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MICROBIOLOGICAL AND NUTRIENT STUDIES OF FERMENTED COOKED LIMA BEAN (*Phaseolus lunatus*) SEEDS

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Abstract

Dehulled and ground white cultivar of cooked, pressure-cooked and uncooked Lima bean seeds was fermented in calabashes for nine days. Microorganisms isolated from the samples include the bacteria *Bacillus subtilis, B. megatagium, B. polymyxa B. pumulis, B. licheniformis, Lactobacillus acidophilus, L. fermentum, L plantarum, L. acidophilus, L. brevis, Leuconostoc, Micrococcus luteus, Proteus vulgaris, Staphylococcus aureus* and *S. saprophyticus* and the fungi isolates include *Aspergillus fumigatus, A. niger, Geotrichum candidum, Penicillium italicum, Rhizopus stolonifer* and *Saccharomyces cerevisiae*. The most frequently isolated microorganisms from all the samples included *B. subtilis, B. pumulis, B.megatagium, L. plantarum, A. fumigatus* and *S. cerevisiae*. The highest bacterial and fungal counts were found in cooked sample at 96 and 144 hours of fermentation respectively. Temperature increased initially with the highest and lowest in cooked sample (31.9ºC) and uncooked sample (29.5ºC) at 96 and 144 hours respectively. The pH values increased up to 96 h with the highest and lowest values also in cooked samples (7.3) and uncooked sample (6.91) at 96th and 168th hours while total titratable acidity decreased at the same hours of fermentation respectively. Moisture, fat and ash contents increased while crude fibre and carbohydrate contents decreased throughout the processing period mostly in heat treated samples. Protein content increased up to 120 hours of fermentation with the highest and lowest contents from cooked sample (27.4%) and uncooked samples (21.3%) respectively. Fermented cooked sample was rated best in all the organoleptic parameters tested.

Keywords: *Lima bean, Fermentation, microorganisms, Nutrients.*

Introduction

Legumes play an important role in the traditional diets of many regions throughout the world. They contain high protein contents and other nutrients which promote good health of human being. Besides they serve as an important and alternative source of cheap protein in developing countries such as Nigeria and some countries where animal protein is expensive (Esenwah and Ikenebomeh, 2008; Murthy, 2011). More recently, some of these legumes have been noted for their soluble fibre contents (Costa *et al*., 2006).

In spite of an urgent effort to meet the nutritional requirements of the ever increasing populations, some of these available cheap protein resources are not acceptable by the consumers and thus remain relatively under-utilised (Thangadurai *et al*., 2006). This may be due to processing procedure such as long hours of cooking and undesirable characteristics such as the presence of flatulence producing factors as well as other anti-nutritive constituents (Olson *et al.*, 1981; Liener, 1994; Ajayi *et al*., 2010; Mugendi *et al.*, 2010).

Lima beans (*Phaseolus lunatus*) belong to the family Fabaceae. It is also known by various names such as butter beans, Chad beans and 'papala' by Yoruba tribe of Nigeria. Lima bean is mainly grown in Peru and later introduced to Europe and African countries (Ezeagu and Ibegbu, 2010). The pod of the Lima bean is flat, oblong and slightly curved, averaging about three inches in length. Within the pod are the two to four flat kidney-shaped seeds. The legume is an annual herbaceous plant with epigeal germination and is self-pollinated and propagated from seeds. Lima beans are twining vines or herbaceous bushes, perennial in nature, but usually grown as annuals, even in the tropics. The crop could be intercropped with tubers, banana and vegetables in the humid forests sorghum and millet in the savanna regions (Ezeagu and Ibegbu, 2010).

Despite the rich nutrient composition of these seeds, some of them usually require long cooking periods which require the use of scarce expensive fuels (Uzogara and Ofuya, 1992). Several researches have been conducted on the fermentation of leguminous plants and results proved their significance as good sources of protein for the less affluent societies (Odunfa, 1985; Oyewole and Odunfa, 1990; Ouoba *et al*., 2003; Dakwa *et al*., 2005).

Legumes based fermented products such as *iru*, *ugba* and *tempe* are used in the human diet for various purposes and also as animal feed supplements. They also serve as flavouring agent for foods such as soya sauce in Japan. The popularity of legume based fermented foods is due to desirable changes in their final texture and organoleptic characteristics (especially elimination of beany flavours, improvement indigestibility), enhancement in keeping quality of the product, removal of anti-nutritional factors, increase in nutritional contents and reduction in cooking time (Steinkraus, 1997). These changes have been attributed to the fermenting microorganisms such as bacteria and fungi which are naturally present in these substrates. *Bacillus*, *Lactobacillus*, *Saccharomyces* and *Rhizopus* have been isolated in some fermented legumes and reported to be responsible for some of these desirable changes (Nwagu *et al.*, 2011).

Although work has been done on the nutritional values and different methods (chemical and thermal) of detoxification of Lima beans as well as its usefulness as food supplements (Ologhobo and Fetuga, 1988; Ologhobo, 1992), not much work has been reported on the fermentation of the seeds. This work is to provide alternative methods of processing (fermentation) Lima beans which could bring about better acceptable products in terms of nutritive values and physical parameters.

Materials and Methods

Preparation of the Samples

Lima beans used for this research work were brought from Ayegunle-Ekiti, Ekiti State, Nigeria. The clean and healthy seeds were sorted and washed with clean water, drained and soaked in hot water for 2 hours to enhance easy removal of the testa from the cotyledons. The cotyledons were washed and rinsed with several changes of sterile water and finally dried using a drying cabinet. Lima bean cotyledons were ground using a blender sterilized with 90% ethanol and rinsed with several changes of sterile water and then divided into three parts. Some samples were cooked for 6h, some pressure-cooked for 40 mins and the last sample fermented raw. One kilogram each of the pastes was fermented in calabash es and plastic buckets each in duplicates for a period of 10 days.

Microbiological Analyses

The samples (10 g) were homogenized with 90 ml sterile peptone water solution and further serially diluted as appropriate. Enumeration of the total bacteria, lactic acid bacteria and fungi was carried out on daily basis using plate count agar (Oxoid CM 325, Hampshire, UK), deMan, Rogosa and Sharpe (MRS) agar (Oxoid CM 361) and Sabouraud dextrose agar (SDA, Oxoid CM 41) respectively. Fungal plates were incubated at 25°C for 2 to 5 days while bacterial cultures were incubated at 37°C for 1 to 2 days. MRS agar plates were incubated under anaerobic conditions. The isolates were sub-cultured by repeated streaking on their respective media until pure cultures were isolated. The isolates were characterized by cultural, morphological and biochemical tests (Olutiola *et al.,* 2000).

Temperature, Total Titratable Acidity and pH

The temperature of the fermenting paste was monitored on daily basis for 10 days by aseptically inserting a thermometer sterilized with 75% ethanol into the fermenting samples. The % total titratable acidity was determined by diluting 10 g of the sample in 90 ml of sterile distilled water. The mixture was homogenized and allowed to settle from which 20 ml would be titrated against 0.1 N NaOH using phenolphthalein as indicator. The pH was determined using a pH meter (Crison Basic model 20) calibrated with standard buffer (pH 7.0 and 4.0).

Proximate Composition

The proximate composition of the fermenting Lima beans was monitored on daily basis for the fermentation period (A.O.A.C., 2006). The parameters monitored include crude protein, crude fats, crude fibre, moisture and carbohydrate contents.

Sensory Evaluation

The fermented samples were served 20 untrained judges to evaluate the sensory qualities (odour, colour, texture, and overall acceptability) using a five-point hedonic scale (1 and 5 representing extremely dislike and extremely like, respectively).

Statistical Analysis

Data obtained were analyzed by ANOVA and significant differences between means were compared using Duncan (Duncan 1955) multiple range test with the aid of SAS/STAT program (SAS 1998)

Results

Thirty one bacteria (6 genera) and 18 fungi (5 genera) were isolated from ground Lima beans fermented in both calabashes and plastic buckets for ten days. Bacterial isolates include *Bacillus subtilis, B. megatagium, B. polymyxa B. pumulis, B. licheniformis, Lactobacillus acidophilus, L. fermentum, L plantarum, L. acidophilus, L. brevis, Leuconostoc, Micrococcus luteus, Proteus vulgaris, Staphylococcus aureus* and *S. saprophyticus* while the fungi include *Aspergillus fumigatus, A. niger, Geotrichum candidum, Penicillium italicum, Rhizopus stolonifer* and *Saccharomyces cerevisiae*.

There were initial increases in bacterial counts in all the samples up to 96 hours in cooked and pressure cooked samples and 120 hours in uncooked samples after which they started to decline. The highest and the lowest optimum counts of 9.12 log cfu/g and 6.45 log cfu/g were observed in pressure cooked and uncooked sample respectively (Figure 1).

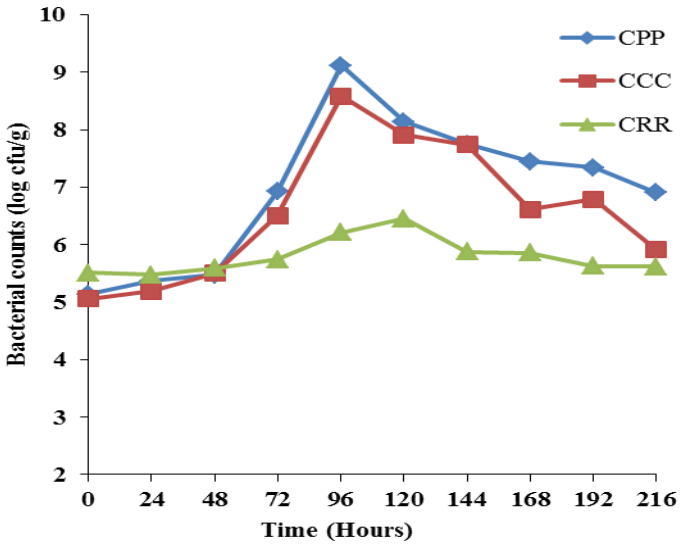

Figure 1: Bacterial counts of the fermenting ground Lima beans.
CPP =Pressure cooked, CCC= Cooked, CRR = Uncooked

Fungal colonies started to appear after 48 hours in uncooked samples and 72 hours of fermentation in cooked and pressure cooked samples. Cooked sample had the highest fungal count at 144 hours (3.92 log sfu/g) while pressure cooked and uncooked samples had 3.82 log sfu/g and 3.55 log sfu/g counts respectively at the end of the fermentation (Figure 2).

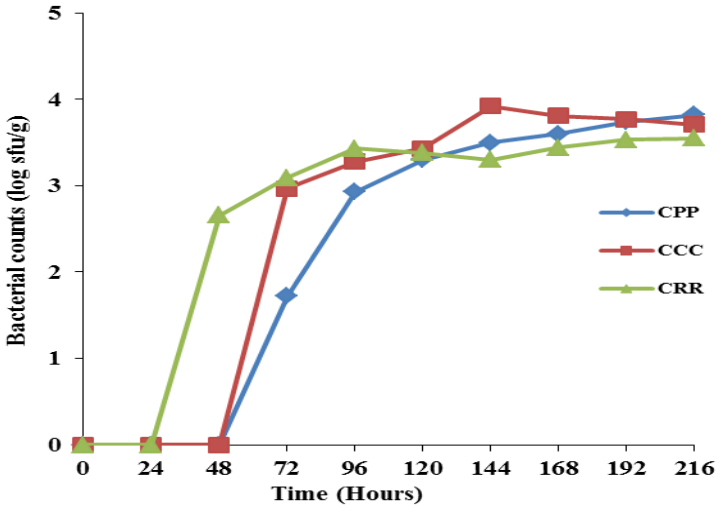

Figure 2: Fungal counts of the fermenting ground Lima bean seeds.
CPP =Pressure cooked, CCC= Cooked, CRR = Uncooked

Bacillus subtilis, *B. pumulis*, *L. plantarum*, *A. fumigatus* and *S. cerevisiae* were the most frequently isolated and the most abundant microorganisms during the fermentation period. *Bacillus* species were the most abundant at the earlier days of fermentation while *Lactobacillus* and the fungi were isolated after several hours of fermentation. *Staphylococcus aureus*, *S. saprophyticus*, *Micrococcus luteus* and *Proteus vulgaris* were usually isolated within the first two days of fermentation (Tables 1-3).

Table 1: Occurrence of bacterial isolates from ground cooked Lima bean seeds during fermentation

Isolates	Days 0	1	2	3	4	5	6	7	8	9
Bacillus subtilis	+	+	+	+	+	+	+	+	+	-
Bacillus polymyxa	-	+	+	+	+	+	+	+	-	-
Bacillus pumilus	+	+	+	+	+	+	+	+	-	-
Lactobacillus acidophilus	-	-	-	-	-	-	+	+	+	+
Lactobacillus plantarum	-	-	-	+	+	+	+	+	+	+
Micrococcus luteus	+	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	+	+	-	-	-	-	-	-	-	-
Saccharomyces cerevisiae	-	-	-	-	+	+	+	+	+	+
Rhizopus stolonifer	-	-	-	-	+	+	+	+	-	-

Table 2: Occurrence of bacterial isolates from ground pressure-cooked Lima bean seeds during fermentation

Isolates	Days 0	1	2	3	4	5	6	7	8	9
Bacillus licheniformis	-	+	+	+	+	+	+	+	-	-
Bacillus subtilis	-	+	+	+	+	+	+	+	+	-
Bacillus pumilus	+	+	+	+	+	+	+	+	-	-
Lactobacillus brevis	-	-	-	-	-	+	+	+	+	-
Micrococcus luteus	+	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	+	+	-	-	-	-	-	-	-	-
Aspergillus niger	-	-	-	-	-	+	+	+	+	+
Geotrichum candidum	-	-	-	-	+	+	+	+	+	+
Saccharomyces cerevisiae	-	-	-	-	-	+	+	+	+	+
Penicillium italicum	-	-	-	-	-	-	+	+	+	+

Table 3: Occurrence of bacterial isolates from ground uncooked Lima bean seeds during fermentation

Isolates	Days 0	1	2	3	4	5	6	7	8	9
Bacillus subtilis	+	+	+	+	+	+	+	+	+	+
Bacillus megaterium	-	-	+	+	+	+	+	+	+	+
Bacillus pumilus	+	+	+	+	+	+	+	+	-	-
Lactobacillus fermentum	-	-	-	+	+	+	+	+	+	+
Lactobacillus plantarum	-	-	-	+	+	+	+	+	+	+
Leuconostoc	-	-	-	-	-	-	-	+	+	-
Proteus vulgaris	+	+	-	-	-	-	-	-	-	-
Staphylococcus aureus	+	+	-	-	-	-	-	-	-	-
Staphylococcus saprophyticus	+	-	-	-	-	-	-	-	-	-
Aspergillus fumigatus	-	-	-	-	-	-	-	+	+	+
Aspergillus niger	-	-	-	-	-	+	+	+	-	-
Geotrichum candidum	-	-	-	-	-	+	+	+	+	-
Saccharomyces cerevisiae	-	-	-	-	-	-	+	+	+	+

Initial progressive increases were observed in the temperatures of the samples; cooked and pressure cooked samples were found to have highest values of 31.9^{o}C and 30.8^{o}C at 96th hour respectively while that of uncooked sample was 29.5^{o}C after which the temperature started decline (Figure 3).

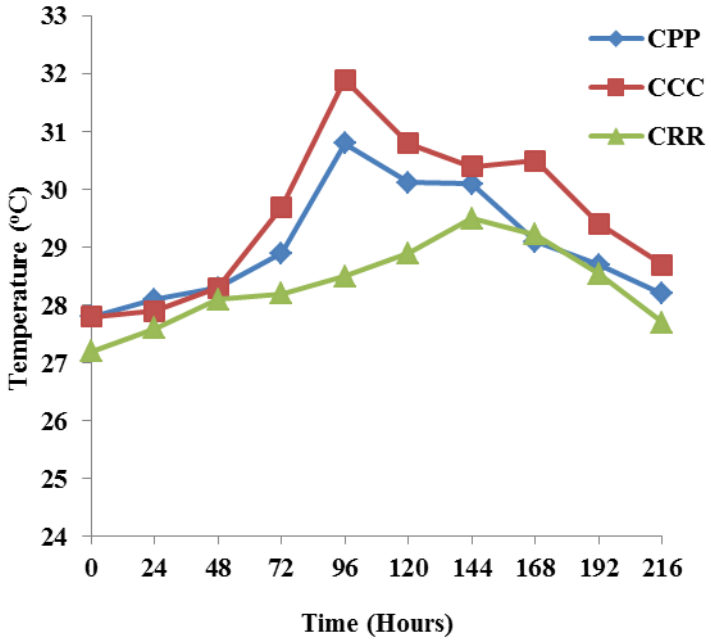

Figure 3: Changes in temperature of the fermenting ground Lima bean seeds.
CPP =Pressure cooked, CCC= Cooked, CRR = Uncooked

The highest pH value of 7.3 and 7.22 were observed in cooked and pressure cooked samples respectively after 96 hours of fermentation while uncooked sample had the highest value of 6.61 at 168 hours of fermentation (Figure 4).

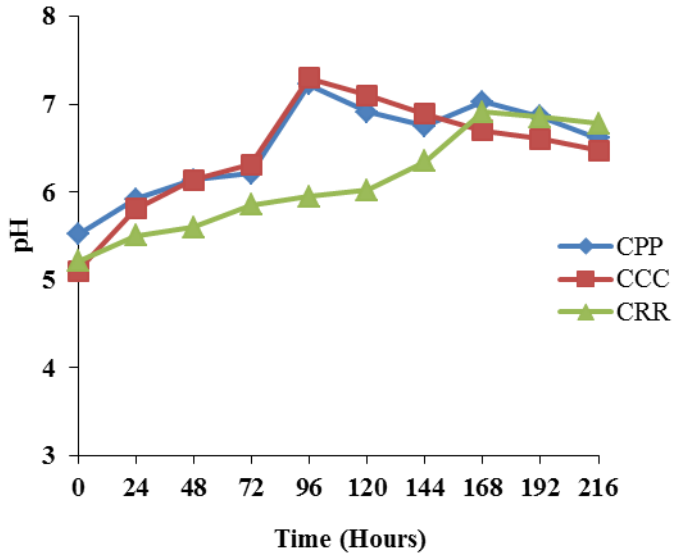

Figure 4: Changes in pH of the fermenting ground Lima bean seeds
CPP =Pressure cooked, CCC= Cooked, CRR = Uncooked

The total titratable acidities (TTA) decreased initially in all the samples. cooked and pressure cooked sample decreased to 0.21% at 96th hour while uncooked sample decreased to 0.26% at 168 hours of fermentation after which they started to increase (Figure 5).

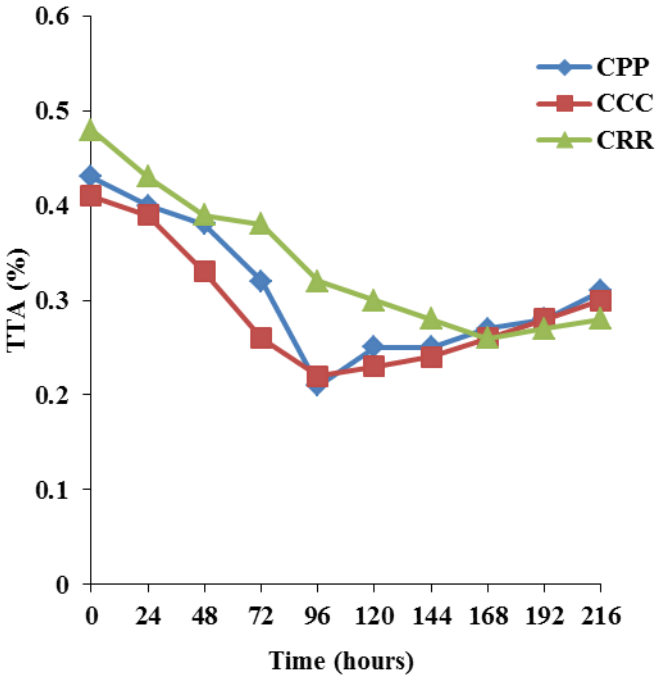

Figure 5: Changes in total titratable acidity of the fermenting ground Lima bean seeds.
CPP =Pressure cooked, CCC= Cooked, CRR = Uncooked

Moisture contents increased throughout the fermentation period. Pressure cooked sample had the highest moisture content (42.3%) closely followed by cooked sample (41.2%) while the lowest content was found in uncooked sample (34.2%). The highest protein content was obtained in each sample at the 5th day of fermentation. Cooked sample contained the highest of 27.4% which was not significantly higher than pressure cooked sample having 25.2% but significantly higher than uncooked sample which contained 21.3%. fat content increased throughout the fermentation period only in the heat treated samples with the highest from pressure cooked sample (4%) while the uncooked sample had the highest content of 3.1% at the fifth day of fermentation and reduced to 2,4% at the end of the fermentation. Fibre contents however reduced in all the sample with the highest reduction in cooked sample followed by the pressure cooked sample while slight reduction was observed in uncooked sample. Ash contents also reduced from 5.3 to 4.4%, 5,2 to 4.6% and 5.2 to 4.4% in pressure cooked, cooked and uncooked samples respectively. higher significant reductions were observed in carbohydrate

contents of pressure cooked sample (24.6%), and cooked sample (25.5%) than the uncooked sample (34.7%) after fermentation (table 5).

Figure 5: Proximate composition of the fermenting Lima beans

Sample	Moisture			Protein			Fat			Fibre			Ash			Cho		
	1	5	9	1	5	9	1	5	9	1	5	9	1	5	9	1	5	9
PP	27.3^{a}	35.3^{ab}	42.3^{a}	16.3^{b}	25.2^{ab}	21.2^{b}	1.8^{c}	3.9^{a}	4.0^{a}	4.4^{a}	3.9^{a}	3.5^{b}	5.3^{a}	4.9^{a}	4.4^{a}	44.9^{b}	26.8^{b}	24.6^{b}
CC	29.4^{a}	37.8^{a}	41.2^{a}	15.6^{b}	27.4^{a}	21.7^{a}	1.9^{b}	3.7^{a}	3.8^{b}	4.4^{a}	3.7^{a}	3.2^{c}	5.2^{a}	4.9^{a}	4.6^{a}	43.5^{b}	22.5^{b}	25.5^{b}
CR	9.8^{b}	29.4^{c}	34.2^{b}	19.3^{a}	21.3^{c}	20.2^{c}	2.1^{a}	3.1^{b}	2.4^{c}	4.4^{a}	4.1^{a}	4.1^{a}	5.2^{a}	4.5^{b}	4.4^{a}	59.2^{a}	37.6^{a}	34.7^{a}

Values with the same superscript letter(s) down a column are not statistically significantly (P>0.05) different.
PP =Pressure cooked, CC= Cooked, CR = Uncooked

Fermented ground cooked Lima bean seeds were rated best based on appearance while cooked sample was rated best based on sliminess, texture and odour of the fermented products. However, uncooked sample was scored lowest in all the parameters (Table 6).

Table 6: Physical changes of the fermented Lima bean seeds

Fermented Substrates	Appearance	Sliminess	Texture	Odour
Cooked	2.6^{a}	3.2^{a}	3.4^{a}	3.6^{a}
Pressure cooked	2.7^{a}	2.9^{ab}	3.2^{ab}	3.3^{a}
Uncooked	2.4^{a}	2.3^{c}	2.3^{c}	1.4^{c}

Values with the same superscript letter(s) down a column are not statistically significantly (P>0.05) different.

Discussion

All the genera of microorganisms identified from this research work have been implicated in the fermentation of some leguminous and other fermented food products. David and Aderibigbe (2010) isolated *Bacillus*, *Micrococcus Leuconostoc* and *Lactobacillus* species while fermenting melon seeds for *ogiri* paste. Sanni et al (2000) *B. subtilis, B. licheniformis , B. megaterium , B. pumulus* from iru, ugba and ogiri. The sources of these organisms could be from the substrates, the handlers, water used for processing, utensils used for fermenting the substrates such as calabashes, banana leaves and even from the air (Nwagu, *et al.,* 2011). The inability of pathogenic organisms to grow and disappearance of some organisms such as *Micrococcus* and *Staphylococcus* at the earlier stage could be attributed to production of antimicrobial substances produced by the predominant organisms isolated during the fermentation processes. *Bacillus subtilis* has been reported to produce antibiotic bacitracin which is inhibitory against *Micrococcus* species (British Pharmacopoeia, 1993).

The increases and subsequent declines in the microbial loads were similar to the results of David and Aderibigbe (2010). Inability of pathogenic microorganisms to grow in the substrate could be due to the fact that the predominant microorganisms in the substrate were inhibitory to these pathogens.
The initial increases in pH of Lima bean seeds during fermentation could also have contributed to the poor growth of *Lactobacillus* and fungi, after which they started to proliferate while the pH values were declining. *Lactobacillus* and fungi had been reported to be aciduric (Aderiye and Ojo, 1987). Increase in pH during fermentation of protein-rich oil seeds has been reported by several authors (Onukwo, 1992; Aderibigbe and Adebayo, 2002; David and Aderibigbe, 2010). The slimy texture observed as the fermentation proceeded was probably due to the growth of *Bacillus* species which produce slimy capsules (Ogueke and Aririatu, 2004).

The increase in the moisture content with increase in fermentation time was also observed by Omafuvbe et al. (2004) while fermenting African locust bean and melon seeds. Ekachai and Primprao (2011) attributed the increase in moisture contents to boiling in water followed by further soaking in water.

The initial progressive increases in crude protein contents observed during this research work have been reported by some authors while working on fermented legumes. Udensi and Okoronkwo (2006) observed progressive increases in % protein contents while studying the effects of fermentation and germination on the physicochemical properties of *Mucuna cochinchinensis* protein isolate flours fermented for 72 h. The increases in crude protein values observed during the fermentation could be due to the action of extracellular enzymes produced by the fermenting microorganisms. It has also been reported that *Bacillus* species implicated in some fermented legumes are important producers of extracellular proteases which could hydrolyze complex plant proteins to amino acids and short chain peptides, thereby causing an increase in total nitrogen content (Fogarty and Griffin, 1973).

The increase in fat content of Lima bean seeds during and after fermentation was in agreement with (Osman, 2007) while studying the effect of different processing methods on nutrient composition, antinutritional factors of Dolichos lablab bean (*Lablab purpureus*). This increase might be due to the fact that some microorganisms could produce microbial oil (Akindumila and Glatz, 1998). Besides, it might be due to the reports of Esenwah and Ikenebomeh (2008) and Effiong and Umoren (2011) on the protein–lipid or carbohydrate-lipid linkages. Also the higher fat contents in samples which were fermented in calabashes might also be influenced by the fermenting containers. It was also suggested that fermented foods that had higher fat contents could be as a result of fat from dead microflora or the fermenting microflora did not use fat from these foods as source of energy (Onoja and Obizoba, 2009).

The slight decreases in the ash contents of fermented Lima bean seeds was in agreement with the results obtained by Esenwah and Ikenebomeh (2008) while fermenting African locust bean seeds. Loss in ash contents may be due to leaching of soluble inorganic salts into the processing water during the fermentation period (Osman, 2007; Ogbonnaya *et al.*, 2011) or the fermenting microflora used it for their metabolism (Reebe *et al*., 2000). However, this is contrary to the results of Enujuigha (2003) who observed increase in ash from 2.1 to 2.9% dry weight within 72 hours during oil bean fermentation. Effiong and Umoren (2011) attributed the increase in ash content to the destruction of antinutritional factors which bound some of these minerals.

The total carbohydrate contents of samples also decreased significantly by after thermal treatments of the substrates. This was further decreased significantly by fermentation throughout the fermentation period. This result was in agreement with results of earlier workers (Addy *et al*., 1995; Omafuvbe *et al*., 2004; Osman, 2007). Loss in carbohydrate during soaking and boiling may be due to leaching of soluble carbohydrates like sugars into the soaking and cooking water; while loss in carbohydrate during fermentation may be as a result of the utilization of some of the sugars by fermenting organisms for growth and metabolic activities and also as a result of apparent increase in protein contents of the samples.

Conclusion

The results from this research work showed that all the dominant microorganisms isolated from all substrates during fermentation were not pathogenic. The fermentation techniques have been able to improve the protein contents significantly. Besides, the results also showed that five-day fermentation period was enough for nutritional improvements of the substrates particularly the protein contents. If well processed under hygienic and controlled conditions, fermented Lima bean seeds could be used as a protein supplement in food as well as a potential condiment in the future.

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