) and inoculated samples (Figure 2) at 24 h fermentation. NFZ and NFS sample counts increased 9 log units in 24 h, while LFZ and LFS increased 1 log unit. Through the fermentation process, a greater variation was observed in LF cassava samples (Figure 2) after 24 h compared to NF samples (Figure 1).

Lactobacillus plantarum (homofermenting population), as facultative anaerobic LAB, could develop from the onset of retting, when oxygen was still present in the medium, and thanks to their great growth rates on the fermentable sugars present (sucrose, glucose and fructose), could overcome the other flora (Brauman and others 1996).

Chemical analyses

The chemical composition of the cassava flours (moisture, ash, nitrogen, protein and starch) was determined and expressed on a wet weight basis. Pro-vitamin A fortified cassava sample showed the greatest moisture (9.15±0.06%) and ash content (4.86 ±0.15%). No significant differences in ash content were found between WT and Z ($p>0.05$). In relation to the nitrogen content, Z had the greatest value (1.28 ±0.09%) that is correlated with the greatest soluble protein concentration (9.52 ±0.0073%). No significant differences were observed between the soluble protein content of WT, SPRO and PRO fortified flours with an of 1.41±0.0004%, 3.63±0.003%, 2.14±0.004%, respectively ($p>0.05$). Regarding nitrogen, values of 0.28%, 0.76% and 0.67% were found for WT, SPRO and PRO, correspondingly. The soluble protein content of the S sample was 6.83±0.003% that was significantly lower than the content of the Z fortified cassava flour.

Concerning the starch content, we expected high protein flours to have reduced amount of starch. However there were no significant differences between WT (55.83±3.71%) and S (63.31±2.91%) samples ($p>0.05$). Also, no significant differences in the starch content were found between WT, Z, SPRO and PRO cassava flours with values of 55.83±3.71%, 63.31±2.91%, 50.98±1.57% and 51.56±2.55%, respectively. According to Santisopasri and others (2001), there are several factors that influence the cassava starch properties such as variety, growth conditions and harvest time. They found, for example, that cassava plants subjected to water stress conditions contained a low content of starch (1.2-3.5% at 6 months). Greater values were obtained for plants without initial water stress (20.4-25.9% at 6 months). Other studies reported 84% starch in fresh cassava roots

imported from Costa Rica and 74.2-81.4% dry basis in cassava flour from Venezuelan varieties (Rodríguez-Sandoval and others 2008; Mejía-Agüero and others 2011). On the other hand, our results were similar to the findings of Niba and others (2002), who reported a starch content range from 62.8 to 75.7 g/100 g dry weight in Nigerian cassava varieties.

Through the fermentation process a reduction of the protein content was observed for each cassava sample (Figures 3-4). No significant differences were observed on the protein content as a function of addition of the starter culture *Lactobacillus plantarum* at all fermentation times ($p>0.05$). Protein content of all fermented cassava flours decreased more than 60% at 96 h of fermentation. NF PRO showed the greatest reduction (78%) as a result of the 96 h fermentation time. During this investigation, the protein level of NFWT decreased from 0.25% at the beginning of fermentation to 0.09% at 96 h. Oyewole and Odunfa (1989) determined and increase of the total protein content to 1.2% at the end of the 96 h of fermentation following a traditional method for fufu processing. During gari production, Ahaotu and others (2011) also found an increase of protein content due to specific microorganisms used during the fermentation process. The sample fermented with mixed fungi showed the greatest amount of protein. The high yield of crude protein is associated with the ability of the organism to convert carbohydrate to protein during the fermentation. Hence, the low protein content observed during fermentation was possibly due to a non efficient utilization of the carbohydrates in the cassava flours (Ahaotu and others 2011).

According to Westby and Twiddy (1992), LAB showed only a weak ability to hydrolyze starch during fufu processing. *Bacillus and Pseudomonas* are known to hydrolyze starch, but these microorganisms form only a small portion of the total population of microbes found in cassava fermentation. LABs in general are not amylolytic. According to Kostinek and others (2005), none of the predominant LAB isolated from fermented cassava showed α-amylase activity. Since cassava generally contains about 84% carbohydrates as starch representing an important potential energy source for the LAB, this is surprising as pointed out by Kostinek and others (2007).

Table 1 shows the pH and titratable acidity (TA) of the unfermented cassava flours. All samples had a pH between 6.31 and 6.72 with S having the greatest value. Regarding acidity, SPRO showed the highest value (0.73% lactic acid) and the lowest was WT (0.37% lactic acid). Tables 2 and 3 show the pH and TA data, respectively of the NF and LF cassava flours at 5 different fermentation times. The fermentation process resulted in an increase of acidity of the cassava mash. The level of acidification increased with increasing period of fermentation. LF samples recorded the greatest values of titratable acidity (Table 3) expressed as lactic acid. Ogunbanwo (2005) cited that lactic acid is a powerful compound that creates pH values below 4.5 and has antimicrobial activity.

Lactobacillus plantarum caused a decrease in the pH in all cassava samples and at all fermentation times. Z sample showed a decrease of pH from 6.02 (0 h) to 3.25 at 96 h for NF and from 5.15 (0 h) to 3.28 (96 h) for LF (Table 2). A similar trend was observed in all samples. The lowest pH was reached at 72 h of fermentation in LF samples. These results agreed with Brauman and others (1996) who pointed out that LAB produced high amounts

of lactic acid, leading to a rapid drop in pH to around 4.5. The environment then became selective against less acid-tolerant microorganisms, as occurs during sauerkraut fermentation.

A similar scenario was observed for TA where all samples reached a maximum value at 72 h of fermentation. When *Lactobacillus plantarum* starter culture was added, the decrease of pH and increase of TA occurred more rapidly after 24 h when compared with no addition of starter culture. This agrees with Kostinek and others's (2005) findings who determined a high capability of the obligate homo- and facultatively heterofermentative group (mostly *L. plantarum*) of lowering the pH of the medium. Acid production and the subsequent decrease of pH extends the lag phase of sensitive organisms including foodborne pathogens as emphasized by Kostinek and others (2007).

The addition of the starter culture *Lactobacillus plantarum* BFE 6710 rapidly decreased the pH at 24 h of fermentation (Table 2) when compared with spontaneous fermentation. Edward and others (2011) determined that spontaneous fermentations generally take a long time (96 h) to obtain the desired characteristics such as pH and sensory characteristics. Initiation of the process can take a relatively long time (24-48 h) and there is also the risk that contaminating microorganisms compete with the desirable microorganisms. Therefore, adding a starter culture notably reduced fermentation times, as demonstrated in this study, by a fast decrease in pH, and noticeable increase in acidity when compared to samples at 0 h of fermentation. This helps reduce the risk of growth of undesiderable microorganisms and contributes to more control over aroma, texture and flavor of the final cassava product.

Consideration must to be taken at selection of the specific LAB strains to use as starter cultures. Some LAB strains associated with fermented foods can degrade antinutritional factors, such as phytic acid and phenolic compounds (Holzapfel 2002). Thus, incorporation of these microorganisms may help to increase the nutritional content of foods. In addition, selected strains may enhance the general benefits of spontaneous fermentation such as improved protein digestibility and micronutrient bioavailability, and contribute more specifically to biological enrichment through the biosynthesis of vitamins and essential amino acids.

Physical analyses

Particle size distribution of cassava flour (PSDC)

There were distinctive differences in particle size distribution among the different cassava flours. For all cassava flours, PSDC > 420 μm was in the range of 13.23-33.09% while PSDC at 125-149 μm was 9.70–12.27%. PSDC at 149–177 μm and 177–250 μm was 9.95–14.44% and15.09–17.64%, respectively. PSDC at 250-420 μm was 3.13-28.29% with WT cassava flour presenting the highest value (28.29%). PSDC < 125 μm (fines) was 10.29-13.31% (Table 4). There was not a significant difference of PSDC > 420 μm among Z, S, SPRO and PRO samples ($p>0.05$) while WT cassava flour had the lowest percentage (13.23%). As reported by Iwuoha and Nwakanma (1998), fines caused a high viscosity and dense cassava pastes. Consequently, particle size will influence the final texture of the final product when cassava flours with the mentioned PSD are used.

Considering that cassava flour is not normally used as a starter material for fufu production, PSD should be taken into account considering intrinsic characteristics of regular fufu products. Servais and others (2002) mentioned that PSD optimization for food production is further constrained by the sensitivity of the palate and an excess of a maximum particle size may result in a product with undesiderable characteristics. As an example, the maximum particle size in chocolate is in the range of 35 μm. A "gritty" or "coarse" chocolate may result if the particle size is out of range.

Starch granule

The properties of starches prepared from different sources vary considerably (Swinkels 1985). Cassava starch granules have a round-oval, truncated on side shape with a diameter range of 4-35 μm (Swinkels 1985; Cui 2005) as it is observed in Figures 5 and 6. No differences on the starch granule morphology were observed between WT, Z, S, SPRO and PRO due to the fortifying process. In addition, no differences in the morphological structure of the starch granules were observed in all fermented cassava flours at all fermentation times (Data not shown). This suggests that starch was not the main substrate for the microorganisms of the cassava mash. In relation to cassava starch granule morphology, Oyewole and Ogundele (2001) determined that the water retention capacity of fufu particles was not significantly affected by the period of fermentation and they concluded that the microstructure of the extracted starch granules was not affected by fermentation suggesting no amylolytic activity.

Pasting properties

Table 5 shows the pasting properties of the protein and pro-vitamin A fortified and wild type cassava flours. According to Swinkels (1985), the pasting temperature is the temperature at which the viscosity of the stirred starch suspension begins to rise. As shown in Table 5, there were significant differences in the pasting temperature among most cassava flours ($p \leq 0.05$). The pasting temperature ranged from 80.23 – 90.4 °C, with WT showing the greatest value. No significant differences of the pasting temperature were found between SPRO and PRO flours ($p>0.05$). When evaluating the pasting properties of several cassava varieties in Nigeria, Maziya-Dixon and others (2007) obtained lower pasting temperature values possibly due to the diverse cassava genotypes. On the other hand, the differences in pasting temperature of cassava flour might be attributed to high fiber content or other components in the flours besides starch. The presence of neutral and water soluble substances contribute to increased starch pasting temperature (Whistler and BeMiller 1997; Shittu and Adedokun 2010). In addition, Akoja and Mohammed (2011) evaluated the pasting properties of fufu flour enriched with pigeon pea flour to increase fufu flour's nutritional value. As a result, they found that the blend with the greatest addition of pigeon pea flour (40%) showed the greatest pasting temperature (79.53 °C) that is similar to 80.23 °C found in this study for Z sample.

Peak viscosity indicates the water-binding capacity of the starch and it is often correlated with the final product quality and ease of handling of the paste. Also, a higher peak viscosity indicates the greater thickening power of the starch (Maziya-Dixon and others 2007; Adegunwa and others 2011; Shittu and Adedokun 2010).

The trough viscosity that refers to hot paste stability ranged from 120 to 157.44 RVU with the lowest values obtained for S and SPRO samples. Breakdown is the process of rupture of the granules, due to the increase of temperature that is held for a period of time; and polymer alignment due to shear thinning. When the gel is formed and the viscosity increases to the final viscosity, retrogradation is involved and it is referred to as the setback region. Thus, the setback is the increase in viscosity during cooling and has been related to texture of various products (Cui 2005; Maziya-Dixon and others 2007). In this study, significant differences were found for peak viscosity, breakdown viscosity and setback viscosity ($p \leq 0.05$). The values for peak viscosity ranged from 305.78 to 471.22 RVU. Thus, WT cassava flour with the smallest peak viscosity may be the most easily handled especially during cooking. The breakdown viscosity ranged from 153.31 to 313.78 RVU and setback viscosity ranged from 74.5 to 127.92 RVU. The greatest setback viscosity was found in the PRO sample that means that PRO had the greatest tendency to retrograde and produce a better consistent dough after cooling (Table 5). As mentioned by Maziya-Dixon and others (2007) a high setback is also associated with syneresis or weeping during freeze/thaw cycles.

Final viscosity indicates the ability of the material to form a viscous paste or gel after cooking and cooling as well the resistance of the paste to shear force during stirring (Maziya-Dixon and others 2007). Final viscosity ranged from 194.50 to 269.94 RVU. No significant differences were found between final viscosities of WT and PRO cassava flours.

Color evaluation

Determination of cassava flour color is important to determine the acceptability of the final product. In this investigation, the color measured in the cassava flours depended on the flour fortification. In this study, cassava flours lightness (L*), greenness to redness (a*) and blueness to yellowness (b*) ranged from 88.01 to 91.17, -0.46 to 0.44, and 8.06 to 15.80, respectively (Table 6). One way ANOVA showed that the cassava flours differed significantly in terms of the flour's lightness (L*) and greenness to redness (a*) ($p \leq 0.05$). No significant differences were found for lightness between Z and S samples. WT showed the greatest lightness value (91.17). Regarding a*, only SPRO and PRO showed low positive value indicating red on the hue axis, whereas the other cassava flours presented negative a* value (green on hue axis). All samples had positive b* value (indicating hue on yellow axis). WT and Z samples blueness to yellowness (b*) did not significantly differ ($p>0.05$). SPRO sample showed the greatest b* value (15.80) due its yellow color provided by the pro-vitamin A fortification. This value was followed by the PRO sample b* value (15.13) that also contained pro-vitamin A.

Wang and others (2012) determined an increase of a* and b* values, a more red and yellow color respectively, in cassava crackers with green banana flour incorporated due oxidized phenol compounds. Concerning the color deviation with respect to the WT cassava flour, the SPRO sample showed the highest deviation having a ΔE value of 8.24 followed by the PRO sample with ΔE value of 7.31 (Table 6). These values suggest that the pro-vitamin A containing samples are less likely to be acceptable.

WT had the greatest whiteness value as observed in Figure 7a. The majority of the cassava flours presented negative hue values that reflected yellow-greenish hue, with the exception of SPRO and PRO samples that presented positive hue values, which reflected yellow-orange hue. The hue angle value in SPRO sample was the highest followed by the PRO sample (Figure 7b). Chroma is the quantitative component of the color associated with the color purity in the CIELAB space (Matos and Rosell 2012). Both SPRO and PRO sample showed chroma values higher than the other samples, which revealed its higher purity of color related to major intensity of the yellow component (Figure 7c) because of their pro-vitamin A fortification.

Volatile compounds

Volatile emissions from fermented cassava product similar to fufu have not been studied extensively. Ohochuku and Ballantine (1983) identified butanoic acid, propanoic acid, and acetic acid as responsible for the obnoxious odor induced by aqueous fermentation of cassava for 4 days. Iyer and others (2010) were able to identify and quantify 18 volatile compounds from cassava roots that included 8 ketones, 1 cyanohydrin, 6 aldehydes, and 3 alcohols. Among these volatiles, ketones were found in the highest amount.

Volatile compounds reported in fermented cassava flours with and without the addition of the starter culture at 5 different fermentation times are shown in Tables 7-11. The volatile compounds detected in cassava samples at all fermentation times varied in number. On average, 10 volatile compounds were quantified at each fermentation time.

The compounds were 2-propanone, 2-butanone, acetic acid, hexanal, nonanal, decanal, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol and 2-decenal but not all were present in all samples.

The number of volatile compounds varied depending on the cassava sample, addition of the starter culture and fermentation time. The only compound that was found in all samples was 2-propanone. Levels of 2-propanone in all NF samples at 0 h fermentation were below their published threshold value (40.9 μg/mL) (Stahl 1973). At 0 h fermentation, 2-propanone was not reduced to levels below their published threshold value in LFWT sample. Production of acetic acid was observed at 0 h of fermentation only in NFS and LFS samples (Table 7). Acetic acid was detected in all samples from 24 to 96 h fermentation with concentrations above the threshold value (24.3 μg/mL) (Stahl 1973). No 2-butanone was observed in the NFS from 0-48 h. 1-hexanol was found in all samples from 24 -72 h of fermentation (Tables 8-10).

Fortified samples were found to be similar to the wild type cassava flour with respect to qualitative volatile composition. As shown in Table 10, LFWT showed the greatest amount of acetic acid production (759.21 μg/mL) followed by the NFWT (324.07 μg/mL) and NF PRO (318.95 μg/mL) but no significant differences were found on the acetic acid concentrations among those samples ($p>0.05$). According to Ohochuku (1983), the acid fraction was responsible for the obnoxious odor during fermentation of cassava. In contrast, a less unpleasant aroma due to low production of acids, such as acetic acid, could be an advantage for utilization of protein and pro-vitamin A fortified cassava flours in fermented products.

As shown in Table 7 significant differences were observed in the 2-propanone content between LFWT (43.11 μg/mL) and LFZ (7.71 μg/mL) samples ($p<0.05$) at 0 h fermentation. At the end of the fermentation cycle (96 h), significant differences in the 2-propanone content were found between NFZ and NFS samples ($p<0.05$).

Some of the compounds identified were common to those found by Iyer and others (2010). Contrary to the findings of Iyer and others (2010), no compounds related to linamarin hydrolysis during the first step of cyanogenesis were identified. The majority of the compounds identified in this study were alcohols (1-hexanol, 1-pentanol, 1-heptanol and 1-octanol). In addition, one volatile acid (acetic acid), one aldehyde (hexanal) and two ketones (2-propanone and 2-butanone) were identified and quantified.

Most of the volatile compounds detected tended to increase at 24 h of fermentation and new compounds were formed as an effect of the fermentation process. Similar results were reported Delgado and others (2010) while characterizing a Spanish soft cheese during ripening.

The formation of 2-propanone and 2-butanone can be attributed to hydroxynitrile lyase (HNL) activity in cassava (Iyer and others 2010). *L. plantarum* can produce diacetyl, acetone and carbon dioxide when grown in complex media. Diacetyl concentration increases when the growth medium has high concentrations of glucose. The presence of carbohydrates may also contribute to other reactions, as sucrose can also be used as a substrate for the formation of polysaccharides. Most organic acids such as malic, tartaric, and acetic acids can be metabolized by *L. plantarum* resulting in the production of carbon dioxide, lactic acid and acetic acid (Todorov and Franco 2010).

A principal component analysis (PCA) was carried out to determine the important volatile compounds on each day of fermentation. The first (PC1) and second (PC2) principal components explained 37.47% and 32.96%, respectively, of the variability. The second principal component (PC2) separated the fermented cassava flours as a function of addition (LF) or not (NF) of a starter culture for fermentation (Figure 8).

Although all the volatiles found in all samples were included in the analysis, only volatile compounds which explained variation of the data have been identified in the figure. LF SPRO samples at 48, 72 and 96 h of fermentation were characterized by higher headspace recovery of 2-propanone and 2-butanone. LF PRO samples at 24, 48, 72 and 96 h of fermentation were characterized for the presence of acetic acid and 1-hexanol mainly while 1-octanol decreased. NF SPRO samples at 24 and 96 h fermentation and LF SPRO at 24 h fermentation were higher in the 1-octanol volatile compound (Figure 8). Sample LFZ at 24 h fermentation was described by 1-octanol to a lesser extent.

CONCLUSIONS

Protein or pro-Vitamin A fortified cassava flours, with or without the addition of *Lactobacillus plantarum* strain 6710, are suitable for fermentation to produce wet fufu. Fortified cassava flours did not influence microbial growth during fermentation. *Lactobacillus plantarum* strain 6710 demonstrated its ability to acidify cassava rapidly. Soluble protein content decreased in most samples through fermentation. The smallest decrease (60%) was found for NF SPRO wet fufu at 96 h. Pasting properties of cassava flours revealed the greatest pasting temperature for the wild type sample. A greater value for yellow color (b*) was observed in the pro-vitamin A containing samples. Four aromatic compounds were mainly detected in all cassava samples at all fermentation times: 2-propanone, 2-butanone, acetic acid and 1-hexanol. *L. plantarum* is feasible for application in production of fufu from both protein and pro-vitamin A fortified cassava flours. Further research should include the microbiological analysis of the initial material and identification of the microbial flora that may have an impact on the further fermentation process and characteristics of the final product.

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Table 1. pH and titratable acidity of cassava flours[1]

Cassava material[2]	pH	Titratable acidity (%)[3]
WT	6.60 ± 0.03[b]	0.37 ± 0.01[d]
Z	6.61 ± 0.06[b]	0.48 ± 0.00[c]
S	6.72 ± 0.03[a]	0.49 ± 0.00[c]
SPRO	6.31 ± 0.02[c]	0.73 ± 0.01[a]
PRO	6.32 ± 0.03[c]	0.66 ± 0.02[b]

[1]Results are reported as the mean of two determinations ± standard deviation. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

[3]Titratable acidity (TA) results are expressed as % lactic acid.

Table 2. pH[1] of fermented cassava flours with (LF) and without (NF) the addition of a starter culture at all fermentation times

Cassava material[2]		Fermentation time (h)				
		0	**24**	**48**	**72**	**96**
LF	**WT**	5.15 ± 0.00^{Acd}	3.48 ± 0.04^{Bf}	3.36 ± 0.00^{Cf}	3.07 ± 0.02^{Ef}	3.16 ± 0.01^{Df}
	Z	5.15 ± 0.01^{Acd}	3.61 ± 0.03^{Be}	3.29 ± 0.01^{Cf}	3.13 ± 0.01^{Def}	3.28 ± 0.01^{Cde}
	S	4.78 ± 0.03^{Ae}	3.71 ± 0.04^{Bde}	3.55 ± 0.08^{Be}	3.27 ± 0.01^{Cd}	3.33 ± 0.01^{Cd}
	SPRO	4.98 ± 0.03^{Ad}	3.79 ± 0.03^{Bd}	3.72 ± 0.01^{Bd}	3.53 ± 0.01^{Cc}	3.72 ± 0.02^{Bbc}
	PRO	5.30 ± 0.10^{Ac}	4.05 ± 0.01^{Bc}	3.91 ± 0.01^{Bc}	3.69 ± 0.00^{Cb}	3.64 ± 0.01^{Cc}
NF	**WT**	6.03 ± 0.00^{Aa}	4.14 ± 0.06^{Bc}	3.61 ± 0.00^{Cde}	3.16 ± 0.06^{De}	3.18 ± 0.07^{Def}
	Z	6.02 ± 0.01^{Aa}	4.57 ± 0.04^{Ba}	3.56 ± 0.00^{Ce}	3.36 ± 0.01^{Dd}	3.35 ± 0.00^{Dd}
	S	6.04 ± 0.09^{Aa}	4.44 ± 0.01^{Bb}	4.41 ± 0.01^{Ba}	4.11 ± 0.00^{Ca}	3.76 ± 0.02^{Db}
	SPRO	5.86 ± 0.06^{Aab}	4.55 ± 0.01^{Bab}	4.47 ± 0.01^{Ba}	4.11 ± 0.01^{Ca}	4.05 ± 0.01^{Ca}
	PRO	5.67 ± 0.01^{Ab}	4.63 ± 0.00^{Ba}	4.13 ± 0.04^{Cb}	3.76 ± 0.00^{Db}	3.66 ± 0.01^{Ec}

[1]Results are reported as the mean of three determinations ± standard deviation. Means within the same column containing different lowercase letters are significantly different ($p \leq 0.05$). Means within the same row containing different uppercase letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 3. Titratable acidity (%)[1,2] of fermented cassava flours with (LF) and without (NF) the addition of a starter culture at all fermentation times

Cassava material[3]		Fermentation time (h)				
		0	24	48	72	96
LF	WT	0.24 ± 0.01^{Dcd}	0.96 ± 0.01^{Cd}	1.20 ± 0.01^{Be}	1.28 ± 0.02^{Ad}	1.18 ± 0.01^{Bg}
	Z	0.30 ± 0.01^{Eb}	1.11 ± 0.00^{Dc}	1.36 ± 0.01^{Bd}	1.47 ± 0.01^{Ac}	1.32 ± 0.01^{Ce}
	S	0.47 ± 0.01^{Ea}	1.20 ± 0.01^{Db}	1.56 ± 0.02^{Cc}	1.80 ± 0.02^{Ab}	1.67 ± 0.02^{Bd}
	SPRO	0.43 ± 0.02^{Da}	1.44 ± 0.00^{Ca}	1.74 ± 0.01^{Bb}	1.91 ± 0.01^{Aa}	1.78 ± 0.01^{Bb}
	PRO	0.44 ± 0.02^{Ca}	1.44 ± 0.00^{Ba}	1.81 ± 0.02^{Aa}	1.82 ± 0.03^{Ab}	1.73 ± 0.01^{Ac}
NF	WT	0.14 ± 0.00^{Ef}	0.49 ± 0.01^{Dh}	0.90 ± 0.01^{Cg}	1.24 ± 0.01^{Bd}	1.29 ± 0.02^{Aef}
	Z	0.18 ± 0.00^{Eef}	0.48 ± 0.00^{Dh}	1.05 ± 0.00^{Cf}	1.27 ± 0.02^{Bd}	1.31 ± 0.00^{Aef}
	S	0.20 ± 0.00^{Dde}	0.68 ± 0.00^{Cg}	0.71 ± 0.01^{Ch}	0.88 ± 0.01^{Bf}	1.27 ± 0.01^{Af}
	SPRO	0.28 ± 0.02^{Cbc}	0.84 ± 0.00^{Bf}	0.87 ± 0.01^{Bg}	1.01 ± 0.01^{Ae}	1.03 ± 0.00^{Ah}
	PRO	0.28 ± 0.00^{Dbc}	0.90 ± 0.01^{Ce}	1.40 ± 0.00^{Bd}	1.83 ± 0.01^{Ab}	1.83 ± 0.00^{Aa}

[1]Titratable acidity (TA) results are expressed as % lactic acid.

[2]Results are reported as the mean of three determinations ± standard deviation. Means within the same column containing different lowercase letters are significantly different ($p \leq 0.05$). Means within the same row containing different uppercase letters are significantly different ($p \leq 0.05$).

[3]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 4. Particle size distribution[1] of cassava flours (% by weight)

Cassava material[2]	< 125 µm	125-420 µm	>420 µm
WT	10.29[b]	76.48[a]	13.23[b]
Z	13.31[a]	59.94[b]	26.74[a]
S	13.21[a]	56.14[b]	30.65[a]
SPRO	11.78[ab]	55.12[b]	33.09[a]
PRO	10.89[ab]	58.98[b]	30.13[a]

[1]Results are reported as the mean of three determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 5. Pasting properties[1] of cassava flours

Cassava material[2]	Peak viscosity (RVU)	Trough (RVU)	Breakdown viscosity (RVU)	Final viscosity (RVU)	Setback viscosity (RVU)	Peak time (min)	Pasting temperature (°C)
WT	305.78 ± 1.13^{e}	152.47 ± 1.09^{b}	153.31 ± 0.24^{e}	268.53 ± 3.61^{ab}	116.06 ± 3.81^{b}	4.24 ± 0.08^{a}	90.40 ± 0.74^{a}
Z	471.22 ± 2.08^{a}	157.44 ± 1.55^{a}	313.78 ± 3.13^{a}	263.86 ± 0.83^{b}	106.42 ± 2.32^{c}	3.18 ± 0.04^{d}	80.23 ± 0.42^{d}
S	413.19 ± 1.34^{b}	120.00 ± 0.90^{d}	293.19 ± 1.88^{b}	194.50 ± 1.71^{d}	74.50 ± 1.73^{e}	3.62 ± 0.04^{c}	84.50 ± 0.39^{c}
SPRO	344.94 ± 4.44^{d}	122.11 ± 0.05^{d}	222.83 ± 4.39^{d}	212.11 ± 2.48^{c}	90.00 ± 2.44^{d}	3.93 ± 0.07^{b}	87.48 ± 0.60^{b}
PRO	389.64 ± 2.88^{c}	142.03 ± 2.01^{c}	247.61 ± 0.91^{c}	269.94 ± 1.05^{a}	127.92 ± 1.02^{a}	3.80 ± 0.00^{b}	86.20 ± 0.05^{b}

[1]Results are reported as the mean of three determinations ± standard deviation. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 6. Color[1] determination of cassava flours

Cassava material[2]	L*	a*	b*	△E
WT	91.17 ± 0.10[a]	-0.33 ± 0.02[d]	8.22 ± 0.18[d]	-
Z	90.10 ± 0.08[b]	-0.09 ± 0.01[c]	8.06 ± 0.06[d]	1.12 ± 0.11[c]
S	90.32 ± 0.03[b]	-0.46 ± 0.03[e]	9.71 ± 0.18[c]	1.73 ± 0.10[c]
SPRO	88.01 ± 0.21[d]	0.33 ± 0.01[b]	15.80 ± 0.13[a]	8.24 ± 0.26[a]
PRO	88.94 ± 0.29[c]	0.44 ± 0.04[a]	15.13 ± 0.17[b]	7.31 ± 0.44[b]

[1]Results are reported as the mean of three determinations ± standard deviation. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 7. Volatile compounds[1] detected in fermented cassava flours with (LF) and without (NF) the addition of a starter culture at 0 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)									
		2-propanone	2-butanone	acetic acid	hexanal	nonanal	decanal	1-hexanol	2-decenal	1-octanol	1-heptanol
LF	WT	43.11^{a}	3.75^{a}	nd	0.022^{b}	0.006^{c}	nd	0.049^{bc}	nd	nd	nd
	Z	7.71^{b}	1.11^{b}	nd	nd	nd	nd	nd	nd	nd	nd
	S	40.16^{ab}	2.05^{b}	68.92^{a}	0.022^{b}	nd	nd	0.106^{ab}	nd	nd	nd
	SPRO	27.68^{ab}	1.88^{b}	nd	0.043^{b}	0.008^{c}	nd	0.188^{a}	0.016^{a}	0.008^{a}	nd
	PRO	10.70^{ab}	1.15^{b}	nd	0.120^{b}	0.031^{b}	nd	nd	0.017^{a}	nd	nd
NF	WT	11.87^{ab}	1.28^{b}	nd	0.042^{b}	0.018^{bc}	0.004^{a}	nd	nd	nd	nd
	Z	11.08^{ab}	1.07^{b}	nd	0.009^{b}	0.007^{c}	nd	nd	nd	nd	nd
	S	25.43^{ab}	nd	172.03^{a}	0.035^{b}	nd	nd	0.024^{bc}	nd	nd	nd
	SPRO	16.30^{ab}	1.51^{b}	nd	0.099^{b}	0.011^{bc}	nd	nd	0.017^{a}	nd	nd
	PRO	22.38^{ab}	1.65^{b}	nd	0.491^{a}	0.068^{a}	0.003^{a}	nd	0.016^{a}	0.011^{a}	0.004

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 8. Volatile compounds[1] detected in fermented cassava flours with (LF) and without (NF) the addition of a starter culture at 24 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)							
		2-propanone	2-butanone	acetic acid	1-hexanol	1-octanol	1-heptanol	1-pentanol	2-decenal
LF	WT	17.06^{b}	1.690^{a}	133.50^{b}	0.031^{d}	nd	nd	nd	nd
	Z	17.87^{b}	1.485^{a}	111.34^{b}	0.049^{cd}	0.393^{a}	nd	nd	nd
	S	16.58^{b}	0.959^{a}	59.43^{b}	0.024^{d}	nd	nd	nd	nd
	SPRO	20.31^{b}	1.400^{a}	133.31^{b}	0.207^{c}	0.007^{c}	0.003	nd	nd
	PRO	21.44^{b}	1.429^{a}	381.35^{ab}	0.565^{b}	0.036^{b}	nd	0.245^{a}	0.017^{a}
NF	WT	16.84^{b}	1.082^{a}	73.04^{b}	0.014^{d}	nd	nd	nd	nd
	Z	6.80^{b}	nd	169.01^{b}	12.944^{a}	nd	nd	nd	nd
	S	62.89^{a}	nd	708.46^{a}	0.101^{cd}	nd	nd	nd	nd
	SPRO	16.47^{b}	1.001^{a}	83.52^{b}	0.040^{d}	0.004^{c}	nd	nd	nd
	PRO	11.89^{b}	nd	217.37^{b}	0.112^{cd}	0.007^{c}	nd	0.064^{b}	0.017^{a}

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 9. Volatile compounds[1] detected in fermented cassava flours with (LF) and without (NF) the addition of a starter culture at 48 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)							
		2-propanone	2-butanone	acetic acid	1-hexanol	1-octanol	1-heptanol	1-pentanol	2-decenal
LF	WT	17.68^{a}	1.495^{ab}	659.50^{a}	0.010^{c}	nd	nd	nd	nd
	Z	16.08^{a}	1.188^{b}	345.55^{a}	0.061^{c}	nd	nd	nd	nd
	S	24.52^{a}	1.505^{ab}	164.71^{a}	0.058^{c}	nd	nd	nd	nd
	SPRO	27.41^{a}	2.184^{a}	297.15^{a}	0.200^{bc}	0.006^{b}	nd	0.036^{a}	nd
	PRO	17.24^{a}	1.029^{a}	229.98^{a}	0.374^{b}	0.018^{a}	0.005	0.103^{a}	0.017
NF	WT	13.14^{a}	0.964^{b}	443.81^{a}	0.020^{c}	0.005^{b}	nd	nd	nd
	Z	10.26^{a}	0.880^{b}	92.97^{a}	12.954^{a}	nd	nd	nd	nd
	S	14.89^{a}	nd	92.05^{a}	0.017^{c}	nd	nd	nd	nd
	SPRO	15.90^{a}	nd	133.72^{a}	0.033^{c}	nd	nd	nd	nd
	PRO	8.71^{a}	nd	111.43^{a}	0.047^{c}	0.003^{b}	nd	nd	nd

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 10. Volatile compounds[1] detected in fermented cassava flours with (LF) and without (NF) the addition of a starter culture at 72 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)							
		2-propanone	2-butanone	acetic acid	hexanal	1-hexanol	1-octanol	1-heptanol	1-pentanol
LF	WT	31.99^{ab}	1.277^{a}	759.21^{a}	nd	0.084^{cd}	nd	nd	nd
	Z	15.56^{ab}	1.275^{a}	152.87^{b}	nd	0.014^{d}	nd	nd	nd
	S	15.74^{ab}	1.351^{a}	164.37^{b}	nd	0.040^{cd}	nd	nd	nd
	SPRO	33.27^{ab}	2.466^{a}	188.2^{b}	nd	0.184^{c}	0.007^{b}	0.003^{a}	nd
	PRO	14.99^{ab}	1.181^{a}	266.31^{b}	0.013	0.398^{b}	0.021^{a}	0.005^{a}	0.150^{a}
NF	WT	29.63^{ab}	1.887^{a}	324.07^{ab}	nd	0.024^{cd}	nd	nd	nd
	Z	12.50^{b}	0.969^{a}	219.46^{b}	nd	12.69^{a}	nd	nd	nd
	S	39.42^{a}	1.777^{a}	184.91^{b}	nd	0.040^{cd}	nd	nd	nd
	SPRO	11.10^{b}	nd	70.99^{b}	nd	0.018^{d}	nd	nd	nd
	PRO	14.77^{ab}	0.815^{a}	318.95^{ab}	nd	0.149^{cd}	nd	nd	0.065^{b}

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 11. Volatile compounds[1] detected in fermented cassava flours with (LF) and without (NF) the addition of a starter culture at 96 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)							
		2-propanone	2-butanone	acetic acid	1-hexanol	1-octanol	1-heptanol	1-pentanol	nonanal
LF	WT	17.89^{ab}	1.313^{c}	140.68^{b}	0.007^{b}	nd	nd	nd	nd
	Z	18.50^{a}	1.465^{bc}	291.19^{ab}	0.100^{b}	nd	nd	nd	nd
	S	38.23^{ab}	nd	635.40^{a}	0.117^{b}	nd	nd	nd	nd
	SPRO	31.22^{ab}	1.889^{a}	272.64^{ab}	0.135^{b}	0.004^{b}	nd	0.036^{b}	nd
	PRO	21.30^{ab}	0.870^{de}	644.69^{a}	0.407^{a}	0.019^{a}	0.005^{a}	0.172^{a}	0.013
NF	WT	27.77^{ab}	1.968^{a}	224.82^{ab}	0.069^{b}	0.007^{b}	nd	nd	nd
	Z	5.06^{b}	nd	69.16^{b}	nd	nd	nd	nd	nd
	S	41.37^{a}	1.756^{ab}	383.80^{ab}	0.044^{b}	nd	nd	nd	nd
	SPRO	13.99^{ab}	0.736^{e}	143.64^{b}	0.037^{b}	0.002^{b}	nd	nd	nd
	PRO	11.94^{ab}	1.219^{cd}	305.34^{ab}	0.156^{b}	0.009^{b}	0.004^{a}	0.083^{b}	nd

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

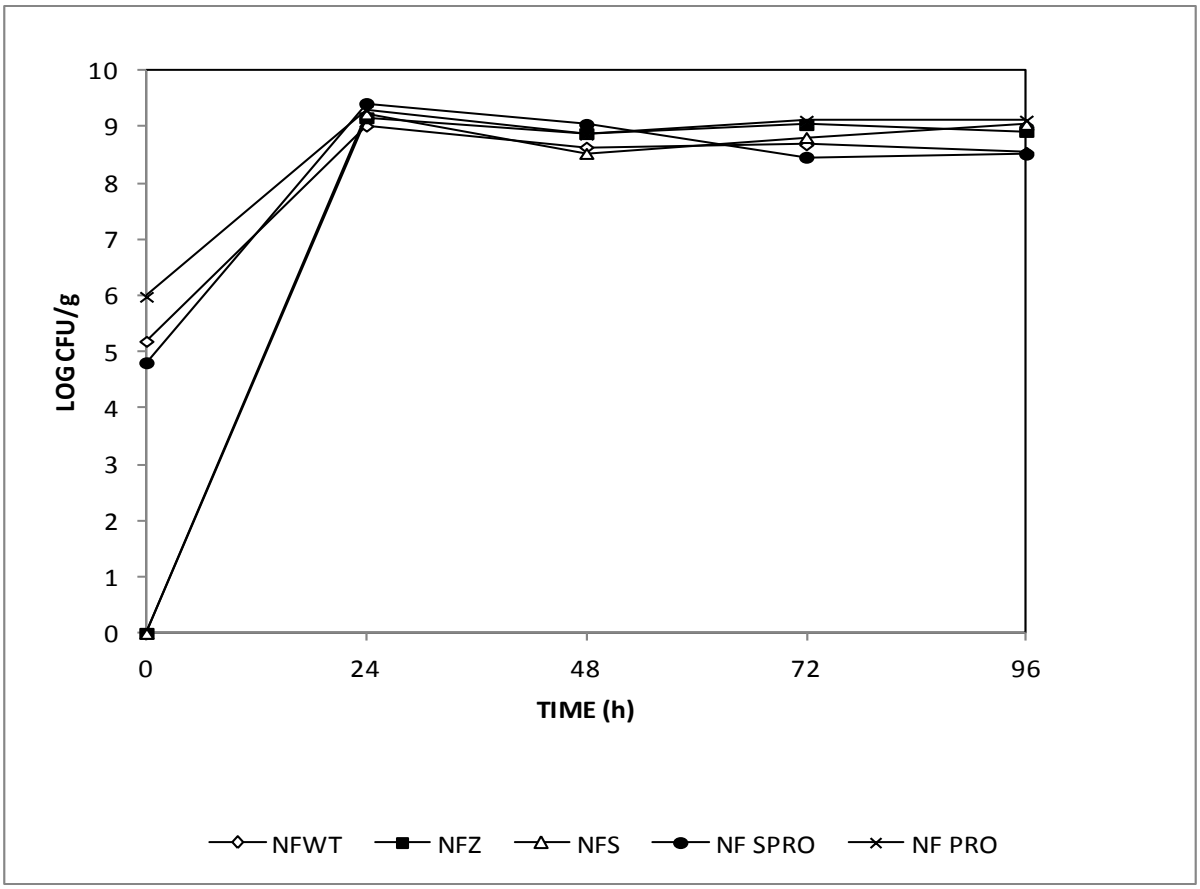


Figure 1. LAB counts (log CFU/g) of naturally fermented (NF) wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cassava flours at different fermentation times.

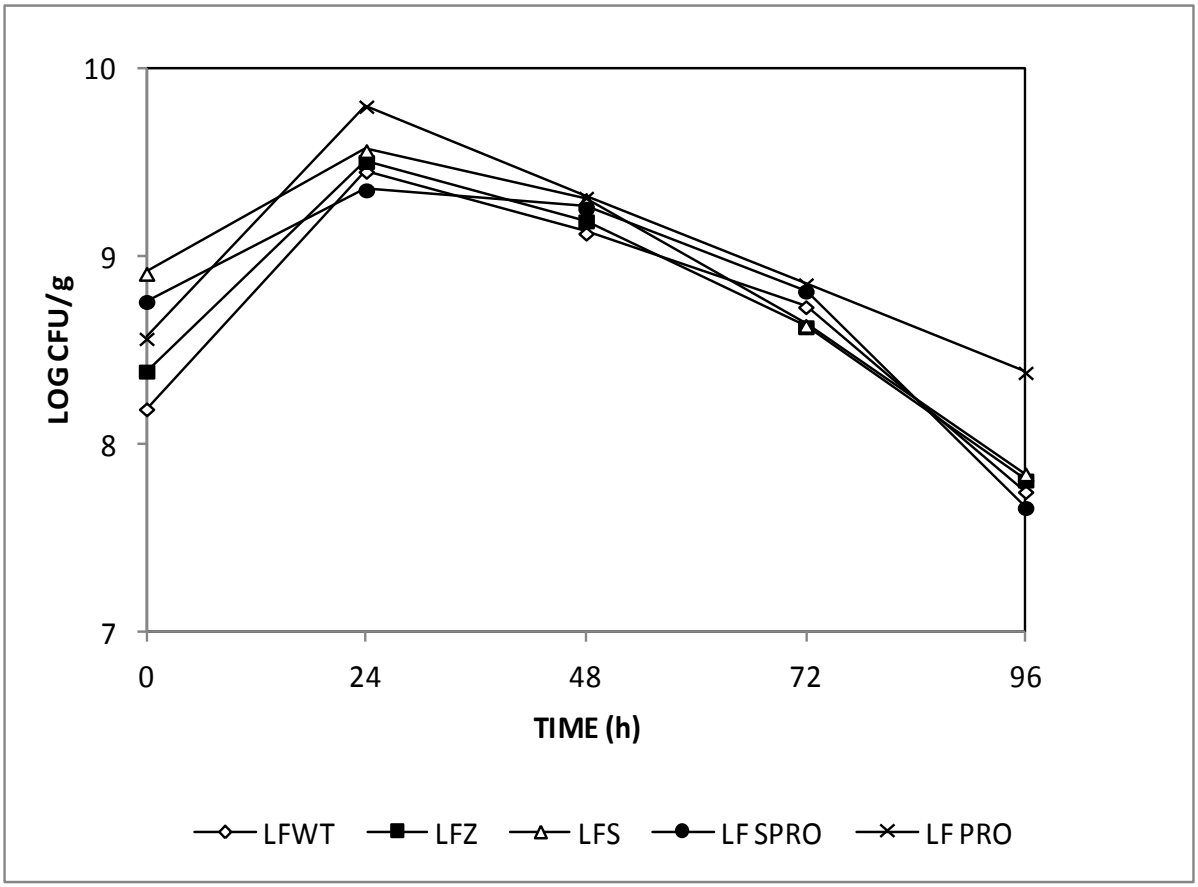


Figure 2. LAB counts (log CFU/g) of *Lactobacillus plantarum* fermented (LF) wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cassava flours at different fermentation times.

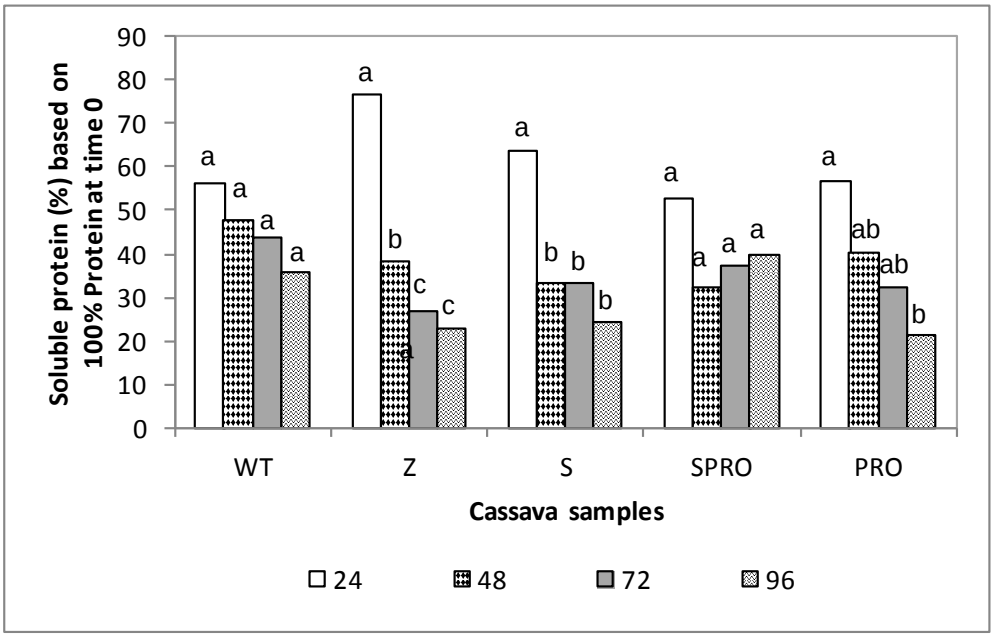


Figure 3. Soluble protein content (%) of naturally fermented wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cassava flours from 24 to 96 h fermentation based on 100% soluble protein content at 0 h of fermentation. Different letters represent significant differences at each sample point ($p \leq 0.05$).

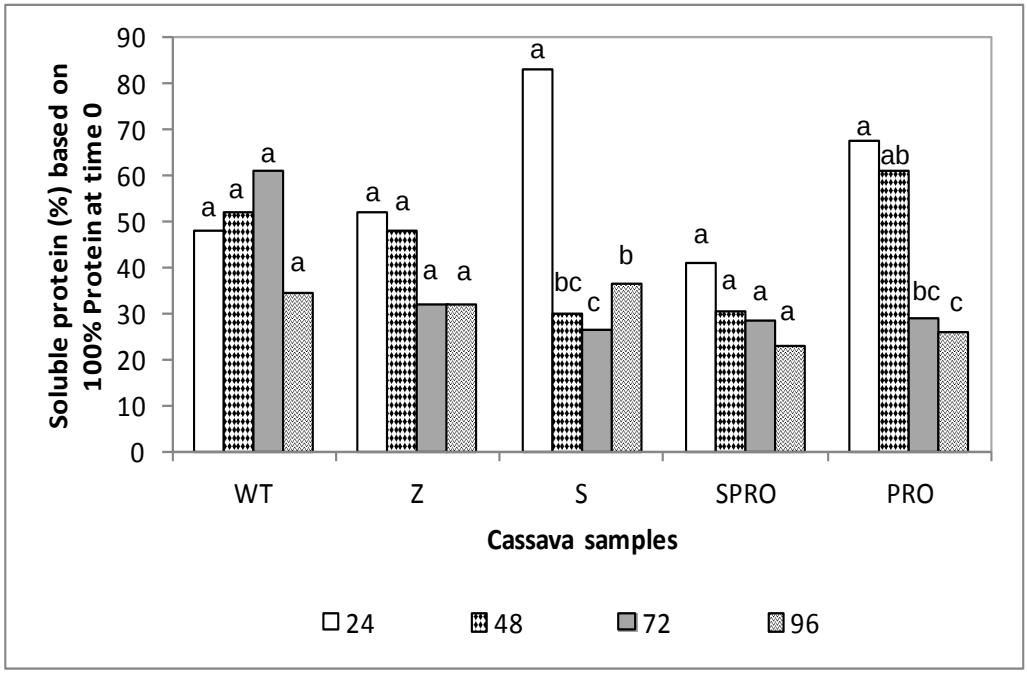


Figure 4. Soluble protein content (%) of *Lactobacillus plantarum* fermented wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cassava flours from 24 to 96 h fermentation based on 100% soluble protein content at 0 h of fermentation. Different letters represent significant differences at each sample point ($p \leq 0.05$).

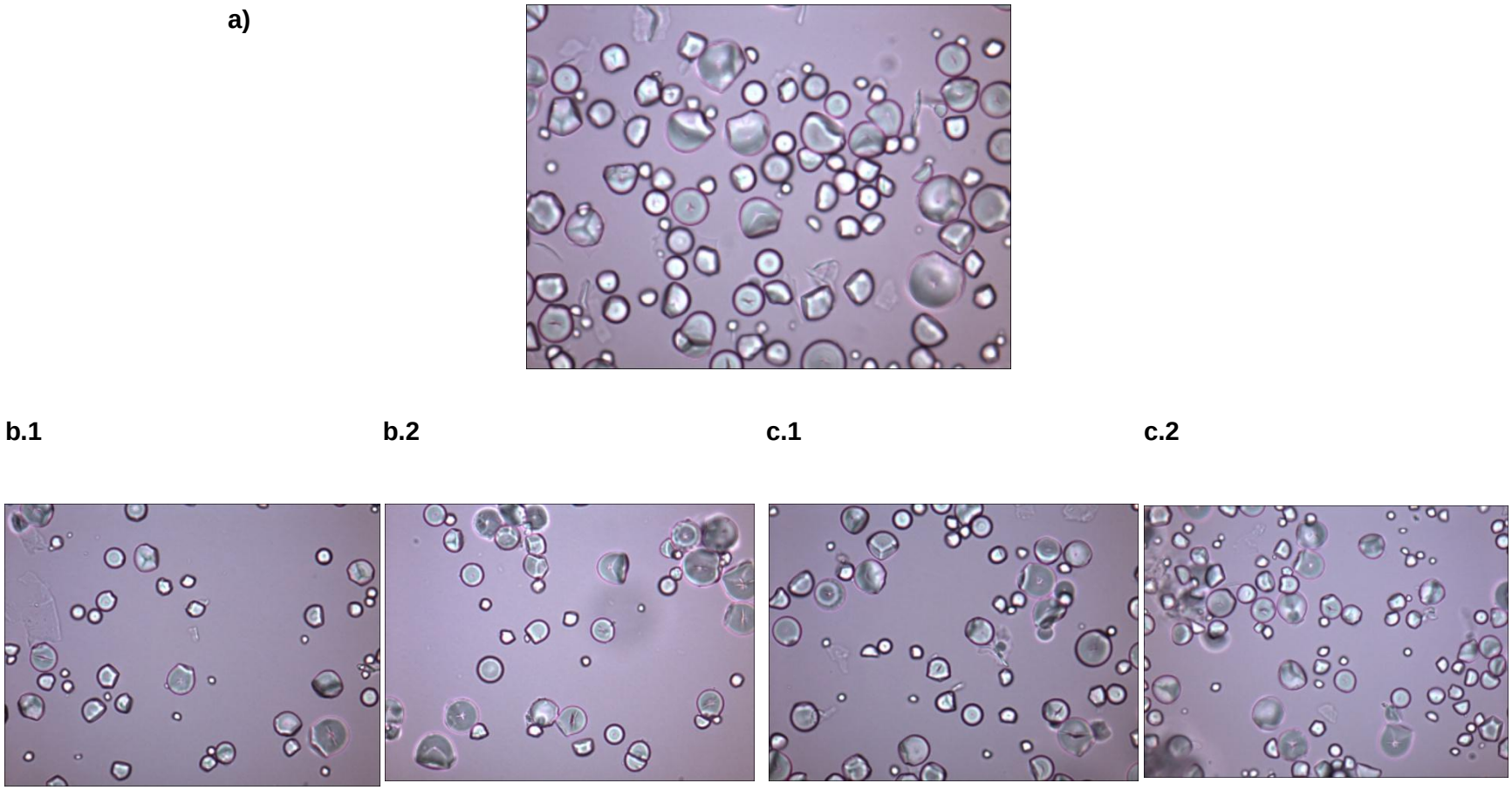


Figure 5. Light microscopy images (50x magnification) of (a) wild type cassava flour (WT), (b.1) NFWT at 0 h of fermentation, (b.2) NFWT at 96 h of fermentation, (c.1) LFWT at 0 h of fermentation, (c.2) LFWT at 96 h of fermentation. NF: Naturally fermented, LF: *Lactobacillus plantarum* fermented.

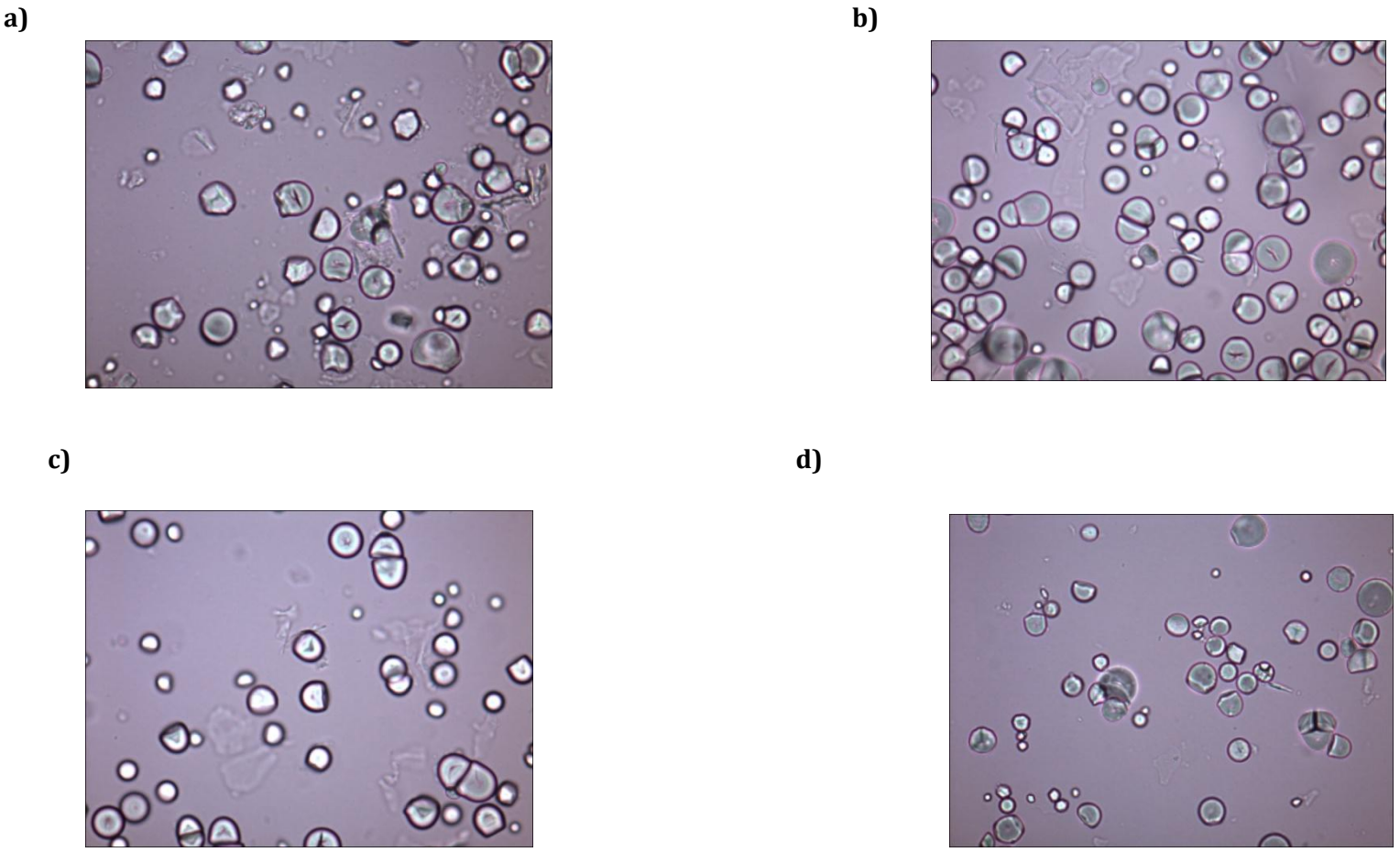
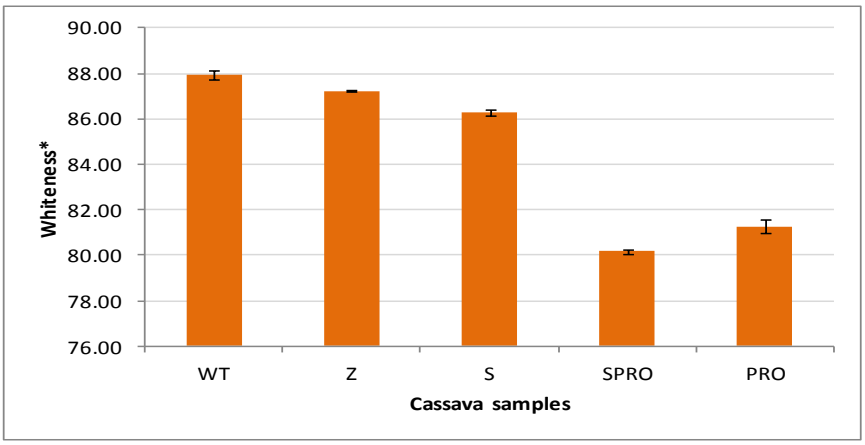
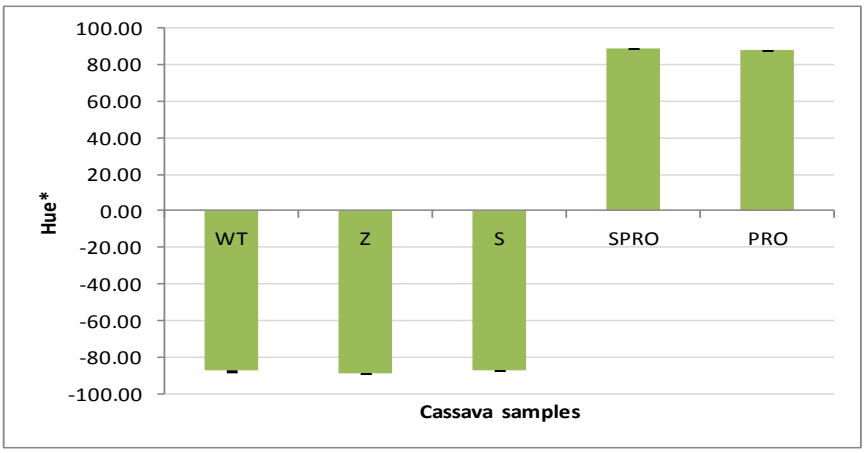


Figure 6. Light microscopy images (50x magnification) of (a) zeolin, (b) sporazein, (c) sporazein plus pro-vitamin A and (d) pro-vitamin A fortified cassava flours.

a)



b)



c)

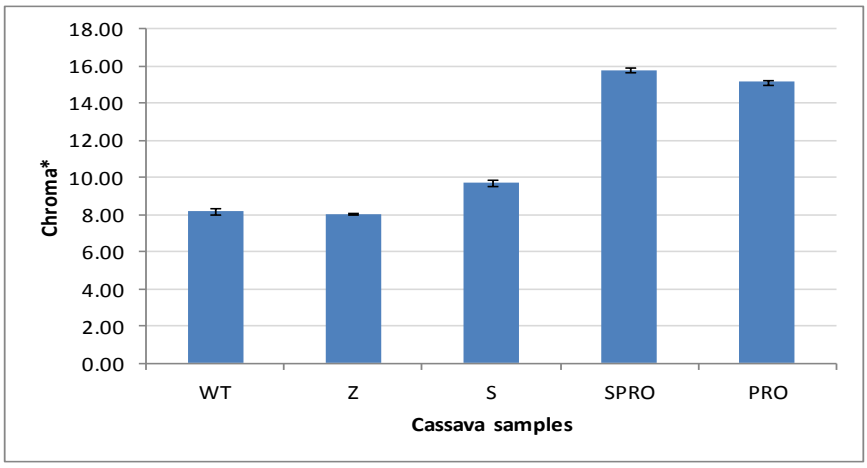


Figure 7. Whiteness (a), hue (b) and chroma (c) of fortified cassava flours zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO), and wild type cassava flour (WT).

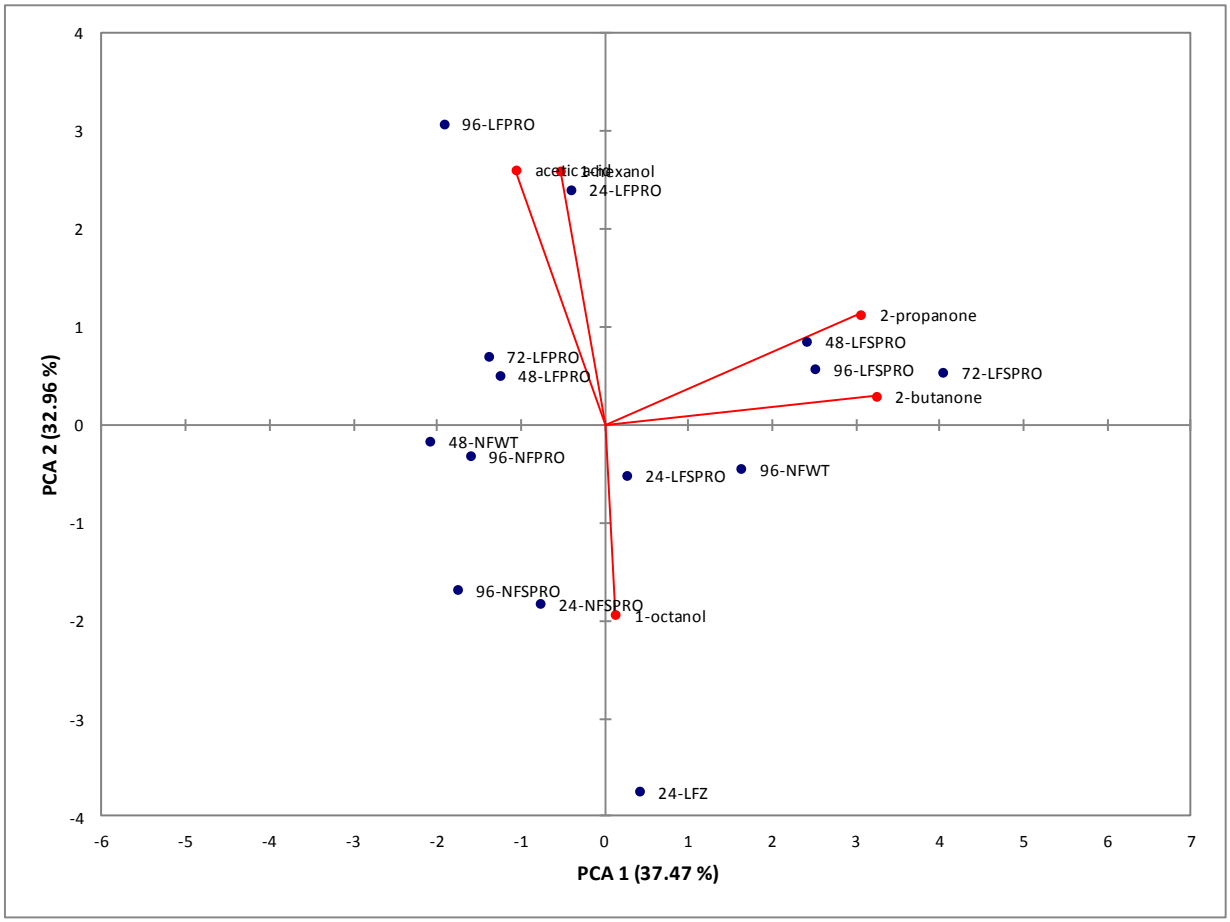


Figure 8. Principal component analysis of the relationship between odorants and wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cassava flours with (LF) and without (NF) the addition of a starter culture at different fermentation times (0, 24, 48, 72, 96 h).

CHAPTER V
PHYSICO-CHEMICAL AND SENSORY EVALUATION OF COOKED FERMENTED PROTEIN FORTIFIED CASSAVA FLOUR (COOKED FUFU)

ABSTRACT

Lactobacillus plantarum strain 6710 was used as starter for the production of cooked fufu, a West African cassava-based staple, using protein and pro-vitamin A fortified and low protein wild type cassava flours. Physico-chemical analyses of fufu flour and cooked fufu were accomplished. A detrimental effect of the cooking process was observed in the starch granule of the cooked fufu by microscopy. Pasting properties determined that non inoculated zeolin fortified cassava flour (NFZ) at 0 h of fermentation, non inoculated wild type (NFWT) and inoculated wild type (LFWT) cassava flours at 96 h fermentation will cook rapidly due to their low pasting temperature. Thermal analysis using a differential scanning calorimeter determined that no significant differences were found in the onset (T_o), peak (T_p), and completion (T_c) gelatinization temperatures, as well as gelatinization enthalpy (ΔH) between fermentation times within each sample. The titratable acidity (TA) of the cooked fufu made of wild type cassava and with *L. plantarum* strain added showed an increase from 0.09 to 0.23% lactic acid at 24 h to 0.63% at 96 h. TA also increased with sporazein, sporazein plus pro-vitamin A and pro-vitamin A samples with the starter added to 1.09, 1.42 and 1.17% at 96 h, respectively. The starter culture

resulted in a rapid reduction in pH to a mean of 3.69 at 24 hours compared to 4.80 for non inoculated samples (controls). Most cooked fufu samples decreased their protein content at 96 h of fermentation. Volatile compounds such as acetic acid, hexanal, nonanal and decanal were detected in most of the cassava samples at all fermentation times and with or without the *L. plantarum* strain added. Pro-vitamin A fortification may influence the degree of lightness (L*) in cooked fufu. A trained sensory panel (n=5) evaluated the different cooked fufu samples. No impact of the sample type was observed on the fufu aroma. Results indicated that sample type and fermentation type influenced the perception of brown color intensity, stale aroma and hardness, adhesiveness and springiness texture attributes. This study showed that it is possible to make a product similar to cooked fufu with cassava flour with or without protein and/or pro-vitamin A fortification.

INTRODUCTION

Over 95% of cassava produced in Nigeria is used as foods for humans for whom roots are the major sources of dietary calories. Among the factors that tend to limit the utilization of cassava without processing are the high amount of toxic compounds and rapid deterioration after harvest (Onwuka and Ogbogu 2007). The cyanogenic glycosides that cassava contains are harmful when hydrolyzed by enzyme and release toxic hydrocyanic acid or free hydrogen cyanide. Toxic compounds can be removed by heat or leached by soaking and washing. Some cassava based products such as gari, fufu and

abacha are based on the latter methodology (Onwuka and Ogbogu 2007).

Fufu is a fermented wet paste that is widely consumed in eastern and south western Nigeria and other parts of West Africa; even though its consumption has decreased due its inherent undesiderable characteristics of poor odor, short shelf life and tedious preparation (Sanni and others 1998). Regular fufu is made by fermenting freshly harvested cassava tubers in water in open containers for days (Onwuka and Ogbogu 2007). Some processors do not peel the cassava before soaking and remove the peel after softening of the roots that usually produces a poor quality product, thus details of fufu preparation vary with locality (Sanni and others 1998, 2006).

The keeping quality of the finished product is greatly affected by the methods of processing. Fufu is traditionally sold in the wet form (moisture about 50%), which renders it highly perishable. The poor or low shelf life is a serious limitation for large-scale processors. A practical approach to improving the shelf life and marketability of fufu is drying. Drying of fufu is aimed at getting reconstitutable fufu dough with physicochemical characteristics of cooked wet paste (Sanni and others 1998, 2006). Several methods have been used for the drying process of fufu. It was determined that a rotary dryer provided a more acceptable product compared to cabinet and sun drying methods, even though the rotary drying method was not cost effective (Adebowale and others 2005). In addition, as reported by Sanni and others (1998), several drying techniques have been reported to reduce the strong odor of fufu but the products were sticky, bland and the quality unacceptable compared to wet fufu.

Qualities of these flour products are not consistent. The main problem appears to be with the texture of the fufu when reconstituted from the flour. Texture is a key desirable sensory attribute of any food product (Bourne 1982) and parameters like finger-feel and mouth-feel are important to the consumer in judging whether the reconstituted fufu flour is acceptable. Therefore, the texture determines the identity of a food product, food quality and its acceptability (Oduro-Yeboah and others 2007).

The objectives of this study were to determine the influence of protein and pro-vitamin A fortified fermented cassava flours on the characteristics of cooked fufu. Physico-chemical characteristics were evaluated including aromatic compounds. A trained panel was used to examine the relationship between the perceived sensory attributes color, aroma and texture with instrumental measurements. Similar characteristics of the cooked fufu made with protein and pro-vitamin A fortified cassava and the wild type cassava may allow consumers to enrich their daily diets without sacrificing the inherent characteristics of commonly consumed fufu. At the same time, considering the perishability of fresh cassava, an alternative is provided for processing of fufu by starting with cassava flour.

MATERIALS AND METHODS

Cassava flours

Four types of protein fortified cassava flours (zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO)) with protein contents (wb) of 9.52, 6.83, 3.63 and 2.14%, respectively and wild type cassava flour (1.41%) were provided by the International Laboratory for Tropical Agricultural Biotechnology (ILTAB) (St. Louis, MO).

Cultures

L. plantarum BFE 6710 strain grown as a stab culture was provided by the Max Rubner-Institut (Karlsruhe, Germany) (Edward and others 2011) and routinely grown in Lactobacilli de Man, Rogosa and Sharpe (MRS) broth (Difco™, Becton Dickinson and Company, Sparks, MD) at 32 ºC for 24 h under aerobic conditions.

Culture propagation and storage

After growth, culture was placed in a cryo tube with a final concentration of 20% glycerol and stored at -72 °C for further use (stock culture). Working cultures were obtained from stock culture. The cells were streaked on MRS agar and incubated at 32 °C for 48 h such that the surface of the bacterial colony was slightly above the surface of the agar and the colony was transferred into MRS broth. Cultures were propagated twice before use. The preculture was centrifugated at 8000 xg at 4 °C for 10 min. The pellet was

washed twice with buffered peptone water (BBL™, Becton Dickinson and Company, Sparks, MD) and centrifuged. The final cell pellet was resuspended into 9 mL of buffered peptone water with an initial concentration of 7 x 10^{10} CFU/mL.

Growth of starter culture in cassava flour

All wild type and fortified cassava flours were preserved in sealed plastic containers at 4 °C. Ninety g of cassava flour were transferred to sterilized plastic containers (under UV light for 15 min). Initial inoculation of all flours by *L. plantarum* was done by transferring a cell pellet suspension with a concentration of 7 x 10^{10} CFU/mL into cassava flour. Sterile water was added to adjust the total moisture content of the cassava sample to the average moisture of the cassava root (68%). A second set of non inoculated flours was also prepared. Due to the limited amount of available cassava flour material, this experiment was done only once. Non inoculated (NF) and inoculated flours (LF) were covered and kept at 32 °C for 96 h fermentation.

Fufu preparation

Fufu flour was prepared as shown in Figure 1. Cassava flour was removed at 0, 24, 48, 72 and 96 h of fermentation and dried for 6.5 h using a tray drier model UOP 8 (Armfield Limited, Ringwood Hampshire, England) set at 70 °C with an air flow of 1.45 m/sec. Temperature profiles for the device are shown in Figure 2. Dried samples were milled using a Tecator Cemotec 1090 sample mill (Foss-Tecator, Eden Prairie, MN), set to 1, the lowest setting available on the mill, and a portion of this fufu flour was set aside before

cooking for further analysis. Milled fermented cassava samples with and without *Lactobacillus plantarum* strain at all fermentation times were mixed with water in a ratio of 1: 3 (cassava: water). Mixes were cooked in a microwave oven Emerson 600 W (Model MW8665W) (Parsippany, NJ) at the highest power setting 10 (100%) in five cycles of 15 s, each followed by a manual mixing of 1 min. Cooked fufu samples were allowed to cool at room temperature (20 °C) for 2 h before sensory evaluation.

PHYSICO-CHEMICAL ANALYSIS

Cassava starch granular morphology characterization by microscopy

Fufu flours and freeze dried cooked fufu products obtained after 0 and 96 h of fermentation were placed on microscope slides. The samples were visually inspected for granule swelling and damage with a Nikon Eclipse E600 microscope (Nikon Inc. Instrument Inc., Melville, NY). Images for fufu flours were evaluated using a 50 X/0.90 oil-immersion lens (Plan; Nikon) and for cooked fufu with a 20X/0.40 lens. Images were captured with the QCapture software (release 2.66.4, Surrey, BC, Canada). Starch granules were visualized microscopically under both normal and polarized light.

Pasting properties

Starch pasting properties were measured in fufu flour obtained with and without the addition of the starter culture *Lactobacillus plantarum* at all fermentation times. The measurements were done with a Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd., Warriewood, Australia) interfaced with a personal computer equipped with Thermocline

software for Windows (release 2.1, Newport Scientific Pty. Ltd., Warriewood, Australia) according to AACC Approved Method 76-21.01 (AACC 2000). The sample was slurried by mixing 3.5 g (14% moisture basis) with 25 mL of water into the RVA canister, stirred manually by rotating the plastic paddle for 10 s to disperse the sample uniformly and to remove lumps, and inserted into the tower of the RVA. The heating and cooling cycles were programmed in the following manner: The samples were brought to 50 °C and held for 1 min, heated from 50 to 95 °C in 4.42 min, held at 95 °C for 2.7 min, cooled to 50 °C in 3.82 min, and held at 50 °C for the remainder of the run. The total run time was 13 min. Parameters estimated were peak viscosity, setback viscosity, final viscosity, pasting temperature and time to reach peak viscosity (Petruccelli and others 1993; Maziya-Dixon and others 2007).

Gelatinization characteristics

Characterization of the gelatinization phenomena of the cassava flours and freeze dried cooked fufu products was performed using a differential scanning calorimeter 2920 Modulated DSC (TA Instruments, New Castle, DE) equipped with a DSC Refrigerated Cooling System using 60 µL stainless Steel Pans, covers and O-rings (Part N° 03190218) (Perkin Elmer). An empty sealed stainless pan was used as a reference in each test.

Flour (10 mg, db) was weighed directly into a stainless steel pan followed by addition of deionized water (20 µL). Pans were sealed, allowed to equilibrate overnight at ambient temperature before analysis, and scanned at a rate of 10 °C/min over a temperature range of 20–180 °C. DSC measurements, onset (T_o), peak (T_p), and completion

(T_c) gelatinization temperatures, as well as gelatinization enthalpy (ΔH) were recorded using the TA Instruments Universal analysis software (version 3.9A). Analysis was performed in duplicate.

pH

pH was determined by a modified AOAC pH of flour method 943.02, sec. 32.1.20 (AOAC 2000b). A 3 g portion of cooked fufu was homogenized with 30 mL of Milli Q water. Homogenized samples were dispersed for 30 min by shaking. An electronic pH-meter AB15 Plus accumet® Basic (Fisher Scientific) was used with pH electrode accu TupH.

Titratable acidity

The titratable acidity (TA) was determined by a modified AOAC glass electrode method 942.15, sec. 37.1.37B (AOAC 2000a). A 3 g portion of cooked fufu was homogenized with 30 mL of Milli Q water. Homogenized samples were dispersed for 30 min by shaking. Sample was titrated to pH 8 with 0.1 M NaOH using a titration unit TitroLine easy Schott Instruments GmbH (Mainz, Deutschland, Germany). The titer volume was multiplied by 0.09 to give %TA as lactic acid.

Soluble protein extraction and measurement

Protein and pro-vitamin A fortified and wild type cooked fufu were extracted with 100 mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM EDTA, 0.2% Triton X-100 with 4% 2-mercaptoethanol. The homogenate was vortexed for 5 min at 2500 rpm and centrifuged at

1500 x g for 10 min at 4 °C. Supernatant was analyzed by the Bradford method (CBX kit) (G-Biosciences, St. Louis, MO) according to manufacturer's protocol. A calibration curve was constructed with bovine serum albumin included in the CBX kit as a standard.

Volatile analysis

The method of Iyer and others (2010) with some modifications was used for the identification of volatile compounds in cooked fufu samples. Optimization of the method was performed to determine the amount of cooked fufu product needed for the analysis based on our experience in GC-analysis.

Briefly, dried fermented cassava flours with and without *Lactobacillus plantarum* strain at all fermentation times were mixed with water in a ratio of 1: 3 (cassava: water). Mixes were cooked in a microwave oven Emerson 600 W (Model MW8665W) (Parsippany, NJ) at the highest power setting 10 (100%) in five cycles of 15 s, each followed by a manual mixing of 1 min. Samples were stored in plastic scintillation vials at -70 °C until analysis.

A cassava sample (0.5 g) was prepared with sodium chloride (0.33 g), and distilled water (1.0 mL) in a 10 mL headspace amber rounded bottom vial (Cat. No. SU860100) (Sigma –Aldrich, Miwaukee, WI) with a magnetic stirring bar. Sample vials were immediately sealed with a screw cap with PTFE/silicone septum (Cat. No. SU860101) (Sigma –Aldrich, Miwaukee, WI). Automation of the procedure was achieved using a CTC CombiPal autosampler (Zwingen, Switzerland), which was programmed using a CycleComposer software version A.01.04 (Agilent Technologies Inc.) and equipped with a sample tray, a temperature controlled agitator tray and a fiber-conditioning device. A SPME

stableflex fiber coated with 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) (57293-U) (Supelco, Bellefonte, PA) was conditioned for 1 h at 270 ºC in the fiber conditioner of the COMBI PAL system before use. Sample was stirred at 250 rpm, the fiber exposed to the headspace of the slurry and the volatile compounds were collected for 60 min based on previous optimization studies done by Cramer and others (2005).

The volatiles adsorbed onto the SPME fiber were thermally desorbed into the injection port of an Agilent Technologies 6890 series gas chromatograph (Agilent, Avondale PA) equipped with a 6890N GC split/splitless injector and data collection provided by Chemstation software version E.02.00.493 and a HP-5MS column (5% Phenyl Methyl Siloxane) (30 m x 0.248 mm x 0.25µm film thickness) (J&W Scientific, Folsom, CA). Helium was used as the carrier gas. The injector and detector temperatures were 200 °C and 250 °C, respectively. The column temperature was initially maintained at 33 °C for 5 min before increasing to 50 °C at a rate of 2 °C/min, and then to 225 °C at a rate of 5 °C/min. The sample was desorbed for 5 min with injector in the splitless mode. Cooked fufu volatile compounds were identified using a mass spectrophotometer MS 5975C (inert XL MSD) and MS spectra was compared against a NIST library. Quantitative data were determined by running known standards and developing response factors based on water matrices. The final values were reported as µg/mL.

Color evaluation

A Minolta colorimeter (Minolta Spectrophotometer CM-2002; Minolta Camera Co., Ltd., Osaka, Japan) was used to measure the color of dried cooked fufu samples. The

instrument was calibrated using an internal white calibration plate. The cooked fufu (approximately 5 g) was placed in a plastic petri dish and the color of the surface of the sample was read on the meter. Values of L*, a*, and b* were recorded, where L* is lightness or darkness, whose value varies from 100 for perfect white to zero for black; +a* is redness (0-60) and –a* is greenness (0 to -60); +b* is yellowness (0-60) and –b* is blueness (0 to -60) (Carreno and others 1995; Francis 1998). The average L, a* and b* values were obtained from two randomly selected points on the surface of the cooked fufu. The color measurement was performed in duplicate.

Instrumental analysis of texture

The texture profile parameters hardness, adhesiveness and springiness for the cooked fufu products, prepared using the fermented protein and pro-vitamin A fortified and wild type cassava flours, were measured using a food texture analyzer TA.XT2 (Texture Technologies, Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) and the data were collected with the software Texture Expert Exceed (Version 2.64). The sample was placed between flat cross heads. A 50 mm diameter and 20 mm high flat aluminum cylinder probe was used for the compression of the products on a flat plate on the HDP/90 heavy duty platform (Figure 3). The force was measured under compression with a trigger force of 0.1 N. The compression distance was set as 50% strain. The cross head speed was 1 mm/s with 0.10 s interval between the first and second compression. Large variations in the textural measurements may result from variation in the shape of the prepared fufu samples. Prior to the textural measurements, fufu sample was cooked, set at room

temperature for 15 min, weighed (5 g), manually shaped as a sphere and kept at room temperature for 2 hours. Texture was evaluated in duplicate samples.

SENSORY ANALYSIS

Sensory Training Panel

Five volunteers (1 male and 4 females), consumers of fermented cassava products, between 18 and 35 years old formed the trained sensory panel. Panelists were recruited from Washington State University and the Pullman community based on their availability and consumption of fermented cassava products at least once a week. A minimum amount of information related to the nature of the study was provided to the panelists to minimize potential bias. Panelists were informed that were assessing the sensory properties of fufu. This project was approved by the Washington State University Institutional Review Board for human subject participation.

Panelists were trained for 7.5 h during which time they learned the definition of the attributes (visual, aroma and texture), descriptors selected and the technique for the evaluation of each attribute. These attributes were selected and defined based upon previous literature (Oyewole 1994; Murray and others 2001; Akissoe and others 2006; Bhattacharya and others 2006; Meilgaard and others 2007; Oduro-Yeboah and others 2007; Sular and Okur 2007). Attributes, definitions, evaluation technique and standards used are presented in Table 1. During this training session, a 15-cm line scale was used for each descriptor, anchored by the terms 'low' (1 cm) and 'high' (14 cm). Commercial fufu flour (Kum-Koum) imported from Cameroon (JKUB, LLC International Foods, Washington,

DC) was used for training.

Panelists' performance was assessed by determining how far each panelist deviated from the mean, and reliability was assessed through duplicate evaluations. Panelists were provided with feedback after each practice session. Two practice evaluations were conducted in the individual sensory booths equipped with laptop computers to allow the panelist to familiarize with the sensory software (Compusense®*five* software (release 5.2, Guelph, ON) that was used for data collection.

Formal Sensory Evaluations

Cooked fufu cassava products with a rounded shape (approximately 5 g) were presented to each panelist over a period of 7 days, with each session held at the same time each day. The cooked fufu products were presented one at the time to the panelists in a covered 2 oz. soufflé cup in a random order at room temperature and were identified with a three-digit code. Panelists rated the perception of intensity of the fermented, stale and fufu aromas; hardness, adhesiveness and springiness for the texture attributes, and the brown color as presented in Table 1. Filtered deionized water was provided for sniffing between samples. Within each session, a total of 15 flights were presented. To minimize adaptation effects, a resting period of 10 min was imposed after the 7^{th} flight to rest the senses and each cooked fufu product was evaluated in a randomized complete block design. All sensory sessions were carried out in separate booths under white light equipped with a computerized system and sensory software (Compusense®*five* software (release 5.2, Guelph, ON) for data collection. All samples were evaluated in duplicate.

DATA ANALYSIS

Physico-chemical data of dried fermented cassava flour (fufu flour) and/or cooked fufu samples were analyzed for significant differences using a three-way analysis of variance (ANOVA). Tukey's multiple comparisons of means were used to analyze both dried fermented cassava flour (fufu flour) and cooked fufu physico-chemical data with XLSTAT (Version 7.5.3, XLSTAT Addinsoft, France) at the $p \leq 0.05$ confidence level. The ANOVA performed on physico-chemical data used sample, fermentation and time as fixed effects. Sensory data of cooked fufu samples were analyzed for significant differences using a three-way analysis of variance (ANOVA) in a randomized complete block using PROC MIXED in SAS 9.1 software (SAS Inst., Cary, NC). The analysis of sensory data using a mixed effects model assumed panelists as a random effect factor and sample, fermentation and time as fixed effect factors. Pearson's correlation test was used to describe the relationship between sensory and instrumental data (XLSTAT, Addinsoft, Paris). Significance level was defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

Starch granule

Gelatinization consists of the disruption of molecular and granular order within starch granules when they are heated in the presence of water (Whistler and BeMiller 1997). Starch gelatinization includes changes in properties such as granular swelling,

crystallite melting, loss of birefringence, viscosity development, and solubilization (Cui 2005). As observed in Figures 4a and 5, NFZ and LFZ at 0 and 96 h fermentation did not lose its organized structure and birefringence when dried at 70°C for 6.5 h which is determined by the presence of the two intersecting bands (Maltese cross) when observed under polarized light. This indicates that the starch granule has a radial orientation of crystallites and there exists a high degree of molecular order within the granule (Cui 2005). A similar situation was observed for all the other dried fermented cassava samples (fufu flours) with or without the addition of the starter culture (data not shown). On the other hand, all fufu flours lost the granular shape and structure after cooking (Figure 4b) as the birefringence as well (data not shown). This occurs due to an increase of temperature of the starch-water suspension that causes a rupture of the intermolecular hydrogen bonds. As reported by Altan and others (2009), the process of gelatinization leads to loss of birefringence and hence a loss of the Maltese cross as it was observed in this study on the samples cooked by microwave.

Pasting properties

Fufu flour is usually cooked into paste before consumption; hence, the pasting properties of fufu flour are an important quality index in predicting the behavior of fufu paste during and after cooking (Sanni and others 2006).

Tables 2-6 show the pasting properties of the fufu flour dried by tray dryer at all fermentation times. The pasting temperature is one of the pasting properties which provide an indication of the minimum temperature required for sample cooking, energy

involved and other components stability as pointed out by Akoja and Mohammed (2011). Regarding the pasting temperature, no significant differences were found between NF SPRO and LFS fufu flours at 0 h fermentation ($p>0.05$) while significant differences in pasting temperature were observed for LFZ compared to LFWT and NFWT fufu flours ($p\leq0.05$) at 96 h fermentation. The greatest pasting temperature (71.08 °C) was determined for LFS fufu flour while the lowest was for NFZ (61.53 °C) at 0 h fermentation (Table 2) which could be related to the starch concentration of the samples. At 96 h fermentation, LFWT showed the lowest pasting temperature while NFS presented the greatest value (Table 6). Based on the lower pasting temperature, the NFZ fufu flour (0 h), NFWT and LFWT (96 h) will cook faster with less energy consumed, thereby saving cost and time compared to other samples. According to Whistler and BeMiller (1997) root (tapioca) starches have weaker intermolecular bonding and gelatinize easily to produce high-viscosity pastes that thin rapidly with moderate shear because their highly swollen granules break easily.

At 0 h fermentation, the mean peak viscosity, defined as the highest viscosity of the sample, was highest for NFZ fufu flour (5969 cP) as opposed to LFS (4285 cP) (Table 2). This observation might have been influenced by greater rigidity of starch granules in NFZ, which in turn may cause instability and consequently disruption upon the heating and stirring treatment. In addition, the higher peak viscosity of the NFZ compared to other fufu flours could be due to apparent small granule size. However, the final viscosity of NFZ flour was substantially lower, likely due to free leaching of amylose and amylopectin from the granules (Leon and others 2006). More significant differences in the peak viscosity of the

samples ($p \leq 0.05$) were observed at 0, 48 and 96 h of fermentation compared to 24 and 72 h (Tables 3 and 5). Peak time is the time at which the peak viscosity occurred in minutes (Sanni and others 2006). Thus, the highest mean peak time was reached by NF SPRO fufu flour at 0 and 96 h of fermentation (Tables 2 and 6).

Breakdown is defined as the process of rupture of the granules and polymer alignment due to stirring and increase of temperature. When the gel is formed and the viscosity increases to a final viscosity, retrogradation is involved (Cui 2005). The higher the breakdown in viscosity, the lower the ability of the sample to withstand heating and shear stress during cooking (Akoja and Mohammed 2011 . Thus, NF SPRO fufu flour might be able to withstand heating and shear stress compared to the other samples because of its low breakdown value at all fermentation times.

The setback region is the phase where some starch molecules start to reassociate during cooling, forming a precipitate or gel and an increase in paste opacity. It therefore reflects retrogradation or reordering of the starch molecules. Setback has been consequently correlated with the texture of the fufu flours. The firmness of the gel depends on the extent of junction zone formation, which can be either facilitated or hindered by the presence of ingredients such as fats, proteins, sugars, acids and the amount of water. Setback is also associated with syneresis or "weeping". Lowest setback value indicates high stability (Whistler and BeMiller 1997; Sanni and others 2006). Here, the highest stability was shown by the NFZ fufu flour followed by LFS sample at 0 h of fermentation. At 96 h of fermentation, NF PRO and LF PRO fufu flours showed the greatest setback viscosity values possibly due to increased hydrogen bonding during cooling and amylose content of the

starch of the sample. This hydrogen bonding leads to the growth of gel micellar regions, hence increase in index of retrogradation, making entrapped water more prone to expression (Akoja and Mohammed 2011). This retrogradation as explained by Whistler and BeMiller (1997) also depends on other variables that include amylose/amylopectin ratio and their structures that will be based on the botanical source, temperature and starch concentration and other ingredients.

The final viscosity gives an indication of the stability of the cooled, cooked paste under low shear (Cui 2005). Final viscosity is used to determine a particular starch-based sample's quality as it indicates the ability of the material to form gel after cooking. NFWT fufu flour showed the highest final viscosity at 0 h fermentation (Table 2) while NFWT and NF PRO fufu flours obtained the highest final viscosity values at 96 h (Table 6).

Thermal properties of cassava flours and cooked fufu

There are a number of physical events occurring in starch during the cooling and heating processes such endothermic or exothermic transitions (Cui 2005). Differential scanning calorimeter (DSC) was used to monitor the thermal events as a function of temperature of cassava flours and freeze dried cooked fufu as depicted in Tables 7 and 8.

The gelatinization onset temperature (T_o) did not show any significant difference between the cassava flours ($p>0.05$). Gelatinization peak (T_p) values show some significant differences between SPRO and WT, S and PRO samples ($p\leq0.05$) while no significant differences were observed in completion (T_c) between S, SPRO and PRO cassava flours ($p>0.05$) (Figure 6). The gelatinization enthalpy ($\triangle H$) that is the measure of the overall

cristallinity (Geera and others 2006), showed the highest value for the WT (8.49 J/g) followed by the S (7.07 J/g) cassava flour (Table 7). The gelatinization enthalpy tended to be smaller in the fortified protein and pro-vitamin A samples compared to the wild type cassava flour. Noisuwan and others (2008) found that the △H of the milk protein/rice starch mixtures decreased when the concentration of milk protein was increased. They suggested that this decrease may be due to the interaction between starch granules and the milk proteins through absorption or adsorption mechanisms. As mentioned by Wang and Kim (1998), a competition for the limited amount of water takes place when protein, starch and other components such as fiber are present.

Previous studies have reported lower gelatinization enthalpies (Pérez and others 1998; Sriroth and others 1999). Sriroth and others (1999), suggest that genetic variation and environmental conditions impact structure of starch. SPRO and PRO samples exhibited a similar trend in the gelatinization enthalpy value. Looking at the mean values, WT presented the highest completion temperature and gelatinization enthalpy, while Z flour exhibited the highest gelatinization onset temperature and SPRO the highest gelatinization peak temperature.

In general, there were no significant effects of starter addition on the thermal properties of cooked fufu at 0 and 96 h ($p>0.05$) (Table 8). One exception was LFS where significant differences were found for T_p ($p\leq0.05$).

The organic acid produced during fermentation may complex with the soluble amylose fraction, thereby leading to an apparent reduction in soluble amylose content. This complexation leads to increased gelatinization temperature, improved starch quality and

reduced stickiness (Ray and Ward 2006). In this study, the gelatinization temperature (T_p) was not influenced by the fermentation time except for the LFS sample where an increase was observed from 58.08 °C (0 h) to 63.66 (96 h) (Table 8). It is necessary to mention that previous studies (Stevenson and others 2005), modified the thermal properties of corn starch using a microwave with controlled conditions. Therefore, more controlled microwave conditions for cooking fufu should include power and wattage.

NFZ fufu flour had the greatest gelatinization enthalpy (△H) at 0 h fermentation compared to other non inoculated samples (Table 8). This is most likely due to the presence of other components in flour such as zeolin that could obstruct the swelling of granules and thus increase the amount of heat required to reach the final swelling. Similar observations have been reported previously (Jane and others 1992). △H is a measure of the loss of molecular order within the granule that increases with a decline of the degree of starch cristallinity (Tester and Morrison 1990). Therefore, the small △H values for NF SPRO, NF PRO, LF SPRO and LF PRO fufu flours were observed at 0 h fermentation. LFZ and LFS had the lowest △H values at 96 h fermentation.

The results showed that the RVA pasting temperatures (temperature at which a measurable viscosity increase was detected upon gelatinization) for all fufu flour samples occurred in the same order as for DSC completion gelatinization temperatures. NFZ fufu flour exhibited higher peak paste viscosity at 0 h fermentation as compared with NF and LF SPRO fufu flours (Table 2). For SPRO fufu flours, the low peak paste viscosity value might be a function of a high degree of starch crystallinity (Wright and others 2002) or its somewhat narrower DSC gelatinization pattern.

pH and titratable acidity

Tables 9 and 10 show the pH and TA, respectively of the NF and LF cooked fufu at 5 different fermentation times. Most naturally fermented samples (NF) reached a minimum value of pH at 96 h of fermentation compared to 72 h for the *L. plantarum* samples (LF). NFS and NF SPRO showed a maximum pH value of 4.34 and 4.51 at 96 and 72 h, respectively. A fast decrease of pH to 3.60 was observed where the lactic acid bacteria strain was added for LFS and LF SPRO at 96 and 72 h of fermentation respectively (Table 9). These results agreed with Brauman and others (1996) who pointed out that LAB produced high amounts of lactic acid, leading to a rapid drop in pH to around 4.5 after the second day of fermentation of cassava roots.

As regards to the TA, the fermentation process resulted in gradual increase of acidity of the NFWT and LFWT cooked fufu up to 72 h of fermentation with a no significant decrease at 96 h ($p \leq 0.05$) (Table 10). An analogous tendency was shown for the NFZ and LFZ. The level of acidification increased with increasing period of fermentation in most of the samples (Table 10). Most samples increased the acidity value until 72 h of fermentation with the exception of NF SPRO sample, with its maximum value found at 48 h.

When *Lactobacillus plantarum* starter culture was added, the decrease of pH or increase of TA occurred more rapidly at 24 h when compared to samples without starter culture. This agrees with Kostinek and others (2005) who determined a high capability of the obligate homo- and facultatively heterofermentative group (mostly *L. plantarum*) of lowering the pH of the medium. Acid production and the subsequent decrease of pH

extends the lag phase of sensitive organisms including foodborne pathogens as emphasized by Kostinek and others (2007).

Soluble protein determination

As shown in Figure 7, NFWT had 56% increase of the soluble protein content from 0 h to 48 h of fermentation. An increase also was observed for NFS at 96 h (24%). On the contrary, Z and PRO showed the greatest decrease on the soluble protein content at 96 h, 67% and 63%, respectively.

For the *L. plantarum* strain containing samples, a decrease of the protein content was observed in all samples from 0 to 96 h of fermentation. The Z samples were the only ones that did not show any significant difference in protein content based on the addition of the starter culture ($p>0.05$). Greater significant differences on the protein content were observed between samples at 0 h and 96 h fermentation time ($p\leq0.05$). Z samples showed the greatest decrease of soluble protein content (77%) from 0 to 96 h of fermentation while S, SPRO and PRO fortified cassava flours decreased 36%, 26% and 37%, respectively. Ezeala (1984) found a decrease of about 45% of the protein content during a traditional fermentation of cassava for gari production. The proteolytic activity of some strains of *L. plantarum* was studied by Fadda and others (1998). They determined that the strains of *L. plantarum* studied grew and utilized the diverse nitrogen sources of the soluble meat extract. As a result, several water-soluble protein bands disappeared when running SDS-PAGE. In another study, Khalid and Marth (1990) also demonstrated the proteolytic activity of some strains of *L. plantarum* when manufacturing cheese. Those strains preferentially

degraded β-casein. Conversely, when active yeast cells (*Saccharomyces cerevisiae*) were added to cassava before fermentation, an increase of crude protein was found compared to fermentation without the addition of yeast cells as inoculums (Antai 1990).

Volatile compounds

For fufu processing, the fermented mashes were dried before milling to powder. The drying process could have driven off the volatiles which give the characteristic offensive odor of traditionally processed fufu (Shittu and Adedokun 2010).

Among some aromatic compounds found by Amoa-Awua and others (1996) in another fermented cassava product (agbelima) from Ghana were 2-methyl- 1 -butanol, 3-methyl- 1 -butanol, 1-propanol, isoamyl alcohol, ethyl acetate, iso butyl acetate, acetoin and a low molecular weight alcohol which was not identified. The aroma profile, determined before the cooking process of the dough, was not influenced by the type of inocula used during the fermentation process.

In this study, only a few aromatic compounds were detected in the cooked fufu samples. A similar profile was obtained for all samples in spite of their specific characteristics. The main compounds detected were acetic acid, hexanal, nonanal and decanal at all fermentation times (Tables 11-15). At 0 h of fermentation, a low concentration of acetic acid was found in all samples (Table 11) and no significant differences were found among treatments ($p>0.05$). Acetic acid concentrations were greater than the published threshold value (24.3 μg/mL) (Stahl 1973) in all cooked fufu samples fermented from 24 to 96 h. High concentrations of acetic acid may affect the

acceptability of the cooked fufu product. Hexanal was detected in most samples at all fermentation times. No significant differences in hexanal concentration were found among samples at 48 h fermnentation (Table 13). Level of hexanal was below the published threshold value (0.03 μg/mL) (Stahl 1973) in most samples. The greatest amount of hexanal was found in NF PRO at 24 h fermentation (0.146 μg/mL) (Table 12).

Due to the cooking process of the fufu flour, small amounts of aromatic compounds were found with exception of the acetic acid which kept increasing during the process of fermentation. When microorganisms were used by Fagbemi and Ijah (2006) to enrich fufu, the aroma of the developed product was preferred compared to the commercial fufu after 24 h and 48 h of fermentation. No determination of aromatic compounds was done. Dougan and others (1983) analyzed the aromatic compounds of gari, a fermented cassava based product, before frying. This group determined several volatile constituents in gari such as hexanal and nonanal (aldehydes) as we found in our cooked fufu. No information of aromatic compounds was reported after cooking the product that is commonly eaten in Africa.

Color evaluation

The color of cooked fufu can be an important quality parameter, which has a direct influence on the acceptability of the developed product as was pointed out by Medoua and others (2008) when studying fermented hardened yam tubers. CIE L*a*b* parameters of cooked fufu with and without the addition of a starter culture from 0 to 96 h of fermentation are presented in Tables 16-18.

Lightness (L*) of NF SPRO and LF SPRO differed significantly ($p>0.05$) at 0 h of fermentation with the latter having the greater value. As shown in Table 16, there was mainly a predominant influence of sample type rather than fermentation type. At 48 h of fermentation there were significant differences between NFZ and LFZ; and NF SPRO and LF SPRO ($p\leq 0.05$). Significant differences in L* were also observed for NFWT between 0 and 96 h fermentation with the latter showing less lightness ($p\leq 0.05$). Similar results were visualized for LFWT. The greatest mean values for lightness were presented by NF SPRO and LF PRO samples at all fermentation times maybe due to the presence of pro-vitamin A that protected the samples for oxidation and loss of lightness. On the other hand, Dziedzoave and others (2000) observed an overall decrease of yellow color in agbelima, a cassava fermented product. They stated that may be due to the izomerization of the regular carotenoids present in cassava roots at the onset of acid formation during fermentation.

As indicated by Medoua and others (2008), a decrease of lightness may be due to browning during fermentation. This browning could have been caused by the oxidation of phenolic compounds. Predominant sugars such as glucose, fructose, and sucrose depending of the specific composition of each one of the cassava flour materials may also have an influence on decreasing of lightness. Higher protein flours in the presence of sugars enhance Maillard reaction or non-enzymatic browning (Rampersad and others 2003).

Regarding a*values, a significant effect of sample type and fermentation type was observed (Table 17). Thus, naturally fermented Z, S and S PRO showed significant differences in a* when compared with their analogous inoculated sample ($p>0.05$) at 0 h fermentation. Similar results were observed at 96 h of fermentation except for PRO sample.

Also, the smallest a* mean value was found for NFZ (-2.16) followed by NFWT (-1.91) at 0h and NFWT (-2.01) followed by NFZ (-1.82) at 96 h fermentation. Consequently, a* values of WT and Z naturally fermented and strain added samples were negative (green). On the contrary, S and S PRO were positive (red). In the case of PRO samples values changed from negative to positive (green to red).

Samples with pro-vitamin A, SPRO and PRO became more yellow with time when the starter culture was not added, as recorded by increases in positive b* values (Table 18). Even though LFS samples did not contain pro-vitamin A, no significant differences were found in b* values between LFS, LF SPRO and PRO samples at 0 h fermentation ($p>0.05$). This trend continued through the fermentation process with no significant differences found between LFS and either LF SPRO or LF PRO samples. Comparing NFS and LFS samples, it seems that addition of the starter culture influenced the degree of yellowness of the sample. Thus, greater b* values were determined when the starter culture was added (Table 18). Carotenoids are fairly resistant to heat, and pH (acid, alkali). The nutritive value is protected during cooking by the carotenoids insolubility in water. However they are very sensitive to oxidation, which results in both color loss and destruction of vitamin A activity (Potter 1986; Srilakshmi 2007). In this study, fortified pro-vitamin A cooked samples showed an increase of yellow color over time that demonstrated lack of detrimental effect of the decrease of pH on color.

Although no instrumental measurements of color were done by Sobowale and others (2007), this group did not find any significant differences on the color and overall acceptability of the naturally fermented and the starter culture strain added fufu flours.

Texture evaluation

The results for the texture attributes measured are displayed in Tables 19-21. In the case of hardness of the cooked fufu samples, no significant differences were observed between samples within each fermentation time ($p>0.05$) (Table 19). Therefore, the type of sample and addition of the starter culture did not play an apparent role in the hardness measurement.

Regarding adhesiveness (Table 20), no significant effect of addition of the starter culture was observed at 0 h fermentation ($p>0.05$). This situation changed when significant differences were observed between all samples at all other fermentation times ($p\leq0.05$). Thus, the composition of each of the cassava flours significantly influenced the degree of adhesiveness. In addition, even though a similar strength was applied during mixing and preparing the cooked fufu samples, it may be possible that mixing intensity could have affected the texture attributes. For example, the breaking up of the gelatinized starch granules in the cooked fufu. According to Oduro-Yeboah and others (2007), the starch leaches out and binds very well with the water added during the pounding process. When the starch granule absorbs water, a good paste is formed and desirable adhesive properties are obtained.

On the other hand, the addition of the starter culture did not influence the adhesiveness of the samples. For example, no significant differences were found on the adhesiveness of the NFWT and LFWT samples at 96 h fermentation ($p>0.05$). Numfor and others (1995) compared the textural properties of starch gels from naturally fermented and inoculated fermented cassava starches. Their results showed that the hardness,

gumminess, cohesiveness and elasticity of flour gels were reduced in fermented products. Gel hardness and gumminess have been linked both to the degree of granule swelling and network formation by leached amylose. A reduction in cohesiveness of fermented products has been explained as being due to failure of starch granules to release sufficient amylose. The improvement of textural quality has also been attributed to production of organic acids that complex with soluble amylose.

Lastly, only slight differences in springiness were observed between samples within each fermentation time (Table 21). Except for a few cases, neither sample type nor fermentation type had a significant effect on springiness. For example, there was a significant difference between the springiness of NFZ and LF PRO samples at 0h fermentation. At 24 h, the springiness of LF PRO was significantly smaller than NFWT. At 96 h of fermentation, NFZ was significantly different from NF SPRO, LFWT, LFS, LF SPRO and LF PRO ($p \leq 0.05$).

Sensory analysis

Sensory properties and nutritional content of foods play an important role in food choices. From the ANOVA results (Table 22), panelists significantly impacted the perception of all cooked fufu attributes ($p \leq 0.05$). There was no significant effect from sample type, fermentation type, fermentation time and interaction between sample type and fermentation time, fermentation type and fermentation time, interaction between sample type, fermentation type and fermentation time on the fufu descriptor for the aroma attribute ($p > 0.05$). This suggests that using either wild type cassava flour or protein – pro-

vitamin A fortified cassava flours, a product with a characteristic fufu aroma is feasible.

In addition, no significant effect of fermentation type on fermented aroma was noted. Similarly, no significant effect of fermentation time was found on stale aroma, adhesiveness and springiness texture attributes ($p>0.05$). However, sample type and fermentation type significantly influenced the perception of texture attributes, hardness, adhesiveness and springiness ($p\leq0.05$). On the other hand, the interaction between fermentation type and fermentation time did not have a significant effect on hardness, adhesiveness and springiness ($p>0.05$) (Table 22).

Ray and Sivakumar (2009) cited Oyewole and Ogundele (2001) who reported that there were no significant differences in functional attributes, texture, color, odor and overall acceptability of fufu fermented for different length of time (12–96 h). This result disagrees with our findings, where we determined that fermentation time had a significant effect on the brown color intensity, fermented aroma and hardness of the cooked fufu products ($p\leq0.05$) (Table 22).

The sensory results indicate that cooked fufu is distinctly different when made from different cassava flour materials in terms of all the sensory attributes measured except for fufu aroma attribute. The characteristic odor of traditionally processed fufu has been attributed to the synthesis of certain organic acids due to certain hetero-fermentative anaerobic bacteria present in fermenting cassava medium (Shittu and Adedokun 2010). Thus, it seems that addition of a strain of *L. plantarum* did not affect the characteristic fufu aroma. In an acceptability study, Sobowale and others (2007), determined that the addition of a starter culture strain reduced the characteristic aroma in fufu, thereby enhancing the

wider acceptability of fufu as compared to the traditional fufu where no culture was added.

According to Sanni and others (1998), fufu is considered to be of good quality when it has a smooth texture, characteristic aroma and has a creamy-white, grey or yellow color. Mean values of trained panel evaluation of visual appearance, aroma and texture attributes of cooked fufu at all fermentation times are shown in Tables 23-27. Based on the color chart used by the panelists (Figure 9), the mean color intensity for brownness rating of NFZ and LFZ were lower than ratings of the other cooked fufu samples at 0 h of fermentation (Table 23). This may suggest that Z sample would have a greater acceptability. Thus, Tomlins and others (2007) determined that to increase the acceptability of fufu flours, they should be creamier in appearance. Later, at 96 hours of fermentation, the NFWT, NFZ, NF PRO, LFWT and LFZ showed lower ratings than the other samples with LF SPRO presenting the highest mean rating for brown color intensity (Table 27).

Regarding hardness, no significant differences were found between NFWT and NF SPRO ($p \leq 0.05$) at 0 h of fermentation (Table 23). At 96 h of fermentation, NF SPRO cooked fufu showed the highest mean rating for hardness while NFWT had a significantly smaller mean ($p \leq 0.05$) (Table 27). No significant differences were observed for adhesiveness at 96 h of fermentation. There were significant differences for springiness between NF SPRO and LFWT cooked fufu at 96 h of fermentation.

Relationship between instrumental and sensory variables

Pearson's correlation analysis was performed for each fermentation time to determine the correlation between sensory attributes and instrumental measurements

related to color, aroma and texture of the cooked fufu as shown in Tables 28-32. Irrespective of the cooked fufu sample type, the brown color intensity perceived by the panelists was highly positively correlated with a* and b* values collected with the colorimeter at all fermentation times (Table 28-32). Regarding the perceived sensory aromas, the fermented aroma was positively correlated with acetic acid determined at 0h while negatively correlated with nonanal and decanal (Table 28). The stale and fufu sensory aromas were highly positively correlated with the hexanal compound measured by GC-MS (Table 28). At 96 h of fermentation, all volatile compounds found in the cooked fufu samples were positively correlated with the fermented, stale and fufu aromas evaluated during the sensory trained panel (Table 32). In relation to the texture sensory attributes, hardness was positively correlated with all the texture measurements obtained through the texture profile analyzer instrument (hardness, adhesiveness and springiness). Conversely, perceived adhesiveness and springiness were only positively correlated with the instrumental measurement of adhesiveness. Several factors may have contributed to the negative relationship between sensory and instrumental measurement of the attributes. Time interval between sensory and instrumental analysis and temperature of the sample are some factors that should be taken into consideration. As determined by Badrie and Mellowes (1991), textural changes in cassava flour extrudates were influenced by extrusion variables such as temperature. Specifically related to texture, Szczesniak (2002) pointed out that some publications have reported good correlations between instrumental and sensory ratings for hardness but correlations for other texture parameters are not as good.

CONCLUSIONS

Lactobacillus plantarum strain 6710 demonstrated its ability to acidify cassava flour rapidly during fufu processing. Pasting temperature of the fufu flours established that NFZ (0 h) and NFWT and LFWT (96 h) will cook faster than the other samples due to their lower pasting temperature. Fufu flour made from NFZ was more stable as indicated by lowest setback viscosity. Starch gelatinization enthalpy tended to be smaller in the protein and pro-vitamin A fortified cassava flours compared to wild type. Based on fermentation time, most samples did not show significant differences in thermal properties between 0 and 96 h of fermentation. Cooked fufu samples generally reached minimum value of pH and maximum acidity at 72 h. Protein content decreased as fermentation time increased in most cooked fufu samples irrespective of the addition of the *L.plantarum* strain. Four main aromatic compounds acetic acid, hexanal, nonanal and decanal were detected in most cooked fufu samples at all fermentation times. Color parameters, springiness and adhesiveness texture attributes were most affected by the flour sample type and fermentation time. The sensory results indicated that cooked fufu is distinctly different when made from different cassava flours in terms of all sensory attributes measured. One exception was the fufu aroma attribute.

Processing of cooked fufu products with protein and pro-vitamin A fortified cassava flours offer an alternative for fufu consumers; even though a consumer acceptability panel would be necessary to indicate the degree of likeness of the developed products.

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Table 1. Attributes, definitions, evaluation technique, standards and intensity values (I) used for sensory evaluation of proposed fufu cassava product

ATTRIBUTE/ Descriptors	DEFINITION	TECHNIQUE	STANDARDS
VISUAL			
Color intensity for brownness	The extent to which the color of the sample is intense. The low end of the scale represents a dull brown color and the high end of the scale represents the brown color	Compare to color chips for brownness	Color chips
AROMA			
Fermented aroma	Intensity of fermented cassava aroma	*Swirl the bottle containing the standard. *Remove lid. *2-3 short, sharp sniffs. *Replace lid.	Shredded cassava solution: 1:3 (cassava:water) with 550 µL acetic acid (I:12)
Stale aroma	Intensity of stale aroma	*Swirl the bottle containing the standard. *Remove lid. *2-3 short, sharp sniffs. *Replace lid.	Corked standard solution (Wine awakening): 1 drop of standard + 200 mL water (I:12.5)
Fufu aroma	Intensity of fufu aroma	*Swirl the bottle containing the standard. *Remove lid. *2-3 short, sharp sniffs. *Replace lid.	Fufu flour (Cameroon) solution: 1:3.75 (fufu flour:water) (I:12)
TEXTURE			
Hardness	Force required to fully compress a sample with the fingers	Compress the sample between the thumb and index finger.	Frankfurter (I:13.5)
Adhesiveness	Degree to which a sample adheres to the fingers	Press the sample between the thumb and the fore-and middle fingers, then release; evaluate the degree of adherence.	Cooked fufu flour (Cameroon) solution: 1:3 (flour:water) (I:14)
Springiness	Degree to which sample returns to original shape after a certain time period	Press the sample by the tip of the forefinger and feel the recovery while releasing the finger.	Bread (I:14.5)

Table 2. Pasting properties[1] of dried fermented cassava flour "fufu flour" with (LF) and without (NF) the addition of a starter culture at 0 h of fermentation

Cassava maaterial[2]		Peak viscosity (cP)	Trough (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak time (min)	Pasting temperature (°C)
LF	WT	4972 ± 8^{c}	1792 ± 10^{d}	3189 ±14^{c}	2735 ± 15^{f}	963 ± 54^{c}	3.85 ± 0.03^{f}	64.58±0.67^{d}
	Z	5518 ± 42^{b}	1628 ± 37^{e}	3899 ±18^{a}	2890 ± 8^{e}	1334 ± 73^{ab}	3.68 ± 0.02^{g}	62.13±0.04^{e}
	S	4285 ± 6^{f}	1672 ± 22^{e}	2620 ± 6^{d}	2588 ±14^{g}	959 ± 23^{cd}	4.36 ± 0.04^{cd}	71.08±0.11^{a}
	SPRO	3667± 32^{g}	1566 ± 13^{e}	2094 ± 8^{g}	2878 ± 16^{e}	1325 ± 21^{ab}	4.46 ± 0.00^{c}	69.78±0.11^{bc}
	PRO	4418 ± 58^{e}	1812 ± 11^{d}	2563 ± 8^{e}	3249 ± 15^{c}	1412 ± 9^{a}	4.26 ± 0.01^{d}	69.05±0.07^{c}
NF	WT	5476 ± 37^{b}	2790 ±13^{a}	2652 ± 25^{d}	3949 ± 47^{a}	1194 ± 12^{b}	4.30 ± 0.05^{d}	69.73±0.04^{bc}
	Z	5969 ± 26^{a}	2359 ± 62b	3648 ± 18^{b}	3057 ± 4^{d}	773 ± 40^{d}	4.02 ± 0.04^{e}	61.53±0.04^{e}
	S	4561 ± 40^{d}	1968 ± 28^{c}	2649 ± 11^{d}	2813 ± 17^{ef}	884 ± 44^{c}	4.63 ± 0.04^{b}	65.35±0.07^{d}
	SPRO	3328 ± 2^{h}	1623 ± 8^{e}	1705 ± 6^{h}	2565 ± 25^{g}	955 ±14^{c}	4.87 ± 0.01^{a}	70.35±0.07^{ab}
	PRO	4269 ± 25^{f}	963 ± 25^{c}	2315 ± 14^{f}	3456 ± 11^{b}	1457 ± 35^{a}	4.25 ± 0.02^{d}	69.08±0.11^{c}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

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Table 3. Pasting properties[1] of dried fermented cassava flour "fufu flour" with (LF) and without (NF) the addition of a starter culture at 24 h of fermentation

Cassava material[2]		Peak viscosity (cP)	Trough (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak time (min)	Pasting temperature (°C)
LF	WT	4206±10^{c}	1683±4^{b}	2528±7^{d}	3153±51^{c}	1462±35de	3.94±0.01^{h}	64.75±0.07^{e}
	Z	5143±59^{a}	1623±12^{b}	3484±20^{a}	2859±54^{d}	1157±47^{f}	4.08±0.02^{fg}	60.33±0.04^{g}
	S	4090±28^{c}	1441±42^{c}	2592±11^{d}	3468±4^{f}	994±9^{g}	4.17±0.05^{ef}	71.00±0.07^{b}
	SPRO	3520±98^{d}	1270±59^{d}	2236±18^{f}	2329±14^{g}	1047±63^{fg}	4.52±0.02^{c}	69.08±0.04^{d}
	PRO	4085±19^{c}	1292±7^{d}	2736±54^{c}	2683±11^{e}	1375±27^{e}	4.23±0.05^{de}	69.33±0.46^{d}
NF	WT	5064±51^{a}	2035±30^{a}	3008±8^{b}	3620±11^{b}	1623±13^{c}	3.91±0.03^{h}	60.58±0.32^{g}
	Z	5195±6^{a}	1623±17^{b}	3539±57^{a}	2781±12de	1172±15^{f}	4.00±0.00^{gh}	60.15±0.07^{g}
	S	4478±32^{b}	1624±14^{b}	2842±62^{c}	3151±11^{c}	1515±20^{cd}	4.34±0.01^{d}	63.35±0.07^{f}
	SPRO	3146±9^{e}	1279±29^{d}	1844±6^{e}	3115±14^{c}	1842±51^{b}	5.24±0.04^{a}	72.33±0.04^{a}
	PRO	4042±42^{c}	1345±18^{cd}	2749±13^{c}	3755±10^{a}	2421±13^{a}	5.00±0.01^{b}	70.13±0.18^{c}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

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Table 4. Pasting properties[1] of dried fermented cassava flour "fufu flour" with (LF) and without (NF) the addition of a starter culture at 48 h of fermentation

Cassava material[2]		Peak viscosity (cP)	Trough (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak time (min)	Pasting temperature (°C)
LF	WT	4752±52^{c}	2328±24^{a}	2416±17^{d}	3259±43^{b}	962±63^{f}	3.93±0.00^{f}	60.98±0.11^{e}
	Z	4888±12^{b}	1492±6de	3382±13^{a}	2682±7^{e}	1177±17de	3.77±0.05^{g}	70.65±0.49^{bc}
	S	3792±11^{e}	1351±5^{g}	2445±11^{d}	2460±6^{f}	1110±10^{e}	4.16±0.03^{e}	70.35±0.00^{cd}
	SPRO	3292±48^{f}	1262±17^{h}	2073±4^{e}	2389±10^{f}	1129±5^{e}	4.88±0.02^{b}	70.83±0.74^{bc}
	PRO	4048±12^{d}	1389±31^{fg}	2682±10^{c}	2840±21^{d}	1442±23^{c}	4.32±0.02^{cd}	69.30±0.42^{d}
NF	WT	4146±6^{d}	1545±10^{d}	2595±6^{c}	3263±7^{b}	1709±11^{b}	4.28±0.02^{d}	70.98±0.04^{bc}
	Z	5098±33^{a}	1690±33^{c}	3443±49^{a}	3077±67^{c}	1374±16^{c}	4.28±0.02^{d}	71.08±0.04^{bc}
	S	3847±12^{e}	1778±16^{b}	2042±42^{e}	2995±10^{c}	1248±16^{d}	4.42±0.04^{c}	72.38±0.04^{a}
	SPRO	3096±20^{g}	1444±8^{ef}	1643±14^{f}	2585±15^{e}	1132±10^{e}	4.79±0.01^{b}	71.73±0.11^{ab}
	PRO	4134±12^{d}	1214±16^{h}	2949±13^{b}	3685±14^{a}	2481±11^{a}	5.06±0.01^{a}	71.00±0.00^{bc}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

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Table 5. Pasting properties[1] of dried fermented cassava flour "fufu flour" with (LF) and without (NF) the addition of a starter culture at 72 h of fermentation

Cassava material[2]		Peak viscosity (cP)	Trough (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak time (min)	Pasting temperature (°C)
LF	WT	5944±12^{a}	2066±13^{a}	3888±13^{a}	3224±24^{b}	1145±8^{f}	3.74±0.00^{g}	62.23±0.04^{g}
	Z	4858±81^{b}	1469±25^{e}	3354±6^{b}	2540±35^{f}	1033±44^{g}	3.68±0.02^{g}	63.93±0.04^{f}
	S	3458±76^{d}	1146±32^{g}	2290±12^{f}	2286±5^{h}	1142±25^{f}	4.35±0.03^{d}	70.30±0.07^{d}
	SPRO	3475±52^{d}	1282±7^{f}	2165±6^{g}	2443±21^{g}	1165±19^{f}	4.80±0.09^{c}	70.40±0.07^{cd}
	PRO	3647±71^{d}	1088±47^{g}	2651±11^{e}	3028±10^{c}	1969±17^{a}	4.12±0.02^{e}	70.63±0.53^{cd}
NF	WT	4670±73^{b}	1718±60^{b}	2954±16^{c}	3243±16^{b}	1535±30^{d}	3.71±0.03^{g}	71.58±0.04^{b}
	Z	4385±8^{c}	1593±5^{cd}	2812±14^{d}	2775±28^{e}	1218±16^{f}	3.89±0.03^{f}	71.60±0.00^{b}
	S	3007±8^{e}	1674±26^{bc}	1317±11^{i}	3383±11^{a}	1712±18^{c}	4.86±0.01^{c}	74.28±0.04^{a}
	SPRO	3145±57^{e}	1489±8de	1629±9^{h}	2859±10^{d}	1386±23^{e}	5.20±0.01^{a}	71.05±0.07^{bc}
	PRO	4445±18^{c}	1611±16^{bc}	2843±16^{d}	3458±28^{a}	1847±45^{b}	5.01±0.01^{b}	69.05±0.07^{e}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 6. Pasting properties[1] of dried fermented cassava flour "fufu flour" with (LF) and without (NF) the addition of a starter culture at 96 h of fermentation

Cassava material[2]		Peak viscosity (cP)	Trough (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak time (min)	Pasting temperature (°C)
LF	WT	4965±18^{b}	2243±22^{a}	2740±21^{c}	3244±24^{bc}	1005±6^{c}	4.14±0.01^{d}	60.83±0.04^{b}
	Z	5179±71^{a}	1731±30^{bc}	3453±48^{a}	2707±16^{e}	996±13^{c}	3.77±0.05^{e}	71.03±0.04^{a}
	S	3480±11^{f}	1280±13^{ef}	2189±8^{e}	2240±20^{g}	924±18^{cd}	4.36±0.04^{c}	72.95±0.07^{ab}
	SPRO	2809±81^{g}	1357±59^{e}	1452±21^{f}	2045±13^{h}	775±50^{e}	4.86±0.01^{b}	70.98±0.04^{ab}
	PRO	3633±11^{e}	1045±17^{g}	2590±10^{d}	3240±9^{bc}	2172±24^{a}	4.85±0.02^{b}	70.18±0.25^{ab}
NF	WT	4571±12^{c}	1779±14^{b}	2818±11^{c}	3303±18^{ab}	1534±18^{b}	5.06±0.01^{a}	62.03±0.04^{b}
	Z	4683±9^{c}	1555±43^{d}	3102±15^{b}	3066±33^{d}	1463±7^{b}	4.03±0.04^{d}	71.60±0.00^{ab}
	S	2830±21^{g}	1633±14^{cd}	1185±18^{g}	3181±7^{c}	1551±11^{b}	5.08±0.02^{a}	75.45±0.07^{ab}
	SPRO	2682±40^{g}	1681±21^{bc}	1051±10^{h}	2489±3^{f}	852±37de	5.14±0.01^{a}	74.23±0.04^{ab}
	PRO	3985±18^{d}	1239±14^{f}	2766±33^{c}	3339±42^{a}	2150±13^{a}	5.05±0.07^{a}	71.03±0.04^{ab}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 7. Thermal characteristics[1] of protein and pro-vitamin A fortified cassava flours

Cassava material[2]	Thermal Transition Temperatures (°C)			Gelatinization enthalpy,
	T_o	T_p	T_c	ΔH(J/g)
WT	70.92 ± 0.28^{a}	76.00 ± 0.28^{b}	93.33 ± 0.28^{a}	8.49 ± 0.49^{a}
Z	71.36 ± 0.11^{a}	76.67 ± 0.23^{ab}	86.23 ± 0.35^{c}	4.15 ± 0.53^{d}
S	70.52 ± 0.38^{a}	76.29 ± 0.23^{b}	88.60 ± 0.21^{b}	7.07 ± 0.07^{b}
SPRO	70.86 ± 0.37^{a}	77.62 ± 0.54^{a}	89.49 ± 0.08^{b}	5.20 ± 0.11^{c}
PRO	70.74 ± 0.18^{a}	76.10 ± 0.16^{b}	89.33 ± 0.47^{b}	5.15 ± 0.15^{c}

[1]Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

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Table 8. Thermal characteristics[1] of cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[2]		Fermentation time (h)	Thermal Transition Temperatures (°C)[3]			Gelatinization enthalpy, △H(J/g)
			T_o	T_p	T_c	
LF	WT	0	50.32 ± 1.00^{cd}	58.22 ± 0.37^{bcd}	66.73 ± 1.29^{abcde}	1.70 ± 0.11^{b}
		96	50.68 ± 2.43^{cd}	56.54 ± 0.36^{cd}	63.26 ± 4.26^{bcde}	2.10 ± 0.76^{b}
	Z	0	52.32 ± 0.21^{abcd}	58.19 ± 0.41^{bcd}	70.59 ± 4.82^{abc}	3.50 ± 0.03^{ab}
		96	55.84 ± 2.39^{ab}	59.75 ± 2.61^{b}	63.85 ± 3.09^{abcde}	0.24 ± 0.04^{b}
	S	0	51.76 ± 0.30^{abcd}	58.08 ± 0.64^{bcd}	65.11 ± 1.00^{abcde}	0.88 ± 0.24^{b}
		96	56.30 ± 0.03^{a}	63.66 ± 0.69^{a}	71.56 ± 0.66^{a}	0.33 ± 0.02^{b}
	SPRO	0	51.92 ± 0.30^{abcd}	56.27 ± 0.10^{cd}	61.60 ± 0.14^{e}	0.53 ± 0.02^{b}
		96	49.46 ± 1.24^{cd}	55.20 ± 0.39^{d}	62.93 ± 0.55^{cde}	1.56 ± 0.49^{b}
	PRO	0	52.03 ± 0.90^{abcd}	56.15 ± 0.60^{cd}	61.00 ± 1.46^{e}	0.35 ± 0.20^{b}
		96	51.82 ± 0.43^{abcd}	56.01 ± 0.04^{cd}	61.17 ± 1.39^{e}	0.43 ± 0.12^{b}
NF	WT	0	50.86 ± 0.72^{bcd}	57.68 ± 0.16^{bcd}	69.79 ± 0.47^{abcd}	3.73 ± 0.40^{ab}
		96	54.25 ± 1.68^{abc}	58.51 ± 0.39^{bc}	64.10 ± 0.23^{abcde}	0.61 ± 0.33^{b}
	Z	0	48.76 ± 1.73^{d}	58.40 ± 0.40^{bc}	71.05 ± 2.89^{ab}	8.89 ± 0.24^{a}
		96	49.94 ± 0.57^{cd}	58.00 ± 1.29^{bcd}	70.25 ± 2.73^{abcd}	3.72 ± 0.26^{ab}
	S	0	51.47 ± 1.06a^{bcd}	57.89 ± 0.30^{bcd}	67.13 ± 0.73^{abcde}	1.98 ± 0.80^{b}
		96	51.53 ± 1.65^{abcd}	57.28 ± 0.19^{bcd}	65.29 ± 1.07^{abcde}	1.62 ± 0.85^{b}
	SPRO	0	51.61 ± 0.08^{abcd}	56.28 ± 0.69^{cd}	60.78 ± 0.49^{e}	0.51 ± 0.02^{b}
		96	48.92 ± 0.69^{d}	55.32 ± 0.33^{d}	65.26 ± 0.80^{abcde}	2.54 ± 0.06^{ab}
	PRO	0	51.80 ± 1.85^{abcd}	56.01 ± 0.36^{cd}	60.17 ± 1.55^{e}	0.38 ± 0.23^{b}
		96	50.25 ± 1.31^{cd}	56.10 ± 0.33^{cd}	62.19 ± 0.79^{de}	0.57 ± 0.03^{b}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$). [2]T_o, T_p and T_c are the onset, peak and conclusion gelatinization temperatures, respectively. [3]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) materials.

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Table 9. pH[1] of cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[2]		Fermentation time (h)				
		0	24	48	72	96
LF	WT	4.52 ± 0.01^{Ad}	3.51 ± 0.01^{Bd}	3.47 ± 0.08^{Bd}	3.39 ± 0.04^{Bc}	3.39 ± 0.05^{Bc}
	Z	4.76 ± 0.09^{Ad}	3.62 ± 0.01^{Bcd}	3.56 ± 0.00^{Bcd}	3.50 ± 0.04^{Bbc}	3.51 ± 0.01^{Bbc}
	S	4.87 ± 0.09^{Ad}	3.63 ± 0.06^{Bcd}	3.61 ± 0.09^{Bcd}	3.58 ± 0.10^{Bbc}	3.60 ± 0.13^{Bbc}
	SPRO	4.74 ± 0.04^{Ad}	3.71 ± 0.01^{Bcd}	3.64 ± 0.03^{BCcd}	3.60 ± 0.02^{Cbc}	3.77 ± 0.05^{Bbc}
	PRO	5.48 ± 0.09^{Ac}	3.98 ± 0.06^{Bc}	3.85 ± 0.05^{Bbc}	3.79 ± 0.03^{Bb}	3.79 ± 0.06^{Bbc}
NF	WT	5.97 ± 0.04^{Aab}	4.42 ± 0.01^{Bb}	3.85 ± 0.04^{Cbc}	3.66 ± 0.02^{Dbc}	3.50 ± 0.02^{Ebc}
	Z	5.91 ± 0.02^{Aab}	4.99 ± 0.01^{Ba}	3.89 ± 0.10^{Cbc}	3.84 ± 0^{Cb}	3.71 ± 0.03^{Cbc}
	S	6.15 ± 0.20^{Aa}	4.81 ± 0.20^{Ba}	4.80 ± 0.21^{Ba}	4.54 ± 0.23^{Ba}	4.34 ± 0.24^{Ba}
	SPRO	5.76 ± 0.25^{Ab}c	5.04 ± 0.26^{ABa}	4.62 ± 0.16^{Ba}	4.51 ± 0.23^{Ba}	4.53 ± 0.25^{Ba}
	PRO	5.75 ± 0.00^{Abc}	4.74 ± 0.01^{Bab}	4.18 ± 0.01^{Cb}	3.89 ± 0.00^{Db}	3.81 ± 0.02^{Eb}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different lowercase letters are significantly different ($p \leq 0.05$). Means within the same row containing different uppercase letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 10. Titratable acidity[1,2] (%) of cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times[b]

Cassava material[3]		Fermentation time (h)				
		0	**24**	**48**	**72**	**96**
LF	**WT**	0.18 ± 0.01^{Cbcde}	0.48 ± 0.01^{Bcd}	0.72 ± 0.01^{Acd}	0.76 ± 0.11^{Acd}	0.59 ± 0.01^{ABe}
	Z	0.22 ± 0.01^{Dabc}	0.59 ± 0.07^{Cc}	1.07 ± 0.04^{ABb}	1.24 ± 0.11^{Aa}	0.94 ± 0.06^{Bcd}
	S	0.24 ± 0.05^{Cabc}	0.84 ± 0.06^{Bb}	1.08 ± 0.10^{ABb}	1.14 ± 0.05^{Aab}	1.09 ± 0.01^{ABbc}
	SPRO	0.30 ± 0.01^{Ca}	1.09 ± 0.02^{Ba}	1.29 ± 0.05^{ABa}	1.22 ± 0.05^{ABa}	1.42 ± 0.12^{Aa}
	PRO	0.21 ± 0.01^{Babcd}	1.03 ± 0.02^{Aa}	1.10 ± 0.01^{Aab}	1.22 ± 0.24^{Aa}	1.17 ± 0.12^{Aabc}
NF	**WT**	0.09 ± 0.00^{Ce}	0.23 ± 0.01^{BCe}	0.38 ± 0.04^{Be}	0.73 ± 0.07^{Ad}	0.63 ± 0.06^{Ae}
	Z	0.13 ± 0.00^{Ede}	0.33 ± 0.05^{Dde}	0.51 ± 0.02^{Cde}	0.83 ± 0.02^{Abcd}	0.69 ± 0.00^{Bde}
	S	0.14 ± 0.00^{Ccde}	0.35 ± 0.03^{Bde}	0.50 ± 0.05^{ABe}	0.49 ± 0.05^{ABd}	0.65 ± 0.06^{Ae}
	SPRO	0.25 ± 0.04^{Bab}	0.38 ± 0.06^{Bde}	0.72 ± 0.03^{Acd}	0.60 ± 0.02^{Ad}	0.65 ± 0.06^{Ae}
	PRO	0.17 ± 0.02^{Ebcde}	0.42 ± 0.00^{Dcd}	0.73 ± 0.06^{Cc}	1.12 ± 0.02^{Babc}	1.32 ± 0.07^{Aab}

[1] Titratable acidity (TA) results are expressed as % lactic acid. [2] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different lowercase letters are significantly different ($p \leq 0.05$). Means within the same row containing different uppercase letters are significantly different ($p \leq 0.05$).

[3]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

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Table 11. Volatile compounds[1] detected in cooked fufu with (LF) and without (NF) the addition of a starter culture at 0 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)			
		Acetic acid	Hexanal	Nonanal	Decanal
LF	WT	21.96	nd	0.007	0.003[b]
	Z	38.86	0.033[c]	0.010	0.002[b]
	S	34.05	nd	0.005	0.002[b]
	SPRO	41.58	0.045[c]	0.008	0.002[b]
	PRO	20.38	0.086[a]	0.014	0.002[b]
NF	WT	30.67	0.017[d]	0.011	nd
	Z	32.93	0.013[d]	0.021	0.006[a]
	S	35.19	nd	0.004	nd
	SPRO	32.14	0.035[c]	0.011	0.002[b]
	PRO	42.10	0.069[b]	0.015	0.002[b]

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 12. Volatile compounds[1] detected in cooked fufu with (LF) and without (NF) the addition of a starter culture at 24 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)			
		Acetic acid	Hexanal	Nonanal	Decanal
LF	WT	91.99^{b}	nd	0.004	0.002
	Z	187.43^{b}	0.006^{d}	0.006	0.002
	S	92.63^{b}	nd	0.005	0.002
	SPRO	133.06^{b}	0.026^{cd}	0.007	0.003
	PRO	196.55^{b}	0.083^{abc}	0.012	0.003
NF	WT	117.96^{b}	0.039^{bcd}	0.012	0.002
	Z	298.65^{b}	0.022^{cd}	0.006	0.002
	S	189.48^{b}	0.013^{d}	0.007	0.003
	SPRO	330.46^{b}	0.095^{ab}	0.010	0.002
	PRO	867.01^{a}	0.146^{a}	0.009	0.002

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 13. Volatile compounds[1] detected in cooked fufu with (LF) and without (NF) the addition of a starter culture at 48 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)			
		Acetic acid	Hexanal	Nonanal	Decanal
LF	WT	109.16^{c}	nd	0.003	nd
	Z	176.17^{bc}	nd	0.005	nd
	S	119.01^{bc}	nd	0.003	nd
	SPRO	134.55^{bc}	0.015	0.004	nd
	PRO	255.81^{bc}	0.089	0.009	nd
NF	WT	240.96^{bc}	0.016	0.006	nd
	Z	462.07^{b}	0.018	0.004	nd
	S	433.59^{bc}	0.016	0.005	nd
	SPRO	467.95^{b}	0.057	0.008	nd
	PRO	900.12^{a}	0.061	0.005	nd

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 14. Volatile compounds[1] detected in cooked fufu with (LF) and without (NF) the addition of a starter culture at 72 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)			
		Acetic acid	Hexanal	Nonanal	Decanal
LF	WT	101.70^{f}	0.008^{c}	0.003	nd
	Z	221.71^{c}	0.005^{c}	0.009	0.002
	S	201.78^{ef}	nd	0.003	nd
	SPRO	127.48^{bc}	0.014^{bc}	0.004	nd
	PRO	300.78^{bc}	0.033^{bc}	0.007	nd
NF	WT	381.71^{cde}	0.019^{bc}	0.007	nd
	Z	521.33^{bcd}	0.031^{bc}	0.006	0.002
	S	607.77^{bc}	0.014^{bc}	0.004	0.002
	SPRO	613.42^{b}	0.046^{b}	0.005	nd
	PRO	912.69^{a}	0.081^{a}	0.011	nd

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 15. Volatile compounds[1] detected in cooked fufu with (LF) and without (NF) the addition of a starter culture at 96 h of fermentation

Cassava material[2]		Volatile compound concentration (µg/mL)			
		Acetic acid	Hexanal	Nonanal	Decanal
LF	WT	124.73^{e}	nd	0.003^{b}	nd
	Z	249.87^{de}	0.007^{c}	0.007^{ab}	0.002
	S	160.64^{e}	nd	0.006^{ab}	nd
	SPRO	175.56^{e}	0.065^{ab}	0.006^{ab}	nd
	PRO	345.50^{cd}	0.045^{abc}	0.010^{ab}	0.002
NF	WT	480.43^{bc}	0.006^{c}	0.007^{ab}	0.002
	Z	505.09^{b}	0.017^{bc}	0.006^{ab}	0.002
	S	957.69^{a}	0.028^{abc}	0.008^{ab}	nd
	SPRO	513.40^{b}	0.062^{ab}	0.011^{ab}	nd
	PRO	950.97^{a}	0.070^{a}	0.018^{a}	0.002

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

nd: No detectable

Table 16. Average values of L*[1] related to cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[2]		Fermentation time (h)				
		0	24	48	72	96
LF	WT	48.92 ± 0.18^{Abcd}	46.11 ± 0.39^{Be}	44.91 ± 0.36B^{Cf}	45.60 ± 1.48^{CDg}	44.39 ± 0.19^{Def}
	Z	49.54 ± 1.05^{Abc}	48.77 ± 1.02A^{Bd}	48.38 ± 0.65^{Bde}	48.82 ± 1.11^{ABef}	46.85 ± 1.84^{Cde}
	S	49.45 ± 0.25^{Bbc}	51.19 ± 0.16^{Ac}	48.13 ± 0.32^{Cde}	49.84 ± 0.37^{Bef}	48.38 ± 0.61^{Cde}
	SPRO	53.72 ± 0.52^{Ba}	53.60 ± 0.72^{Bb}	54.88 ± 0.51^{Ab}	53.16 ± 2.29^{Bcd}	42.59 ± 2.52^{Cf}
	PRO	54.80 ± 0.49^{Ba}	54.57 ± 1.64^{Bab}	56.18 ± 1.13^{Aab}	56.24 ± 0.99^{Aab}	56.11 ± 0.14^{Aab}
NF	WT	49.85 ± 2.21^{Ab}	48.29 ± 1.96^{Bde}	46.86 ± 2.11^{Def}	47.00 ± 1.09^{CDfg}	47.66 ± 1.73^{BCde}
	Z	50.38 ± 0.87^{Bb}	50.03 ± 0.07^{Bcd}	51.66 ± 1.22^{Ac}	48.04 ± 0.25^{Cefg}	49.90 ± 0.60^{Bcd}
	S	47.18 ± 0.39^{Dcd}	55.47 ± 0.51^{Aab}	50.44 ± 1.04^{Ccd}	50.56 ± 1.21^{Cde}	52.66 ± 0.08^{Bbc}
	SPRO	46.53 ± 1.92^{Dd}	54.07 ± 0.44^{Ab}	51.46 ± 0.44^{Bc}	54.35 ± 0.51^{Abc}	50.29 ± 3.73^{Ccd}
	PRO	54.88 ± 0.34^{Ca}	56.51 ± 0.67^{Ba}	58.61 ± 1.99^{Aa}	58.38 ± 1.51^{Aa}	57.88 ± 1.60^{Aa}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different lowercase letters are significantly different ($p \leq 0.05$). Means within the same row containing different uppercase letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 17. Average values of a*[1] related to cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[2]		Fermentation time (h)				
		0	24	48	72	96
LF	WT	-1.42 ± 0.04^{Cde}	-2.04 ± 0.14^{De}	-1.33 ± 0.01^{Cfg}	-1.06 ± 0.09^{Ae}	-1.18 ± 0.27^{Be}
	Z	-1.37 ± 0.18^{Bd}	-1.59 ± 0.08^{Cde}	-1.40 ± 0.10^{Bfg}	-0.94 ± 0.09^{Ae}	-0.92 ± 0.09^{Ae}
	S	0.61 ± 0.12^{Bab}	0.51 ± 0.26^{Bb}	1.68 ± 0.21^{Ab}	0.60 ± 0.24^{Babc}	1.50 ± 0.23^{Ab}
	SPRO	0.15 ± 0.05^{Db}	1.30 ± 0.05^{Ba}	0.84 ± 0.10^{Cc}	0.94 ± 0.26^{Cab}	2.89 ± 0.14^{Aa}
	PRO	-0.36 ± 0.19^{Dc}	0.79 ± 0.31^{Aab}	0.11 ± 0.17^{Bd}	-0.05 ± 0.44^{Cd}	-0.05 ± 0.46^{Cd}
NF	WT	-1.91 ± 0.06^{Def}	-1.68 ± 0.18^{Cde}	-1.10 ± 0.43^{Af}	-1.54 ± 0.39^{Be}	-2.01 ± 0.09^{Df}
	Z	-2.16 ± 0.12^{Ef}	-1.40 ± 0.23^{Bd}	-1.74 ± 0.08^{Cg}	-1.08 ± 0.05^{Ae}	-1.82 ± 0.01^{Df}
	S	-0.52 ± 0.04^{Dc}	0.41 ± 0.23^{Cb}	1.58 ± 0.17^{Ab}	0.48 ± 0.32^{Cbcd}	0.60 ± 0.27^{Bc}
	SPRO	0.70 ± 0.49^{Da}	0.66 ± 0.29^{Db}	2.86 ± 0.33^{Aa}	1.11 ± 0.16^{Ca}	1.64 ± 0.40^{Bb}
	PRO	-0.66 ± 0.28^{Dc}	-0.52 ± 0.14^{Cc}	-0.44 ± 0.08^{Ce}	0.23 ± 0.04^{Acd}	-0.31 ± 0.04^{Bd}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different lowercase letters are significantly different ($p \leq 0.05$). Means within the same row containing different uppercase letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 18. Average values of b*[1] related to cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[2]		Fermentation time (h)				
		0	**24**	**48**	**72**	**96**
LF	**WT**	8.74 ± 0.28^{Acd}	4.03 ± 0.21^{Ef}	7.74 ± 0.11^{Bef}	6.93 ± 0.27^{Cde}	5.96 ± 0.87^{De}
	Z	8.60 ± 0.39^{Ccd}	6.55 ± 0.71^{De}	9.54 ± 0.63^{Be}	10.24 ± 0.49^{Ac}	9.16 ± 0.51^{Bd}
	S	14.41 ± 0.11^{Cab}	16.29 ± 1.30^{Bb}	18.37 ± 0.49^{Ab}	14.65 ± 1.37^{Cb}	16.23 ± 0.32^{Bab}
	SPRO	16.64 ± 0.76^{Ca}	19.62 ± 0.38^{Aa}	18.20 ± 0.23^{Bb}	18.56 ± 0.41^{Ba}	16.29 ± 2.23^{Cab}
	PRO	15.68 ± 0.78^{Ba}	16.85 ± 0.33^{Ab}	15.68 ± 1.95^{Bc}	14.37 ± 0.20^{Cb}	14.73 ± 0.49^{Cbc}
NF	**WT**	8.41 ± 3.83^{Acd}	5.95 ± 0.34^{Ce}	6.74 ± 0.41^{Bf}	4.60 ± 2.95^{De}	3.81 ± 0.28^{Ef}
	Z	7.77 ± 0.14^{Bd}	8.48 ± 0.82^{Ad}	6.27 ± 0.35^{Cf}	9.05 ± 0.45^{Acd}	5.12 ± 0.28^{Def}
	S	11.32 ± 0.58^{Bbc}	13.15 ± 1.19^{Ac}	12.87 ± 1.27^{Ad}	11.12 ± 0.68^{Bc}	13.21 ± 0.54^{Ac}
	SPRO	16.15 ± 1.61^{Ca}	16.40 ± 0.81^{Cb}	20.49 ± 0.17^{Aa}	17.16 ± 1.44^{Bab}	17.31 ± 0.42^{Ba}
	PRO	14.99 ± 0.40^{Ba}	12.73 ± 0.61^{Dc}	13.94 ± 0.81^{Ccd}	16.34 ± 0.66^{Aab}	15.22 ± 0.15^{Bb}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different lowercase letters are significantly different ($p \leq 0.05$). Means within the same row containing different uppercase letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 19. Instrumental hardness (N)[1,2] analysis of cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[3]		Fermentation time (h)				
		0	24	48	72	96
LF	WT	2.79 ± 0.65	2.29 ± 0.49	2.44 ± 0.58	2.52 ± 0.54	3.05 ± 1.67
	Z	2.89 ± 0.88	2.51 ± 0.89	2.85 ± 0.82	2.05 ± 0.40	2.58 ± 0.88
	S	3.38 ± 0.59	2.98 ± 1.02	3.20 ± 0.50	2.90 ± 1.23	3.18 ± 1.32
	SPRO	2.51 ± 0.70	3.12 ± 1.14	2.54 ± 0.85	2.61 ± 0.20	2.71 ± 0.24
	PRO	3.42 ± 1.81	3.10 ± 0.28	3.21 ± 0.65	2.25 ± 0.73	2.09 ± 0.45
NF	WT	2.45 ± 0.41	1.81 ± 0.41	2.20 ± 0.24	2.98 ± 1.04	1.63 ± 0.42
	Z	2.53 ± 0.92	2.79 ± 0.72	2.37 ± 0.84	2.05 ± 0.36	2.09 ± 0.42
	S	2.75 ± 0.46	2.04 ± 0.59	2.92 ± 0.27	2.20 ± 0.004	2.13 ± 0.34
	SPRO	2.90 ± 0.12	2.27 ± 0.25	3.06 ± 0.81	2.69 ± 1.22	2.48 ± 0.86
	PRO	2.34 ± 0.37	1.73 ± 0.42	1.72 ± 0.55	2.85 ± 0.32	2.06 ± 0.48

[1] Results are reported as the mean of two determinations ± standard deviation. All samples within each column were not significant different from each other ($p>0.05$). [2]Units: Newton.

[3]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 20. Instrumental adhesiveness[a] (N.mm)[1,2] analysis of cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[3]		Fermentation time (h)				
		0	24	48	72	96
LF	WT	-1.39 ± 0.57^{a}	-0.18 ± 0.09^{a}	-0.18 ± 0.11^{a}	-0.35 ± 0.09^{a}	-0.21 ± 0.09^{a}
	Z	-1.63 ± 0.49^{a}	-1.19 ± 0.54^{cd}	-1.14 ± 0.21^{bc}	-1.02 ± 0.21^{cd}	-1.03 ± 0.23^{cd}
	S	-1.14 ± 0.27^{a}	-0.86 ± 0.11^{bc}	-0.81 ± 0.19^{bc}	-0.81 ± 0.20^{abcd}	-0.69 ± 0.42^{abcd}
	SPRO	-1.06 ± 0.44^{a}	-0.75 ± 0.12^{bc}	-0.85 ± 0.23^{bc}	-0.83 ± 0.28^{abcd}	-0.48 ± 0.16^{abc}
	PRO	-1.21 ± 0.59^{a}	-0.62 ± 0.11^{ab}	-0.73 ± 0.15^{bc}	-0.64 ± 0.15^{abc}	-0.56 ± 0.21^{abc}
NF	WT	-1.34 ± 0.52^{a}	-0.52 ± 0.25^{ab}	-0.64 ± 0.34^{ab}	-0.46 ± 0.35^{ab}	-0.29 ± 0.14^{ab}
	Z	-1.85 ± 0.49^{a}	-1.52 ± 0.28^{d}	-1.25 ± 0.20^{c}	-1.28 ± 0.31^{d}	-1.18 ± 0.32^{d}
	S	-1.61 ± 0.55^{a}	-1.00 ± 0.23^{bc}	-0.95 ± 0.22^{bc}	-0.71 ± 0.18^{abc}	-0.79 ± 0.16^{bcd}
	SPRO	-1.16 ± 0.30^{a}	-0.98 ± 0.13^{bc}	-0.81 ± 0.37^{bc}	-0.64 ± 0.19^{abc}	-0.47 ± 0.20^{ab}
	PRO	-1.52 ± 0.50^{a}	-0.76 ± 0.07^{bc}	-0.84 ± 0.16^{bc}	-0.93 ± 0.04^{bcd}	-0.65 ± 0.13^{abcd}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).[2]Units: N.mm.

[3]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 21. Instrumental springiness (dimensionless)[1,2] analysis of cooked fufu with (LF) and without (NF) the addition of a starter culture at different fermentation times

Cassava material[3]		Fermentation time (h)				
		0	**24**	**48**	**72**	**96**
LF	**WT**	0.34 ± 0.01^{ab}	0.48 ± 0.01^{ab}	0.47 ± 0.02^{a}	0.47 ± 0.02^{a}	0.47 ± 0.03^{a}
	Z	0.35 ± 0.01^{ab}	0.37 ± 0.03^{ab}	0.37 ± 0.02^{ab}	0.36 ± 0.01^{ab}	0.38 ± 0.02^{ab}
	S	0.32 ± 0.03^{ab}	0.36 ± 0.02^{ab}	0.37 ± 0.01^{ab}	0.32 ± 0.01^{ab}	0.38 ± 0.02^{a}
	SPRO	0.26 ± 0.01^{ab}	0.30 ± 0.02^{ab}	0.28 ± 0.01^{ab}	0.30 ± 0.02^{ab}	0.30 ± 0.01^{a}
	PRO	0.25 ± 0.01^{a}	0.31 ± 0.01^{a}	0.29 ± 0.02^{a}	0.28 ± 0.01^{ab}	0.29 ± 0.02^{a}
NF	**WT**	0.33 ± 0.04^{ab}	0.42 ± 0.04^{b}	0.44 ± 0.02^{b}	0.46 ± 0.04^{ab}	0.45 ± 0.03^{ab}
	Z	0.31 ± 0.01^{b}	0.33 ± 0.02^{ab}	0.38 ± 0.03^{b}	0.38 ± 0.02^{b}	0.37 ± 0.02^{b}
	S	0.28 ± 0.02^{ab}	0.33 ± 0.02^{ab}	0.35 ± 0.01^{ab}	0.32 ± 0.02^{ab}	0.33 ± 0.01^{ab}
	SPRO	0.28 ± 0.01^{ab}	0.25 ± 0.01^{ab}	0.34 ± 0.01^{ab}	0.29 ± 0.01^{ab}	0.31 ± 0.03^{a}
	PRO	0.23 ± 0.01^{ab}	0.22 ± 0.00^{a}	0.25 ± 0.02^{ab}	0.29 ± 0.02^{ab}	0.27 ± 0.02^{ab}

[1] Results are reported as the mean of two determinations ± standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 22. Degrees of freedom and F-ratios from ANOVA results of trained panel evaluation (n=5) for visual appearance, aroma and texture attributes of cooked fufu with and without *Lactobacillus plantarum* added

Source of Variation	df	Visual	Aroma			Texture		
		Color intensity for brownness	Fermented	Stale	Fufu	Hardness	Adhesiveness	Springiness
Panelist	4	9.59*	294.77***	121.76***	69.39***	234.71***	15.32**	261.31***
Sample Type	4	134.75***	12.65***	4.33**	1.74	15.67***	2.89*	14.03***
Fermentation Type	1	26.60***	0.34	8.72**	0.14	28.25***	7.11**	27.95***
FermentationTime	4	6.40***	6.46***	1.59	1.76	4.96***	1.99	0.26
Sample Type* Fermentation Type	4	5.64**	1.74	0.82	4.45**	3.11*	5.66***	1.16
Sample Type* FermentationTime	16	5.63***	1.21	0.61	0.20	2.65***	1.77*	0.51
FermentationType* FermentationTime	4	6.29***	0.76	0.35	0.43	1.76	1.37	0.28
SampleType*Fermentation Type*FermentationTime	16	3.23***	0.78	0.52	0.67	3.44***	2.40**	0.87

*, **, *** Indicate significant $p \leq 0.05, 0.01, 0.001$ respectively.

Table 23. Mean values[1] of trained panel evaluation of visual appearance, aroma and texture attributes of cooked fufu[2] with (LF) and without *Lactobacillus plantarum* added (NF) as evaluated by panelists (n=5) at 0 h of fermentation

Attributes	NF					LF				
	WT	Z	S	SPRO	PRO	WT	Z	S	SPRO	PRO
VISUAL Color intensity for brownness	4.62^{bc}	2.40^{d}	5.91^{b}	8.06^{a}	3.82^{cd}	3.95^{cd}	2.63^{d}	5.77^{b}	6.25^{b}	5.76^{b}
AROMA										
Fermented	6.47^{ab}	4.95^{b}	6.71^{ab}	7.64^{a}	6.87^{ab}	6.63^{ab}	6.62^{ab}	7.18^{a}	7.94^{a}	6.40^{ab}
Stale	5.61^{a}	4.53^{a}	5.44^{a}	5.36^{a}	6.02^{a}	4.57^{a}	4.92^{a}	5.31^{a}	4.65^{a}	5.50^{a}
Fufu	7.44^{a}	7.35a	7.50^{a}	8.19^{a}	7.99^{a}	7.90^{a}	7.80^{a}	7.97^{a}	7.68^{a}	8.09^{a}
TEXTURE										
Hardness	8.69^{a}	6.97^{ab}	7.91^{ab}	8.24^{a}	7.90^{ab}	6.38^{ab}	7.80^{ab}	6.75^{ab}	5.60^{b}	7.18^{ab}
Adhesiveness	7.02^{b}	9.29^{ab}	8.18^{ab}	8.37^{ab}	8.56^{ab}	9.97^{a}	9.03^{ab}	9.05^{ab}	10.13^{a}	6.82^{b}
Springiness	8.65^{ab}	8.37^{a}b	8.62^{ab}	7.68^{b}	7.49^{b}	9.97^{a}	9.50^{a}	8.78^{ab}	8.91a^{b}	7.39^{b}

[1]Results are reported as mean value on a scale of 0 to 15 (0 = low intensity and 15 = high intensity). Means containing different letters within the same row are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 24. Mean values[1] of trained panel evaluation of visual appearance, aroma and texture attributes of cooked fufu[2] with (LF) and without *Lactobacillus plantarum* added (NF) as evaluated by panelists (n=5) at 24 h of fermentation

Attributes	NF					LF				
	WT	**Z**	**S**	**SPRO**	**PRO**	**WT**	**Z**	**S**	**SPRO**	**PRO**
VISUAL **Color intensity for brownness**	3.38^{c}	3.59^{c}	4.71^{bc}	5.60^{ab}	3.30^{c}	2.77^{c}	2.93^{c}	5.68^{ab}	7.11^{a}	6.04^{ab}
AROMA **Fermented**	8.10^{a}	6.52^{a}	7.65^{a}	7.53^{a}	7.38^{a}	7.14^{a}	6.70^{a}	7.84^{a}	7.62^{a}	7.75^{a}
Stale	5.36^{a}	4.78^{a}	4.98^{a}	5.15^{a}	5.77^{a}	4.06^{a}	5.28^{a}	5.03^{a}	4.99^{a}	5.13^{a}
Fufu	7.41^{a}	7.87^{a}	6.95^{a}	8.10^{a}	8.21^{a}	6.62^{a}	7.39^{a}	8.20^{a}	7.18^{a}	7.58a
TEXTURE **Hardness**	5.68^{bc}	8.88^{a}	7.58^{ab}	7.25^{abc}	5.20^{bc}	4.81^{c}	5.82^{bc}	7.19^{abc}	6.27^{bc}	7.11^{abc}
Adhesiveness	7.88^{b}	9.65^{ab}	9.58^{ab}	9.04^{ab}	10.86^{a}	10.70^{a}	9.66^{ab}	8.95^{ab}	9.47^{ab}	7.37^{b}
Springiness	9.07^{a}	9.42^{a}	8.06^{a}	7.36^{a}	7.69^{a}	9.32^{a}	8.99^{a}	8.82^{a}	8.40^{a}	8.40^{a}

[1]Results are reported as mean value on a scale of 0 to 15 (0 = low intensity and 15 = high intensity). Means containing different letters within the same row are significantly different ($p \leq 0.05$)

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 25. Mean values[1] of trained panel evaluation of visual appearance, aroma and texture attributes of cooked fufu[2] with (LF) and without *Lactobacillus plantarum* added (NF) as evaluated by panelists (n=5) at 48 h of fermentation

Attributes	NF					LF				
	WT	**Z**	**S**	**SPRO**	**PRO**	**WT**	**Z**	**S**	**SPRO**	**PRO**
VISUAL Color intensity for brownness	3.21^{de}	2.79^{e}	6.63^{bc}	8.72^{a}	3.74^{de}	4.76^{cd}	3.44^{de}	7.32^{ab}	6.93^{ab}	6.14^{bc}
AROMA										
Fermented	7.11^{a}	6.61^{a}	7.73^{a}	7.95^{a}	8.24^{a}	7.75^{a}	7.25^{a}	7.54^{a}	8.10^{a}	7.71^{a}
Stale	4.36^{a}	5.00^{a}	4.94^{a}	4.68^{a}	5.27^{a}	4.68^{a}	4.54^{a}	4.32^{a}	4.44^{a}	5.53^{a}
Fufu	7.43^{a}	6.82^{a}	7.48^{a}	7.92^{a}	8.24^{a}	7.07^{a}	8.28^{a}	7.62^{a}	7.94^{a}	7.04^{a}
TEXTURE										
Hardness	5.00^{b}	6.87^{ab}	8.78^{a}	8.95^{a}	6.21^{ab}	5.07^{b}	6.85^{ab}	7.44^{ab}	6.50^{ab}	6.91^{ab}
Adhesiveness	9.50^{ab}	9.07^{ab}	7.43^{ab}	6.71^{b}	9.43^{ab}	10.20^{a}	9.58^{ab}	7.47^{ab}	9.28^{ab}	6.69^{b}
Springiness	8.74^{ab}	8.74^{ab}	7.81^{b}	7.99^{ab}	7.53^{b}	9.91^{a}	9.23^{ab}	8.70^{ab}	9.00^{ab}	7.83^{b}

[1]Results are reported as mean value on a scale of 0 to 15 (0 = low intensity and 15 = high intensity). Means containing different letters within the same row are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 26. Mean values[1] of trained panel evaluation of visual appearance, aroma and texture attributes of cooked fufu[2] with (LF) and without *Lactobacillus plantarum* added (NF) as evaluated by panelists (n=5) at 72 h of fermentation

Attributes	NF					LF				
	WT	Z	S	SPRO	PRO	WT	Z	S	SPRO	PRO
VISUAL										
Color intensity for brownness	4.37^{ab}	3.85^{b}	5.27^{ab}	5.85^{a}	4.73^{ab}	4.16^{ab}	3.72^{b}	5.580^{b}	5.82^{a}	5.57^{ab}
AROMA										
Fermented	7.63^{a}	6.75^{a}	8.04^{a}	7.98^{a}	8.16^{a}	7.08^{a}	7.42^{a}	7.87^{a}	7.47^{a}	7.55^{a}
Stale	5.28^{a}	4.63^{a}	4.91^{a}	4.79^{a}	5.48^{a}	5.18^{a}	4.55^{a}	4.24^{a}	3.60^{a}	4.66^{a}
Fufu	7.20^{a}	7.17^{a}	7.15^{a}	7.77^{a}	7.92^{a}	7.43^{a}	7.73^{a}	7.73^{a}	6.98^{a}	7.43^{a}
TEXTURE										
Hardness	6.96^{ab}	8.25^{a}	7.41^{a}	7.34^{ab}	8.13^{a}	4.86^{b}	7.80^{a}	6.29^{ab}	6.72^{ab}	6.05^{ab}
Adhesiveness	7.67^{a}	7.98^{a}	9.25^{a}	8.68^{a}	7.42^{a}	9.96^{a}	8.37^{a}	9.98^{a}	8.93^{a}	9.48^{a}
Springiness	7.93^{b}	8.93^{ab}	7.96^{b}	8.28^{b}	7.44^{b}	10.06^{a}	9.18^{ab}	8.46^{ab}	8.17^{b}	8.40^{ab}

[1]Results are reported as mean value on a scale of 0 to 15 (0 = low intensity and 15 = high intensity). Means containing different letters within the same row are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 27. Mean values[1] of trained panel evaluation of visual appearance, aroma and texture attributes of cooked fufu[2] with (LF) and without *Lactobacillus plantarum* added (NF) as evaluated by panelists (n=5) at 96 h of fermentation

Attributes	NF					LF				
	WT	Z	S	SPRO	PRO	WT	Z	S	SPRO	PRO
VISUAL										
Color intensity for brownness	2.70^{e}	2.55^{e}	5.72^{c}	7.54^{b}	3.93^{de}	4.10^{de}	3.22^{e}	7.96^{b}	11.04^{a}	5.11^{cd}
AROMA										
Fermented	7.40^{abc}	5.97^{c}	8.08^{ab}	7.73^{abc}	7.47^{abc}	7.60^{abc}	6.27^{bc}	8.33^{a}	6.64^{abc}	8.07^{ab}
Stale	5.08^{a}	4.55^{a}	5.73^{a}	4.83^{a}	5.90^{a}	4.67^{a}	4.56^{a}	4.85^{a}	3.93^{a}	5.25^{a}
Fufu	6.92^{a}	6.85^{a}	7.04^{a}	7.65^{a}	8.20^{a}	6.87^{a}	7.54^{a}	7.78^{a}	6.79^{a}	6.88^{a}
TEXTURE										
Hardness	4.29^{c}	6.04^{abc}	7.00^{ab}	7.84^{a}	7.24^{ab}	5.37^{bc}	7.23^{ab}	7.11^{ab}	5.79^{abc}	5.87^{abc}
Adhesiveness	9.99^{a}	9.59^{a}	7.61^{a}	8.06^{a}	7.69^{a}	10.19^{a}	8.67^{a}	8.42^{a}	9.32^{a}	9.68^{a}
Springiness	9.20^{ab}	9.12^{ab}	7.75^{ab}	7.07^{b}	8.04^{ab}	9.73^{a}	9.14^{ab}	9.06^{ab}	9.03^{ab}	8.80^{ab}

[1]Results are reported as mean value on a scale of 0 to 15 (0 = low intensity and 15 = high intensity). Means containing different letters within the same row are significantly different ($p \leq 0.05$).

[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 28. Correlation matrix between instrumental measurements and sensory attributes for all cooked fufu samples at 0 h of fermentation*

Variables	I- L	I-a	I-b	I-HARD	I-ADH	I-SPRIN	ACETIC ACID	HEXANAL	NONANAL	DECANAL
V-COLOR	-0.164	0.953	0.851	0.320	-0.636	0.823	-0.257	0.296	-0.471	-0.562
A-FER	-0.014	0.890	0.775	-0.027	-0.501	0.609	0.408	0.284	-0.863	-0.912
A-STALE	0.321	0.368	0.504	0.120	-0.617	0.646	0.066	0.744	0.052	-0.523
A-FUFU	-0.028	0.744	0.681	0.495	-0.677	0.884	-0.173	0.654	-0.371	-0.742
T-HARD	-0.463	0.004	-0.147	0.196	0.130	0.154	-0.259	0.043	0.219	-0.147
T-ADH	-0.170	-0.171	-0.255	-0.773	0.554	-0.634	0.635	-0.680	-0.245	0.074
T-SPRIN	-0.255	-0.354	-0.542	-0.212	0.651	-0.666	0.426	-0.590	-0.439	0.059

* **Sensory attributes**: Visual-COLOR (V-COLOR);

Aroma-FERMENTED (A-FER); Aroma-STALE (A-STALE); Aroma-FUFU (A-FUFU);

Texture-HARDNESS; (T-HARD); Texture-ADHESIVENESS; (T-ADH); Texture-SPRINGINESS; (T-SPRIN)

* **Instrumental measurements**: Instrumental-COLOR Lab: I-L; I-a, I-b;

Texture profile analyzer – Instrumental texture: HARDNESS (I-HARD), ADHESIVENESS (I-ADH), SPRINGINESS (I-SPRIN);

GC-MS Volatile compounds: Acetic acid, hexanal, nonanal and decanal

Table 29. Correlation matrix between instrumental measurements and sensory attributes for all cooked fufu samples at 24 h of fermentation

Variables	I- L	I-a	I-b	I-HARD	I-ADH	I-SPRIN	ACETIC ACID	HEXANAL	NONANAL	DECANAL
V-COLOR	0.480	0.928	0.918	0.620	0.320	0.698	-0.335	0.036	0.169	0.742
A-FER	0.308	0.434	0.362	-0.256	0.903	0.111	-0.169	0.261	0.780	0.413
A-STALE	0.191	-0.238	-0.174	-0.646	0.516	-0.139	0.680	0.678	0.385	-0.405
A-FUFU	0.176	-0.154	0.000	-0.213	-0.135	0.225	0.742	0.777	0.245	-0.654
T-HARD	0.000	0.137	0.099	0.420	-0.646	0.195	-0.299	-0.331	-0.272	0.180
T-ADH	0.277	-0.116	-0.049	-0.310	-0.467	-0.304	0.650	0.140	-0.676	-0.287
T-SPRIN	-0.828	-0.656	-0.652	0.295	-0.320	-0.343	-0.481	-0.674	-0.273	-0.160

* **Sensory attributes**: Visual-COLOR (V-COLOR);

  Aroma-FERMENTED (A-FER); Aroma-STALE (A-STALE); Aroma-FUFU (A-FUFU);

  Texture-HARDNESS; (T-HARD); Texture-ADHESIVENESS; (T-ADH); Texture-SPRINGINESS; (T-SPRIN)

* **Instrumental measurements**: Instrumental-COLOR Lab: I-L; I-a, I-b;

  Texture profile analyzer – Instrumental texture: HARDNESS (I-HARD), ADHESIVENESS (I-ADH), SPRINGINESS (I-SPRIN);

  GC-MS Volatile compounds: Acetic acid, hexanal, nonanal and decanal

Table 30. Correlation matrix between instrumental measurements and sensory attributes for all cooked fufu samples at 48 h of fermentation

Variables	I- L	I-a	I-b	I-HARD	I-ADH	I-SPRIN	ACETIC ACID	HEXANAL	NONANAL
V-COLOR	0.088	0.958	0.891	0.742	0.264	0.759	-0.320	0.248	0.262
A-FER	0.609	0.640	0.837	0.059	0.465	0.516	0.210	0.412	0.079
A-STALE	0.655	-0.148	0.054	0.136	-0.180	0.395	0.418	0.723	0.299
A-FUFU	0.355	0.473	0.588	-0.354	0.384	-0.006	0.405	0.075	-0.193
T-HARD	-0.022	0.795	0.530	0.668	-0.289	0.596	0.057	0.121	0.000
T-ADH	-0.022	-0.679	-0.539	-0.898	-0.147	-0.819	0.177	-0.560	-0.616
T-SPRIN	-0.448	-0.345	-0.328	-0.129	-0.171	-0.475	-0.645	-0.683	-0.339

* **Sensory attributes**: Visual-COLOR (V-COLOR);
  Aroma-FERMENTED (A-FER); Aroma-STALE (A-STALE); Aroma-FUFU (A-FUFU);
  Texture-HARDNESS; (T-HARD); Texture-ADHESIVENESS; (T-ADH); Texture-SPRINGINESS; (T-SPRIN)
* **Instrumental measurements**: Instrumental-COLOR Lab: I-L; I-a, I-b;
  Texture profile analyzer – Instrumental texture: HARDNESS (I-HARD), ADHESIVENESS (I-ADH), SPRINGINESS (I-SPRIN);
  GC-MS Volatile compounds: Acetic acid, hexanal, nonanal and decanal

Table 31. Correlation matrix between instrumental measurements and sensory attributes for all cooked fufu samples at 72 h of fermentation

Variables	I- L	I-a	I-b	I-HARD	I-ADH	I-SPRIN	ACETIC ACID	HEXANAL	NONANAL
V-COLOR	0.928	0.988	0.774	0.997	0.853	0.859	0.730	-0.100	-0.803
A-FER	0.972	0.893	0.998	0.857	0.997	0.996	0.195	-0.661	-0.306
A-STALE	0.867	0.958	0.679	0.976	0.774	0.781	0.817	0.037	-0.877
A-FUFU	-0.244	-0.458	0.057	-0.522	-0.081	-0.093	-0.982	-0.745	0.954
T-HARD	-0.967	-0.884	-0.999	-0.847	-0.996	-0.995	-0.177	0.675	0.288
T-ADH	1.000	0.975	0.953	0.956	0.986	0.988	0.424	-0.462	-0.525
T-SPRIN	-0.876	-0.963	-0.692	-0.980	-0.785	-0.792	-0.806	-0.019	0.869

* **Sensory attributes**: Visual-COLOR (V-COLOR);

  Aroma-FERMENTED (A-FER); Aroma-STALE (A-STALE); Aroma-FUFU (A-FUFU);

  Texture-HARDNESS; (T-HARD); Texture-ADHESIVENESS; (T-ADH); Texture-SPRINGINESS; (T-SPRIN)

* **Instrumental measurements**: Instrumental-COLOR Lab: I-L; I-a, I-b;

  Texture profile analyzer – Instrumental texture: HARDNESS (I-HARD), ADHESIVENESS (I-ADH), SPRINGINESS (I-SPRIN);

  GC-MS Volatile compounds: Acetic acid, hexanal, nonanal and decanal

Table 32. Correlation matrix between instrumental measurements and sensory attributes for all cooked fufu samples at 96 h of fermentation

Variables	I- L	I-a	I-b	I-HARD	I-ADH	I-SPRIN	ACETIC ACID	HEXANAL	NONANAL
V-COLOR	0.763	0.921	0.889	0.149	0.314	0.783	0.023	0.681	0.543
A-FER	0.645	0.556	0.577	-0.474	0.851	0.692	0.226	0.565	0.556
A-STALE	0.828	0.570	0.684	-0.373	0.608	0.421	0.782	0.870	0.941
A-FUFU	0.413	0.483	0.563	0.336	-0.041	0.279	0.656	0.601	0.765
T-HARD	0.327	0.599	0.602	0.829	-0.598	0.197	0.230	0.454	0.442
T-ADH	-0.452	-0.535	-0.609	-0.458	0.209	-0.223	-0.625	-0.631	-0.745
T-SPRIN	-0.878	-0.682	-0.798	0.008	-0.186	-0.293	-0.836	-0.961	-0.991

* **Sensory attributes**: Visual-COLOR (V-COLOR);

  Aroma-FERMENTED (A-FER); Aroma-STALE (A-STALE); Aroma-FUFU (A-FUFU);

  Texture-HARDNESS; (T-HARD); Texture-ADHESIVENESS; (T-ADH); Texture-SPRINGINESS; (T-SPRIN)

* **Instrumental measurements**: Instrumental-COLOR Lab: I-L; I-a, I-b;

  Texture profile analyzer – Instrumental texture: HARDNESS (I-HARD), ADHESIVENESS (I-ADH), SPRINGINESS (I-SPRIN);

  GC-MS Volatile compounds: Acetic acid, hexanal, nonanal and decanal

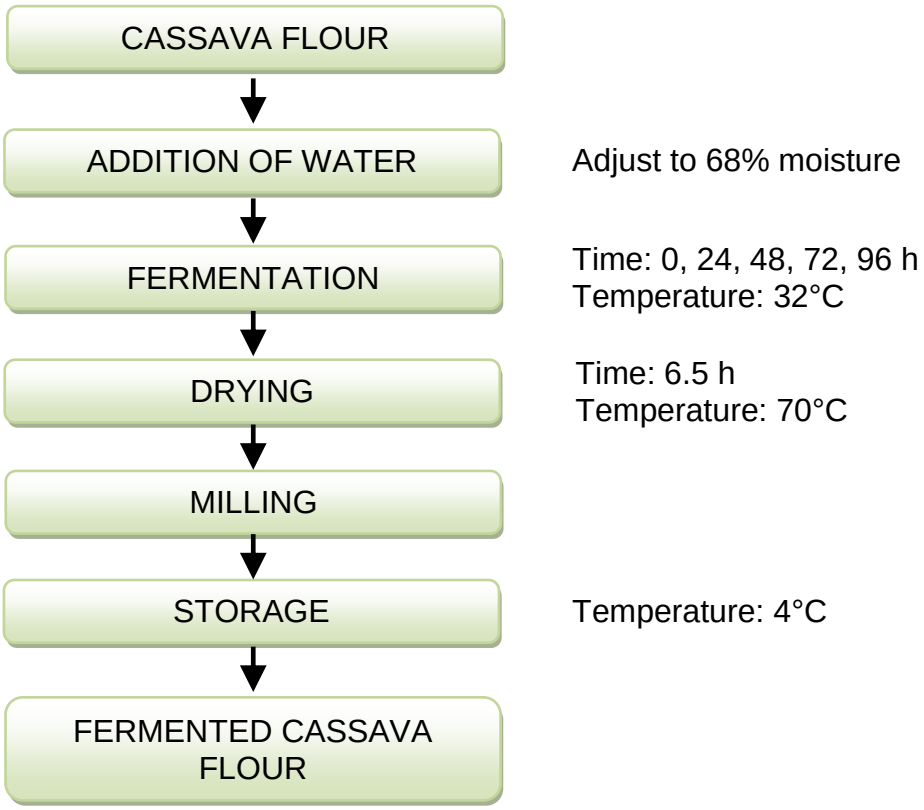

Figure 1. Flow chart for processing of fermented cassava flour.

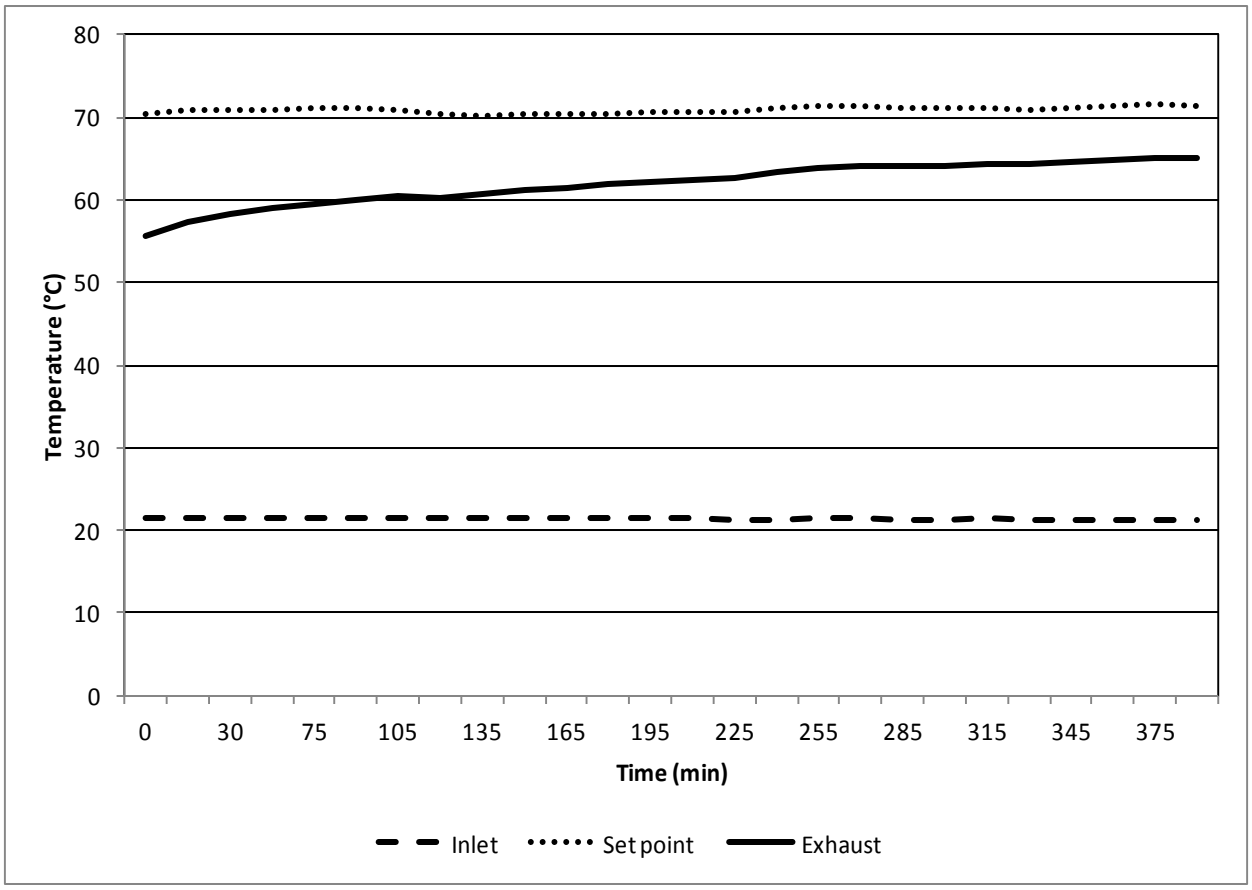

Figure 2. Temperature profile of drying process of protein and pro-vitamin A fortified and wild type fermented cassava flours.

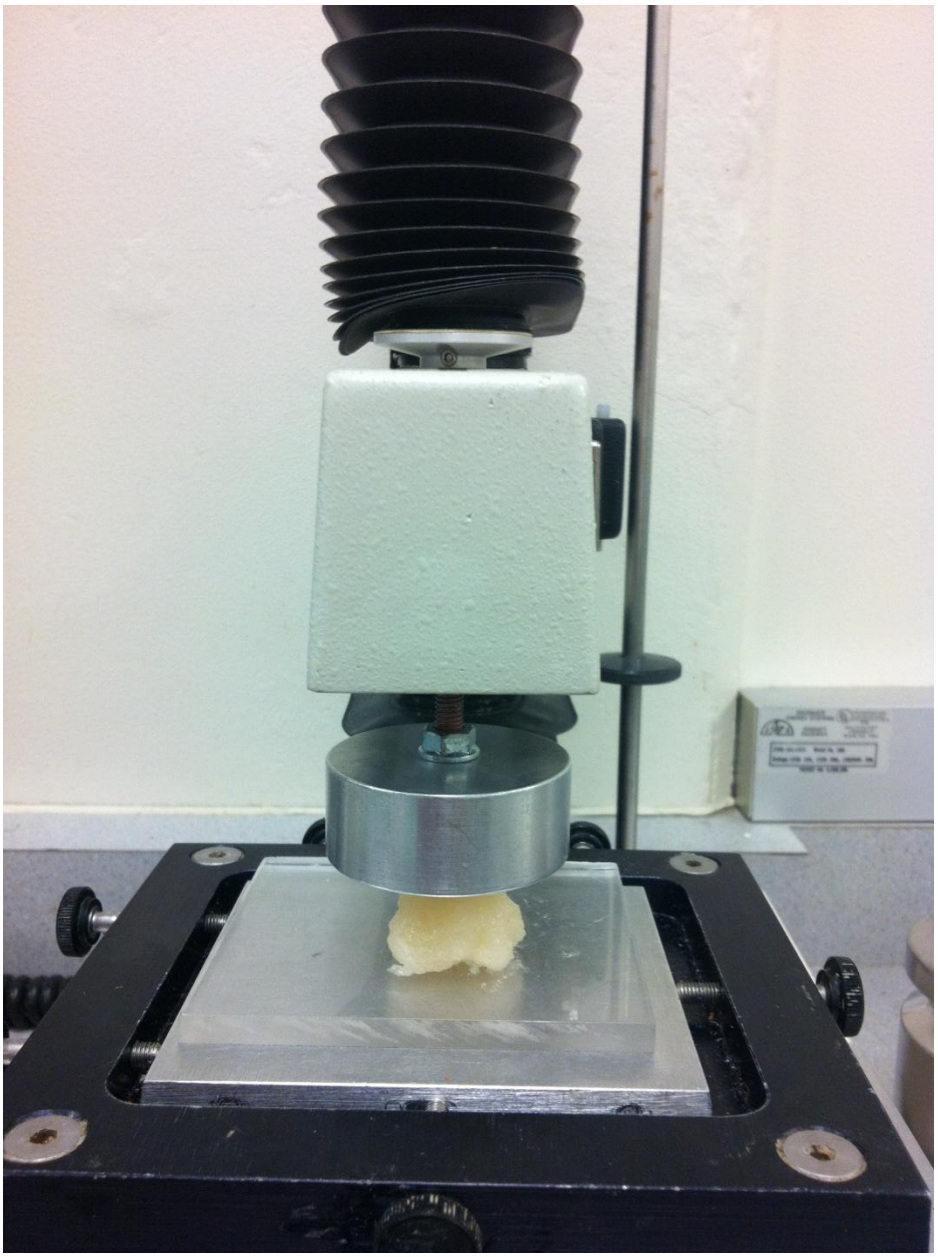

Figure 3. Texture Profiler Analyzer and cooked fufu.

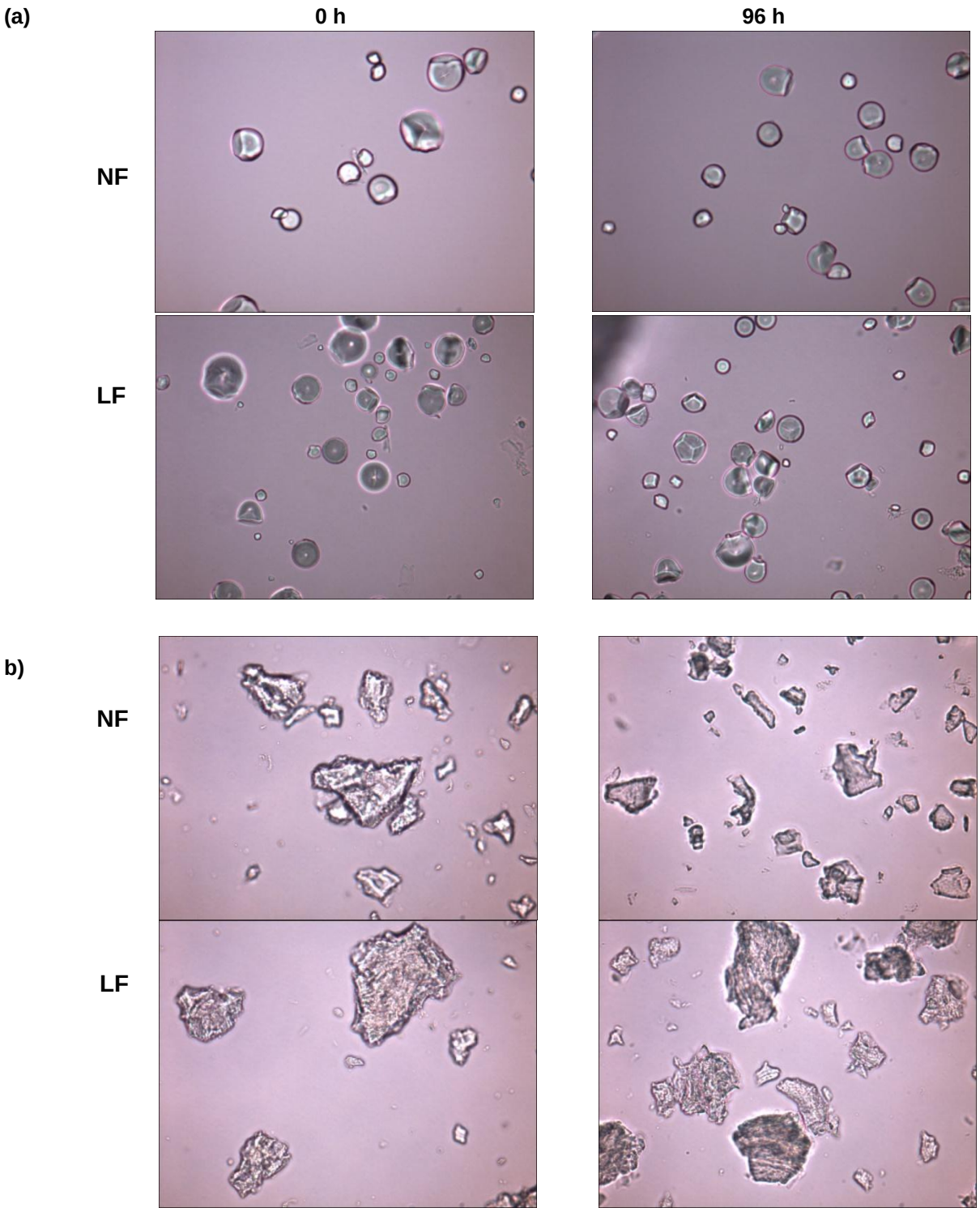

Figure 4. Morphologies of dried fermented zeolin "fufu flour" (a) and "cooked fufu" (b) with (LF) and without (NF) the addition of the starter culture at 0 and 96 h of fermentation. (a) oil immersion (50X) and (b) aqueous media (20X).

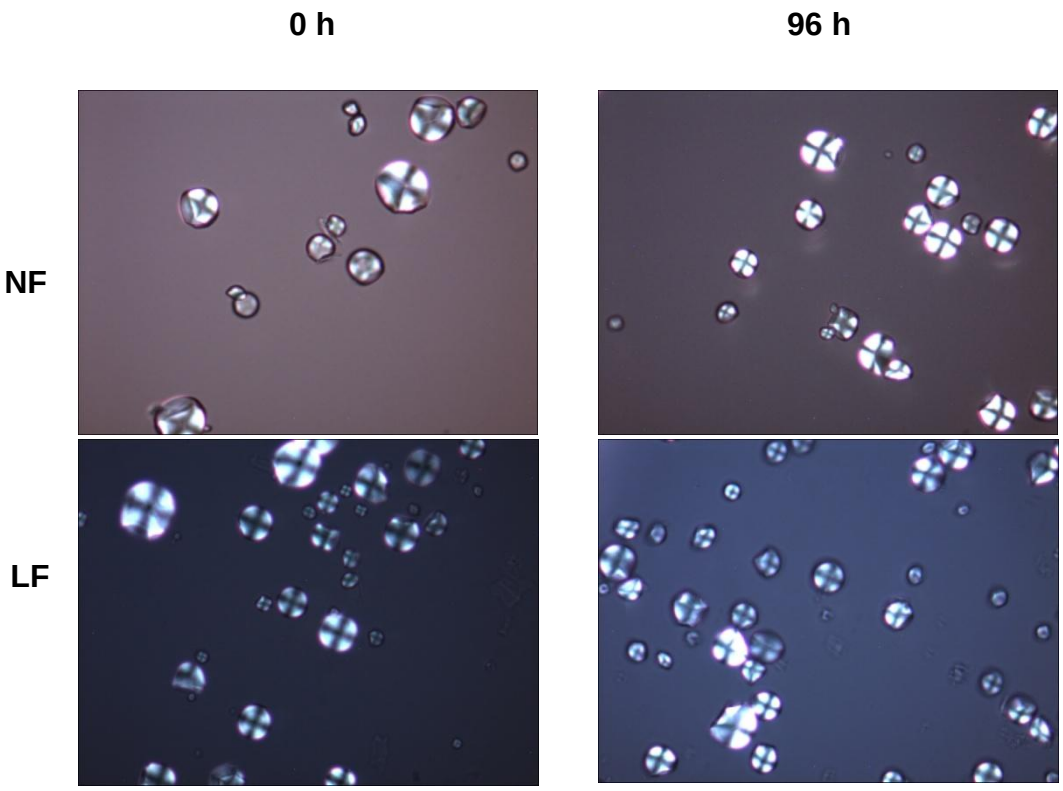

Figure 5. Morphologies of dried fermented zeolin “fufu flour” with (LF) and without (NF) the addition of the starter culture at 0 and 96 h of fermentation with polarized light. (a) oil immersion (50X) and (b) aqueous media (20X).

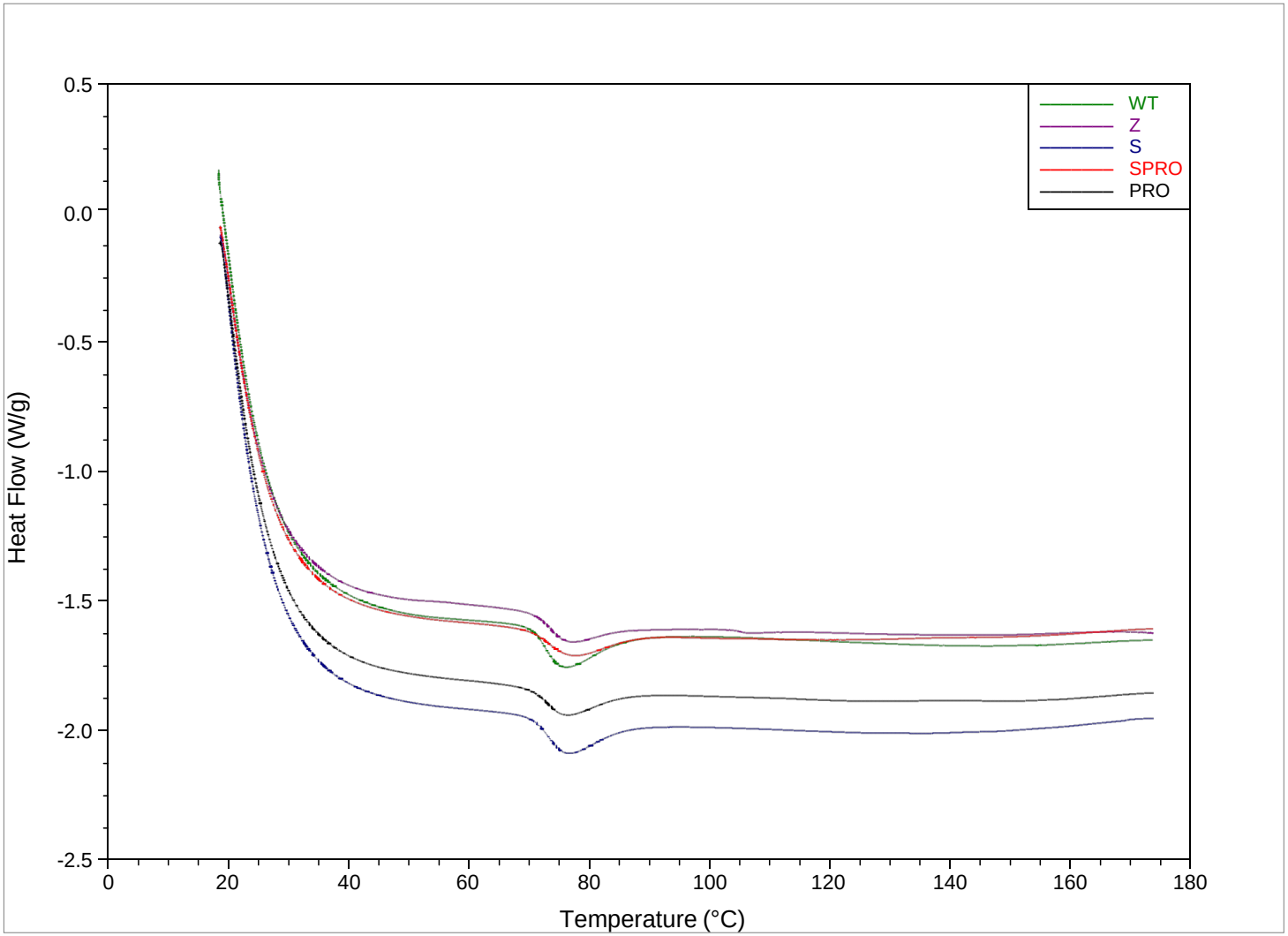

Figure 6. Thermal profile of wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cassava flours.

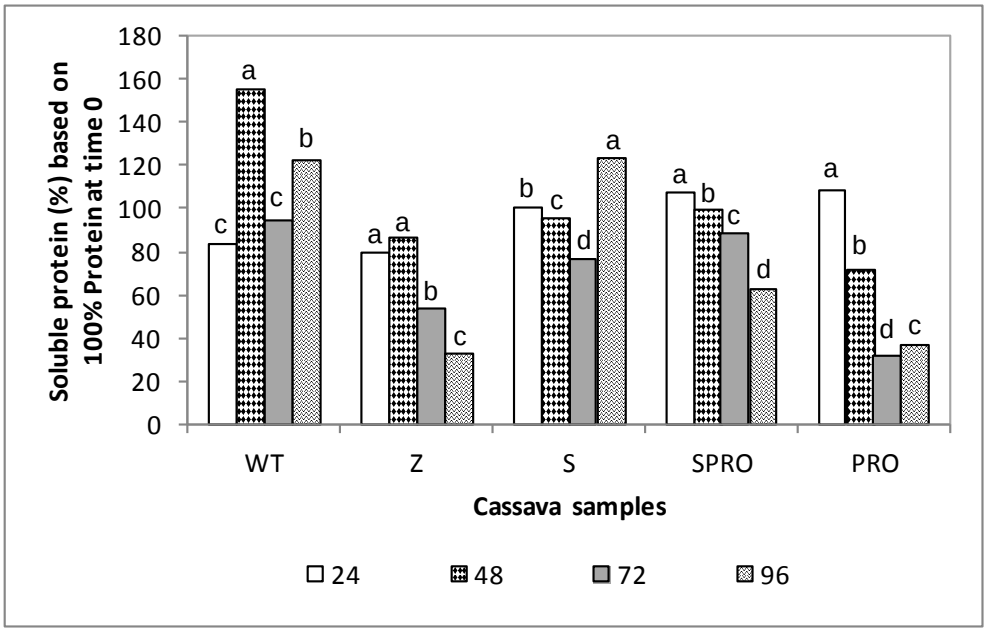

Figure 7. Soluble protein content (%) of naturally fermented wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cooked fufu samples from 24 to 96 h fermentation based on 100% soluble protein content at 0 h of fermentation. Different letters represent significant differences at each sample point ($p \leq 0.05$).

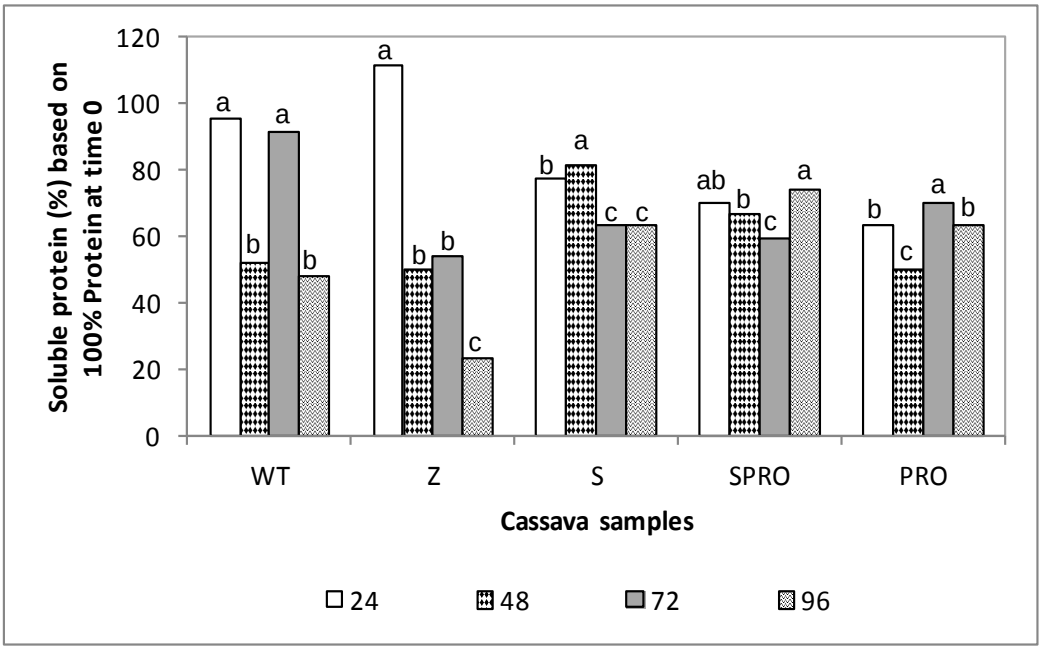

Figure 8. Soluble protein content (%) of *Lactobacillus plantarum* wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) cooked fufu samples from 24 to 96 h fermentation based on 100% soluble protein content at 0 h of fermentation. Different letters represent significant differences at each sample point ($p \leq 0.05$).

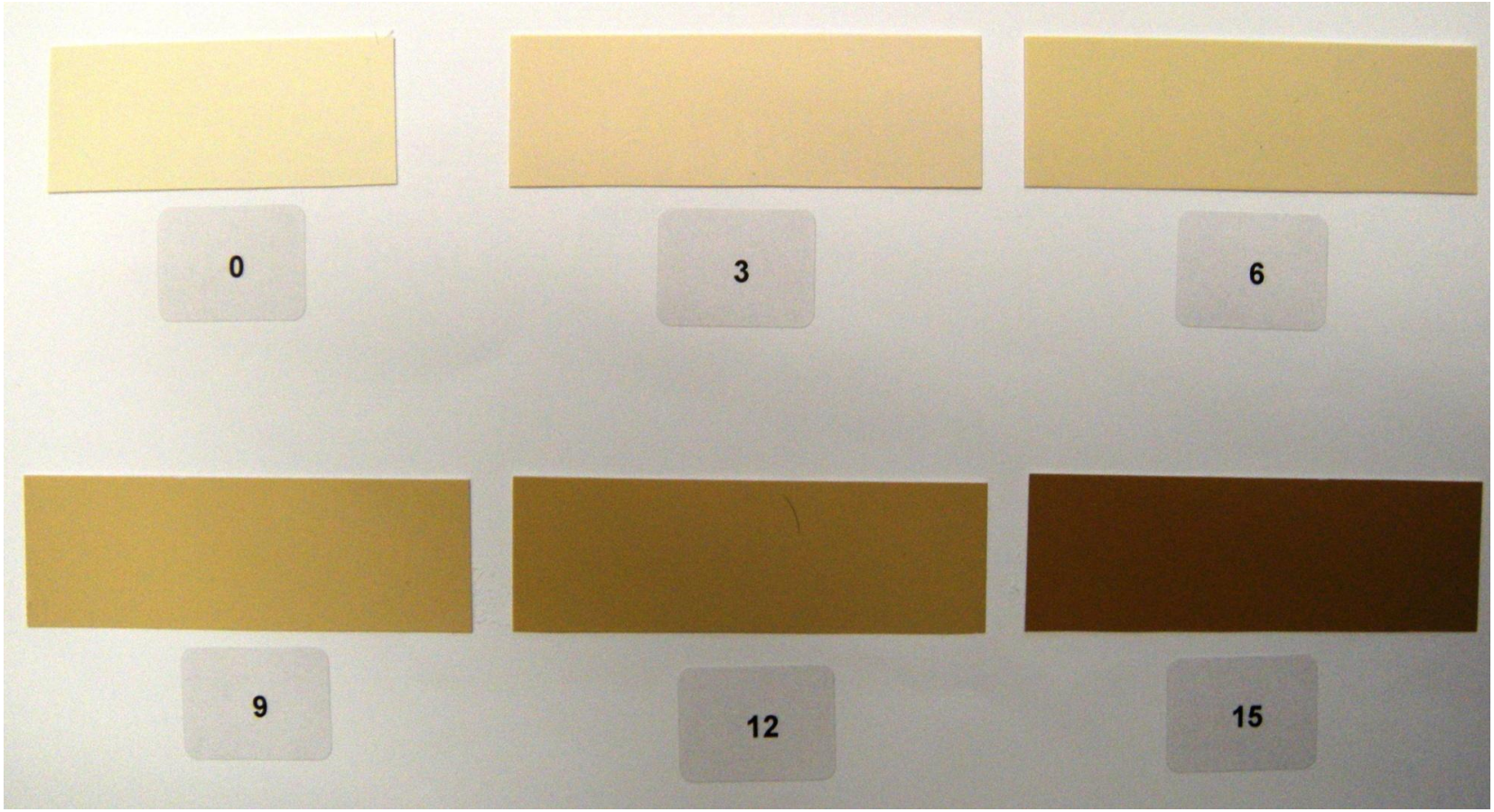

Figure 9. Chart of colors

CHAPTER VI
FUTURE STUDIES

Sporamin is a good candidate to increase cassava protein content. Additionally, fortified cassava flours showed similar characteristics to the wild type flour providing an alternative material for fufu production.

The current study did not identify any significant sporamin protein degradation due to oxidation analogous to cassava postharvest physiological deterioration. However, it is not clear whether the result will remain the same after inclusion of the sporamin gene in cassava roots. Therefore, it is recommended that the study must be repeated with sporamin incorporated into cassava root via gene insertion.

Moreover, additional studies should focus on specific bioactive properties of sporamin that will allow us to elucidate its activity against cross-linking formation. A more purified form of either sporamin A or B as it will be incorporated into cassava will better model the *in vivo* system. Concentration and reaction conditions of the factors involved in PPD should also be varied including the buffer mixture used, pH of the medium, time and incubation temperature, concentration of hydrogen peroxide and scopoletin among others that may provide us with additional information regarding sporamin behavior.

There is a lack of studies on proteolysis of fermented fortified cassava due to the addition of *L. plantarum* strains. A proteolysis study can include analysis of protein cleavage pattern due to strains used. Selection of strains with smaller proteolytic activity will result in greater protein retention during processing. More investigation is needed

related to other strains that can be used for the production of fufu that will degrade antinutritional factors in cassava, such as phytic acid and phenolic compounds.

Further information related to methods for evaluation of fufu volatile compounds would be desirable. Additional investigation on this matter will allow the maximum efficiency of the extraction of the fufu volatile compounds. Variables to study may include: extraction time, type of fiber, and type of GC column among others.

In order to improve the microwave oven method for preparing cooked fufu, an accurate control system of the wattage would be necessary to achieve a better and standardized quality of the final product.

The trained sensory panel found a significant effect of the flour sample type on most of the attributes evaluated. Consequently, a consumer panel to evaluate the degree of liking of the fortified cooked fufu products is necessary. This will allow us to determine the most preferred fortified cooked fufu and the attributes that need improvement.

CHAPTER VII

APPENDIX

Appendix 1. Research Study Consent Form

WASHINGTON STATE UNIVERSITY

School of Food Science, CAHNRS

Study Title: Consumer evaluation of fermented cassava flour
Researchers: Joseph R. Powers, Maria U. Rosales

You are being asked to take part in a research study carried out by researchers in the School of Food Science. This form explains the research study and your part in it if you decide to join the study. Please, read the form carefully, taking as much time as you need. Ask the researchers to explain anything you don't understand. You can decide not to join the study. If you join the study, you can change your mind later or quit at any time. There will be no penalty or loss of services or benefits if you decide to not take part in the study or quit later. This study has been certified for human participation by the Washington State University Institutional Review Board.

What is this study about?
This research study is being conducted to determine the sensory characteristics except for taste of fermented cassava flour. You are being asked to take part in this study because human subjects with knowledge of fermented cassava products are required to evaluate these cassava samples for different sensory properties. Taking part in the study will take 1.5 h per training session for 5 days and 1 h per evaluation session for 7 days. You can not take part in this study if you are under 18.

What will I be asked to do if I am in this study?
If you take part in this study, you will be presented with different fermented cassava samples and asked a series of questions regarding the sensory properties of these samples. You may refuse to answer any question in any test.

Are there any benefits to me if I am in this study?
There is no direct benefit to you from being in this study.

Are there any risks to me if I am in this study?
The potential risk from taking part in this study is becoming fatigued from sample evaluation.

Will my information be kept private?
The data for this study are being collected anonymously. Neither the researcher (s) nor anyone else will be able to link data to you. The data for this study will be kept for 3 years.

Are there any costs or payments for being in this study?
There will not be costs for you for taking part in this study
You will receive a small non-monetary incentive per session per taking part in this study.

Who can I talk to if I have questions?
If you have questions about this study or the information in this form, please contact the researcher(s). If you have questions about your rights as a research participant, or would like to report a concern or complaint about this study, please contact the Washington State University

Institutional Review Board at (509) - 335-3668, or email irb@wsu.edu, or regular mail at: Albrook 205, PO Box 643005, Pullman, WA 99164-3005.

What are my rights as a research study volunteer?

Your participation in this research study is completely voluntary. You may choose not to be a part of this study. There will be no penalty to you if you choose not to take part. You may choose not to answer specific questions or to stop participating at any time.

What does my signature on this consent form mean?

Your signature on this form means that:

- You understand the information given to you in this form.
- You have been able to ask the researcher questions and state any concerns.
- The researcher has responded to your questions and concerns.
- You believe you understand the research study and the potential benefits and risks that are involved.

Statement of consent

I give my voluntary consent to take part in this study. I will be given a copy of this consent document for my records if requested.

_____ _____

Signature of participant Date

_____

Printed Name of Participant

Statement of person Obtaining Informed Consent

I have carefully explained to the person taking part in the study what he or she can expect.
I certify that when this person signs this form, to the best of my knowledge, he or she understands the purpose, procedures, potential benefits, and potential risks of participation.

I also certify that he or she:

- Speaks the language used to explain this research.
- Reads well enough to understand this form or, if not, this person is able to hear and understand when the form is read to him or her.
- Does not have any problems that could make it hard to understand what it means to take part in this research.

_____ _____

Signature of Person obtaining the consent Date

_____ _____

Printed Name of Person obtaining the consent Role in the Research Study

Appendix 2. Demographic questionnaire

Panelist Nº: _____ Date: _____

1. Age range:

   ☐ 18 – 34

   ☐ 35-44

   ☐ 45- 54

   ☐ 55-64

   ☐ 65+

2. Gender:

   ☐ Male

   ☐ Female

   ☐ Prefer not to say

3. Country of Origin: _____

4. How often do you consume cassava in the United States?

   ☐ Three (3) times a day

   ☐ At least once a day

   ☐ Once to few times a week

   ☐ Not so frequently, a few times a month

   ☐ Never

5. How often do you consume cassava in your Home Country?

- ☐ Three (3) times a day
- ☐ At least once a day
- ☐ Once to few times a week
- ☐ Not so frequently, a few times a month

6. How often do you consume fermented cassava products in your Home Country?

- ☐ Three (3) times a day
- ☐ At least once a day
- ☐ Once to few times a week
- ☐ Not so frequently, a few times a month

7. What of these products you consume and/or prefer the most? Select only one.

- ☐ Gari
- ☐ Fufu
- ☐ Lafun
- ☐ Chickwangue
- ☐ Agbelima
- ☐ Attieke
- ☐ Kivunde
- ☐ Other (s): _____

8. How often do you consume the product selected above in your Home Country?

☐ Three (3) times a day

☐ At least once a day

☐ Once to few times a week

☐ Not so frequently, a few times a month

9. What is (are) the attribute (s) of the product selected above you like the most?

_____

_____

_____

10. Where consumers eat the product selected above most often?

☐ Home

☐ Street food

☐ Workplace

☐ Restaurant

☐ Other: _____

11. How do you consume the product (s) you selected in 7?

_____

_____

_____

12. In what form the product selected above is purchased? You can select more than one.

- ☐ Toasted
- ☐ Wet paste
- ☐ Cooked
- ☐ Other: _____

Thank you for your information!

Appendix 3. Description of colors assigned to the chart of colors

Color	Code	L*, a*, b*	Color Chart Score
Honey white	C20-1	92, 2, 12	0
Off White	C20-2	89, 4 12	3
Powdery Mist	C20-3	87, 5, 17	6
Peanut Shell	C20-4	78, 8, 23	9
Cashew Crunch	C20-5	73, 10, 25	12
Peanut Brittle	C20-6	61, 12, 29	15

SOURCE: ACE paint colors for your life (ACE N° 9960485)