Full Length Research Paper

Identification of subdominant lactic acid bacteria in dawadawa (a soup condiment) and their evolution during laboratory-scale fermentation of *Parkia biglobosa* (African locust beans)

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The successive colonization of fermenting African locust beans (*Parkia biglobosa*) by lactic acid bacteria (LAB) was investigated for seven days. The LAB isolated were *Pediococcus pentosaceus, Lactobacillus raffinolactus, Leuconostoc mesenteroides, Leuconostoc* sp*, Pediococcus halophilus, Pediococcus* sp*, Lactobacillus* sp*, Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus brevis.* The first dominant species were *P. pentosaceus,* which was more in abundance*, L. raffinolactus, L. mesenteroides* and another *Leuconostoc* sp. At the end of fermentation, a stable community comprising of *P. halophilus, P. pentasaceus* and *L. brevis* was formed. The dominant genera in this study were *Lactobacillus* and *Pediococcus*. The proximate analysis of the fermenting and fermented mash showed an increase in moisture content from 55.1 to 65.2%, pH from 6.25 to a stable alkaline value of 8.4. The reducing sugar however, decreased from 0.96 to 0.58 mg. The amino acid content increased from an initial value of 8.13 to 35.55 mg for the laboratory-scale product, whereas the value of the control was 13.4 mg. The total viable count increased from an initial value of $\log_{10}2.6$ to $\log_{10}5.8$ and then fell to a final value of $\log_{10}4.1$. The enterococci count increased initially but eventually fell to zero. The total yeast count increased initially from $\log_{10}5.3$ to $\log_{10}6.6$ and thereafter fell to zero. The market sample had a value of $\log_{10}1.5$. No coliforms were found in the course of fermentation as well as in the final product of the laboratory-scale experiment and the market sample.

Key word: Community, fermentation, laboratory-scale, *Leuconostoc, Parkia biglobosa, Lactobacillus, Pediococcus.*

INTRODUCTION AND LITERATURE REVIEW

The African locus bean is a perennial tropical tree legume, *Parkia* sp*ecies.* It was named after the Scottish surgeon "Mongo Park" who explored West Africa in the 1790's following the course of Niger River. Park had mentioned these trees by the local name "nitta", in his

"Travels in the interior district of Africa published in 1799. middle of the traditional "hungry season (Campbell-Plat, 1980). Campbell-Platt (1980) in his analysis has shown the trees are widely spread (Gambia, Sierra Leone, Nigeria and Cameroon) and provide valuable food in the that *Parkia biglobosa* contain up to 60% carbohydrate, 10 -24% of which is sucrose and 291 mg of vitamin C per 100 g of the seeds. Because of its nutritive value *P. biglobosa* is used as food source to feed livestock

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(Campbell-Platt, 1980). *P. biglobosa* have been used as food, medicine and fodder, soil amendment, charcoal and fire wood (Campbell-Platt, 1980).

The fermentation of African locust beans (*P. biglobosa*) by *Bacillus* spp. to produce dawadawa via alkaline fermentation has been discussed by several workers in West Africa (Odunfa, 1986) and in India (Tamang, 1998). Many common foods in West Africa are fermented to increase their nutritive value and keeping quality. Such fermentations are brought about by indigenous micro-organisms, which are present in the substrates. Banke is a fermented cereal product from cassava (*Manihot esculenta*) and maize (*Zea mays*) and gari, a fermented product from cassava (Campbell-Platt, 1980).

Several workers have also mentioned fermentation of different seeds and the fermenting microbes involved. Fermentation of melon seed to form ogiri (*Citrulus vulgaris*), fermentation of oilseed (*Pentacletha macrophylla*) to produce ugba), fermentation of sesame seed (*Sesamin indicum*) to produce ogiri saro, as well as fermentation of castor bean (*Ricinus communis*) seeds to produce ogiri-Igbo (Steinkraus, 1983; Tolminson et al., 1997). *Bacillus* spp. have been described as the initiator of the fermentation of these seeds (Diawara et al., 1992; Odunfa, 1981, 1985; Ouoba et al., 2002; Ouoba et al., 2003; N'dir et al., 1997;).

Studies by Antai and Ibrahim (1986) and Odunfa (1985) have shown that several microorganisms are associated with dawadawa fermentation and noted that the most abundant and the major dominant agent of fermentation after 24 h was *B. subtilis*. They also noted the presence after fermentation of *L. mesenteroides* and *Staphylococcus* species. Odunfa (1985) and Campbell-Platt (1980) also noted a high level of proteolytic activity during dawadawa fermentation, which culminated in the formation of peptides and amino acids. On flavour of dawadawa, Owen et al. (1997) on studying the aroma characteristics of *Bacillus*-fermented soy bean noted the presence of active aldehydes, ketones and acids. Owen et al. (1997) also observed that active microbial metabolism is required in order to bring about the changes observed in locust beans during fermentation.

Microbial fermentation of dawadawa have been found to involve only bacteria since fungi found have been regarded as incidental and does not play any notable role in its fermentation (Ikenebomah and Ingram, 1986). Most bacteria implicated are facultative anaerobes, while approximately 10% are aerobic after 36 h of fermentation. Odunfa (1985) first reported that predominant fermenting organisms was a *Bacillus* possibly *B. subtilis* and other species. However, recently, in 1986, Odunfa and co-workers confirmed the presence of *B. pumilis, B. licheniformis* and *B. subtilis* in the fermenting mash. They also observed that in some Nigerian fermented samples, *Pediococcus* and 2 varieties of *Staphylococcus saprophyticus* were detected. Several other workers have also implicated *B. subtilis* in the alkaline fermentation of locust

beans (Adewuyi, 1982; Campbell-Platt, 1980; Diawara et al., 1992; N'dir et al., 1997; Ouoba et al., 2002; Ouoba et al., 2003). Campbell-Platt (1980) also mentioned lactic acid bacteria especially species of *Lactobacillus* and *Pediococcus* as partakers in the fermentation of dawadawa that gives characteristic properties to the final product. In related studies, Vogel et al. (1999), De Vuyst and Ganzle (2005) working on sourdough isolated a consortium of lactic acid bacteria. Sourdough (mixture of wheat, rye, rice, etc) is a unique food ecosystem in that it selects for LAB strains for sourdough fermentation (Da 'Bello et al., 2005; De Vuyst and Neysens, 2005; Gobbetti et al., 2005). Stolze et al. (1996) observed that in the presence of abundance of maltose and under stress conditions, maltose phosphorylase is constitutively expressed in *Lactobacillus senfranciscensis, Lactobacillus reuteri, Lactobacillus fermentum* and *Lactobacillus pontis.* This enables maltose hydrolysis without expenditure of ATP and extracellular accumulation of glucose, initiating glucose repression in competitors for maltose. Gobbetti et al. (2005) asserted that several sourdough LAB contributes to bread aroma (for instance through production of ornithine as aroma precursor), dough rheology (amino acid metabolism), texture (production of exopolysaccharides) and delay of spoilage (production of acetic acid). De Vuyst and Vancanneyt (2007) studied the biodiversity and identified the lactic acid bacteria involved in sourdough fermentation. De Vuyst and Ganzle (2005) reported the ability of *Saccharomyces cerevisiae* to inhibit the growth of *Oenococcus oeni* during alcoholic fermentation by mechanism other than SO2 production. Osborne and Edwards (2007) reported the inhibition of malolactic fermentation by Saccharomyces during alcoholic fermentation under low and high conditions.

Bacteriocins are antibacterial proteinous substances produced by Gram – positive bacteria that have a narrow spectrum of activity against closely related species (De Vuyst and Vandamme, 1994 a, b; Jack et al., 1994; Oscariz and Pisabarro, 2001). De Vuyst and Ganzle (2005) observed that it is possible that the antibacterial peptide they isolated in their study acts in a similar way as bacteriocin to inhibit bacterial growth. Oscariz and Pisabarro (2001) in their study asserted that bacteriocins act by forming membrane channels or pores that destroy the energy potential of sensitive cells.

The aim of this study was to determine the successive colonization of fermenting locust beans by lactic acid bacteria, the amino acid and reducing sugars concentration during fermentation, the moisture content, the pH as well as the presence of coliform organisms during and after fermentation. It is hoped that the findings from this study will further encourage the use of dawadawa as soup condiment.

To our knowledge there is a dearth of information on the successive colonization of fermented locust beans by lactic acid bacteria as well as the identification of species of LAB involved in the fermentation. Although reports

from several workers have indicated the proteolysis by *Bacillus* species during fermentation, no attempt have been made to determine the daily level of amino acids released during this proteolysis (Campbell-Platt, 1980; Diawara et al., 1992; Odunfa, 1981, 1985, 1986; Ouoba et al., 2002; Ouoba et al., 2003; N'dir et al., 1997).

MATERIALS AND METHODS

African locust bean

The African locust bean used for this study was purchased from Mushin market in Lagos State, Nigeria. The grains were screened, broken ones and those perceived to be of low quality were removed. About 20 gm of the seeds, which are in good condition were dehusked, the cotyledon obtained and cooked in an aluminium pot according to the method of Odunfa (1981) and aseptically transferred to a sterile calabash, covered and allowed to undergo fermentation.

Sample collection

Samples were collected at zero hour and every 24 h of fermentation for a total period (7 days). The calabash harbouring the fermentation mash was aseptically accessed each time sampling was done to prevent termination and contamination of the fermenting mash. Samples for investigation were put in a cellophane bag, sealed and refrigerated to stop fermentation at the end of the 7th day. Samples were subsequently analysed. A market sample was also obtained to serve as control.

Sample preparation and isolation method

1 g of each sample collected was homogenized with 9 ml of sterile distilled water after pounding with mortar and pestle aseptically. The resulting homogenates were diluted appropriately by the method of ICMSF (1988) and Uzuegbu and Eze (2001). 10-fold serial dilution was performed up to a final dilution of 10^{-10}. Desired diluent of each sample was plated using the spread plate method of five (5) different media namely - plate count agar (PCA), malt extract agar (MEA), de Man Rogosa Sharpe Agar (MRS), Kanamycin Agar and VBR agar. These media were used to determine the total viable bacteria, yeast or fungi, *Lactobacillus*, *enterococci* and coliforms respectively. The isolates from the pure plates were subcultured severally until pure cultures were obtained. The pure cultures were preserved and kept for future use. The mean total plate count for viable cells, LAB, yeast and enterococci during the period of fermentation were as shown in Figure 5.

The progressive colonization of fermenting mash by lactic acid bacteria was studied using aliquots of appropriate diluent and spreading on de Man Rogosa Sharpe medium with subsequent incubation according to methods of Tamang (1998) and Odunfa (1986). The emerging colonies were identified by morphological and biochemical methods. The results obtained were as shown in Table 1.

Proximate analysis of fermented locust bean mash

The pH, total reducing sugar, moisture content and amino acid concentration produced daily for 7 days were also determined using standard methods (AOAC, 1984; Collins, 1989; Miller, 1959; Pearson, 1973; Rosen, 1957).

Determination of total amino acid in fermented dawadawa

Total amino acid was determined according to the procedure of Rosen (1957). 1 ml of diluted sample extract was mixed with 0.5 ml each of cyanide-acetate buffer (pH 5.4) and 3% ninhydrin solution of methylcellulose (w/v). The mixture was heated at 100°C for 15 min in a boiling water bath. The mixture was removed from the water bath at the end of 15 min and 5 ml of isopropyl alcohol-water mixture was added to cool at room temperature. The absorbance of the solution was determined at 570 nm wavelength. The total amino acid was determined by extrapolating from readings obtained from standard tubes containing different concentrations of leucine solution. Total amino acid was also determined for the control as shown in Figure 4.

Determination of total reducing sugars

The extracts used were produced by homogenizing 5 g of dawa dawa samples with 25 ml of distilled water. The mixed homogenates were centrifuged at 8000 rpm for 20 min to obtain a clear supernatant used for the analysis. The total reducing sugar content of extract was determined by the dinitrosalicylic acid (DNSA) reagent method of Miller (1959). The reagent was prepared by adding 2 g of 3, 5-dinitrosalicylic acid to 80 ml of 1N NaOH and 6 ml of distilled water. Potassium sodium tartarate (60 g) was added and the mixture was diluted to 200 ml with distilled water. The reducing sugar content was determined by adding 2 ml of 3, 5-dintrosalicylic reagent to 1 ml of the sugar extract. The mixture was heated in boiling water for 5 min and allowed to cool.

Absorbance of the resulting coloured solution was determined spectrophotometrically at 540 nm against blank prepared by substituting the sugar extract with distilled water. The resulting sugar contents were extrapolated by reference to a standard curve of known concentrations of maltose. The total reducing sugar was also determined for the control. The profile for the reducing sugars in the fermenting, final product and control is as shown in Figure 3.

RESULTS AND DISCUSSION

The issue of fermentation of different plants to improve their nutritional flavour, food value and keeping quality have been discussed (De Vuyst and Ganzle, 2005; Campbell-Platt, 1980; Ikenebomah and Ingram, 1986; Odunfa, 1986; Owen et al., 1997; Steinkraus, 1995; Tamang, 1998). It is a known fact that the fermentation of such plants is brought about by a consortium of microbes, and in most cases initiated by *Bacillus* spp with the assistance of lactic acid bacteria (Antai and Ibrahim, 1986; Odunfa, 1985, 1986). However, no effort was made by these workers to study the microbial succession involving the lactic acid bacteria (LAB) and the order in which they appear during fermentation of dawadawa mash.

Our findings (Table 1) show that the genera and species among the LAB group isolated after morphological, biochemical and sugar fermentation tests were *Lactococcus raffinolactus, Pediococcus pentosaceus, Leuconostoc mesenteroides, Lactobacillus brevis, Lactobacillus plantarum, Pediococcus* sp. and *Pediococcus halophilus.* Although several workers have made mention of the presence of some LAB, *Leuconostoc mesenteroides* (Antai and Ibrahim, 1986; Odunfa, 1985);

Table 1. Morphological and biochemical properties of Lactic acid bacteria isolated from locust beans during dawadawa fermentation.

S/N	Isolate code	Colony morphology	Cell shape	Gram reaction	Oxidase	Catalase	Homo-heterofermentation	xylose	Cellobiose	Maltose	Salicin	Lactose	Mannitol	Glucose	Arabinose	Sorbose	Sucrose	Galactose	Raffinose	Suspected organismsi
1	OMRS 3	Small, flat round whitish colony	cocci	+	_	_	Homo	+	+	+	+	+	+	+	_	_	+	+	+	*Lactobacillus raffinolactus*
2	OKA3 OK43 7KA14	Small, round flat and whitish colony	Cocci	+	_	_	Homo	+	+	+	+	+	W	+	+	_	W	+	_	*Pediococcus pentosaceus*
3	OK8 5KA16	Small, whitish grey round and slimy colony	Cocci	+	_	_	Hetero	_	+	+	+	+	+	+	W	_	+	+	_	*Leuconostoc mesenteroides*
4	7KA16 5KA13	Tiny, translucent colony	Rod	+	_	_	Hetero	_	_	+	+	+	W	+	+	_	+	+	+	*Lactobacillus brevis*
5	3KA11 5K9 3KA10	Small, round and translucent colony	Rod	+	_	_	Hetero	_	+	+	+	+	+	+	+	_	+	+	+	*Lactobacillus plantarum*
6	1K4 1K5	White, round and flat colony	Cocci	+	_	_	Homo	_	+	+	+	+	+	+	+	_	+	+		*Pediococcus sp.*
7	3RA9 3RA8 2R25 2RA6	Small, round, raised and whitish colony	Rod	+	_	_	Hetero	W	+	+	+	+	+	+	+	_	+	+	+	*Lactobacillus plantarum*
8	7K10 1KA6 7K13	Tiny translucent colony	Cocci	+	_	_	Homo	_	+	±	+	+	+	+	+	_	+	+	W	*Pediococcus halophilus*
9	OK1	Small, whitish grey and round colony	cocci	+	_	_	Hetero	+	+	+	+	_	_	+	+	_	±	+	_	*Leuconostoc sp.*
10	IRA56 IRA4	Flat, round and whitish colony	Rod	+	_	_	Hetero	_	+	+	+	+	+	+	+	_	+	+	+	*Lactobacillus sp.*
11	IKA7	Flat creamy colony	Cocci	+	_	_	Homo	_	+	+	+	+	+	+	+	_	+	+	_	*Pediococcus sp.*
12	3KA7	Flat, round and whitish colony	Rod	+	_	_	Homo	W	+	+	+	±	W	+	_	_	+	+	+	*Lactobacillus acidophilus*

+ = Positive reaction; - = negative reaction; Homo = homolactic fermentation; Hetero = heterolactic fermentation; W = variable reaction.
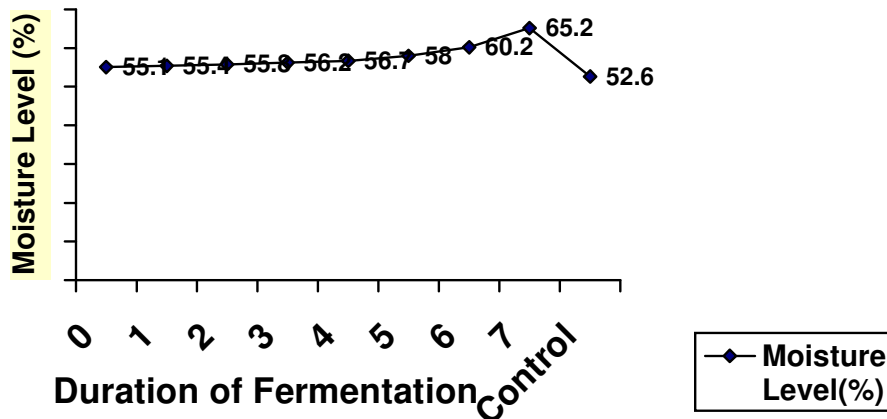
Figure 1. Moisture level of fermenting locust beans (*P. biglobosa*) during production of dawadawa.
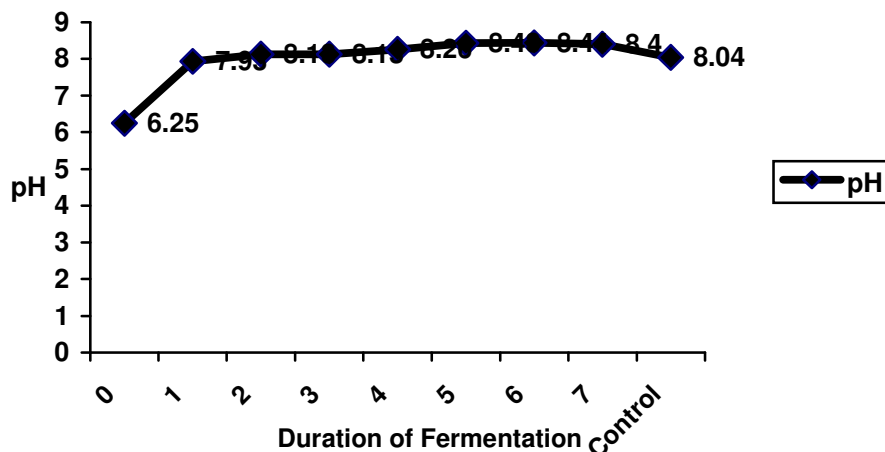

Figure 2. The pH of ferment locuct beans (*P. biglobosa*) production of dawadawa.

Pediococcus sp. (Odunfa and Co-workers; Campbell-Platt, 1980), none have indicated with specificity the occurrence of *Pediococcus* spp. and *Lactobacillus* spp. presented in this work. There was an increase in al., 2003). Campbell-Platt (1980) also mentioned lactic moisture level from 55.1 to 65.2% at the end of fermentation (Figure 1). The moisture level of the control was 52.6%. The pH increased from an initial value of 6.25 to a maximum alkaline value of 8.4 on the fifth day of fermentation and stabilized at this value till the last day of fermentation (Figure 2). The pH of the control was 8.04. The highest mean value obtained for reducing sugar was 1.25 mg at the fourth day of fermentation (Figure 3). The value of the control was 0.64. The maximum value for amino acid concentration was 35.55 mg on the seventh day of fermentation. The value for the control was 13.4 mg (Figure 4), indicating that laboratory fermented dawadawa contain more amino acid than the locally fermented counterpart. The maximum mean viable count was $\log_{10}9.3$, which was obtained in day four of fermentation. This value fell and stabilized at $\log_{10}8.8$ with the control value at $\log_{10}6.9$ (Figure 5). The Lactobacilli count obtained in this study was $\log_{10}5.8$, which stabilized between $\log_{10}4.2$ and $\log_{10}4.1$ at the fifth to the seventh day of fermentation, while the value for the control was $\log_{10}2.5$ (Figure 5). With the high level of LAB, the laboratory fermented dawadawa will have a better keeping quality than the locally fermented product. It was the progressive elimination of enterococci as fermentation extended that was interesting as the population of this species increased from $\log_{10}4.2$ to $\log_{10}6.4$ and thereafter decreased exponentially to zero (0) at the end of fermentation (Figure 5). This may have resulted from the high acidity created by members of LAB or they are poor at competing with the other LAB in a high acid environment. The total yeast count increased initially from $\log_{10}5.3$ to $\log_{10}6.6$ and then decreased exponentially to a value of 0 (Figure 5). However, no coliform was detected in all the samples analysed. An increase that was initially observed for all isolates may be due to the exponential
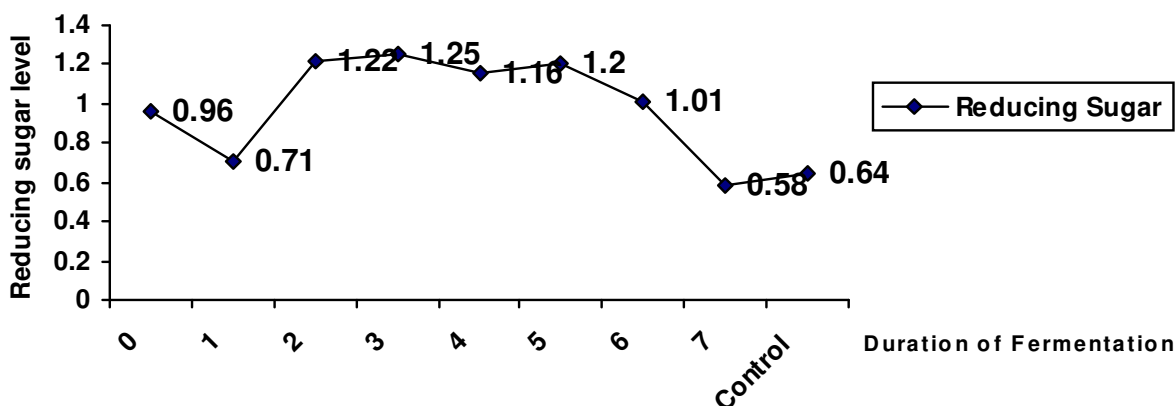
Figure 3. Total reducing sugar level of fermenting locust beans (*P. biglobosa*) during production of dawadawa.
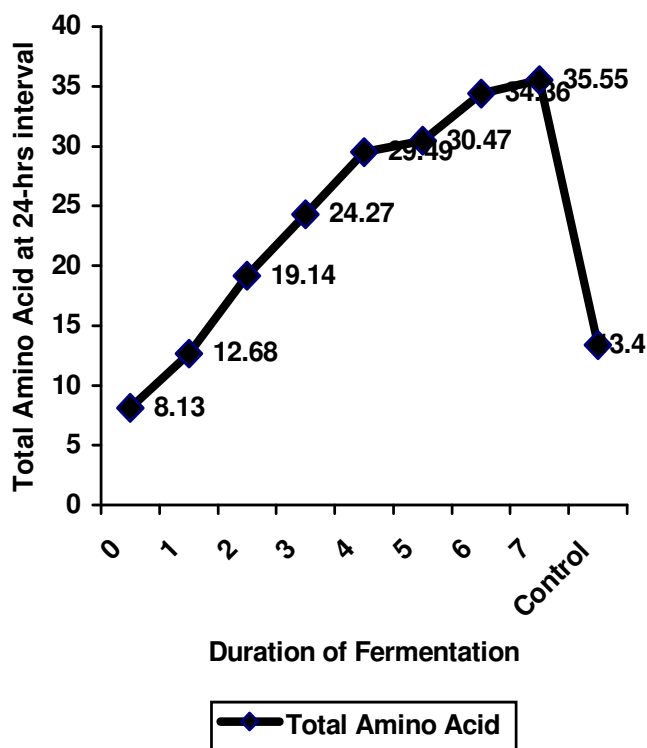

Figure 4. Total Amino Acid level of fermenting locust beans (*P. biglobosa*) during production of dawadawa.

growth of the microbes, which soon fell as a result of nutrient exhaustion and accumulation of metabolic products (Stanier et al., 1976; Uaboi-Egbenni, 2000; De Vuyst and Ganzle, 2005).

Studies on the ecological succession of LAB in fermenting locust bean (*P. biglobosa*) showed that at the 0 h *P. pentosaceus, L. raffinolactus, L. mesenteroides* and another *Leuconostoc* sp. were the first colonizers having a percentage occurrence of 40, 20, 20 and 20, respectively. These were gradually replaced after a day

of fermentation by *Pediococcus* sp., *Lactobacillus* sp. and *P. halophilus* with a percentage occurrence of 50, 33 and 17, respectively. After 2 days of fermentation the next group of LAB isolated were *L. plantarum* with a percentage of occurrence of 67 and *P. pentosaceus* with a percentage of 33. At the third day of fermentation colonizing LAB were *L. plantarum* (80%), *L. acidophilus* (20%). LAB occurring after the fourth day of fermentation were *L. plantarum* (59%) and *L. brevis* (41%). LAB occurring after the fifth of fermentation were *L. plantarum* (33%), *L. brevis* (33%) and *L. mesenteroides* (33%). At the sixth day of fermentation, the emerging LAB were *L. brevis*, *P. halophilus* and *L. mesenteroides*. At the seventh day of fermentation, the LAB at the climax of succession were *P. halophilus* (50%), *P. pentosaceus* (25%) and *L. brevis* (25%). This finding is in line with the observation of Campbell-Platt (1980) who noted percentage occurrence of lactobacilli and *Pediococcus* of final product to be 21%. The LAB succession observed in this study may have stemmed from changes in the mash at different times during fermentation. Such changes may be capable of inhibiting the growth of some LAB species. This ecological competition may then result in survival of the fittest. After 7 days of fermentation of dawadawa a climax of microbial succession in a high alkaline environment had been established and persists in the end product.

Antai and Ibrahim (1986) and Odunfa (1986) isolated *L. mesenteroides* from fermented dawadawa. Odunfa and Co-workers also isolated *Pediococcus* sp from fermenting and fermented dawadawa. Although these groups of workers have mentioned the presence of other *lactobacilli*, they were not actually identified and their successive colonization of fermenting dawadawa was not studied. De Vuyst and Vancanneyt (2005) studied the biodiversity of lactic acid bacteria during fermentation of sourdough and isolated several species of LAB. Fermentation of the seed does not destroy the nutritional and elemental content of the seeds; rather it brings about
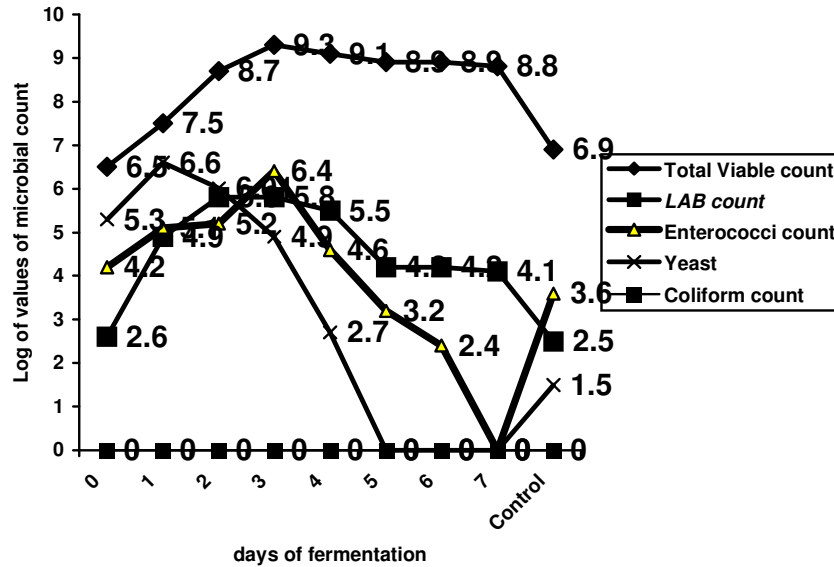
Figure 5. Plot of Log of values of viable counts of different groups of microorganisms against duration of fermentation.

a modification of the nutritional component of the seed for better uptake and utilization. Mohan and Janardhanan (1995) opined that the prohibitive cost of animal proteins in developing countries including Nigeria, calls for extensive exploitation of plant protein sources, which are economically cheaper. There was a continuous increase in the concentration of amino acid with increase in the period of incubation. In a recent study the isolates *B. subtilis* and *B. pumilis* were shown to demonstrate different ability to degrade African locust beans proteins culminating in the release of different profiles of water soluble proteins and free amino acids (Ouoba et al., 2003). In addition, the total elimination of coliform organisms observed early in fermentation makes the ferment healthy for use as condiment in foods. As a result, its present limited use should be expanded into additional opportunities. The ferment is recommended for use as food condiment especially in soups. The concentration of the reducing sugars increased until day 3 and thereafter decreased in value till the last day of fermentation. From this observation it is apparent that fermentation could be completed within the first three days. Further increase in days of fermentation will result in the indigenous microbes using the resulting nutrients in the ferment for their metabolism.

Although LAB do not initiate the fermentation of *P. biglobosa*, their presence in the final product enhance its keeping quality due to the bacteriocin they produce (De Vuyst and Ganzle, 2005). In addition, Gobbetti et al. (2005) observed that several sourdough LAB contributes to bread aroma and delay of spoilage due to the lactic acid they produce. Hence it may be possible that dawa dawa LAB play similar role in the preservation of the final product from African locust beans fermentation.

Conclusion

On the basis of analysis, it was observed that the fermentation of African locust beans does not affect its high nutritional value and the product from its fermentation can be used effectively as seasoning agent as well as fortifying the protein contents of some foods that are protein deficient. Fermentation of the seed does not in any way result in decrease in nutritional and elemental content; rather it brings about a modification of the nutritional component of the seed for better uptake and utilization. With the ever increasing cost of protein sources in Africa and other developing countries, the ferment is recommended for use as food condiment especially in soups to alleviating protein deficiencies and its associated diseases.

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