

Full Length Research Paper

# Biochemical and Microbiological changes during the production of fermented pigeon pea (*Cajanus cajan*) flour

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**An investigation was carried out to study the biochemical and microbiological changes that occur during fermentation of pigeon pea (*Cajanus cajan*) flour. Standard methods were employed for most of the analyses. The pH decreased from 6.8 at 0h to 4.2 at 72 hours of fermentation but increased to 6.5 at 96 hours. Three genera of *Bacillus*, *Staphylococcus* and the yeast *Saccharomyces* were found to be associated with the fermentation of pigeon pea. The nutritive quality of the fermented pigeon pea in terms of crude protein 19.43g/100g, crude fiber 3.6g/100g, and crude fat 1.4 g/100g was found to be superior to those that were not fermented which were 20.65g/100g, 5.2g/100g and 1.62g/100g respectively. Water Absorption Capacity (WAC) was in the range of 3.20g/ml to 5.0g/ml. The highest WAC was recorded for unfermented sample. However, the carbohydrate content was lower in fermented pigeon pea than the unfermented sample. In terms of organoleptic properties of the product 'moinmoin' from the fermented pigeon pea this was more preferred by a 15-man panel using parameters such as color, taste, texture and aroma than "moinmoin" from unfermented pigeon pea.**

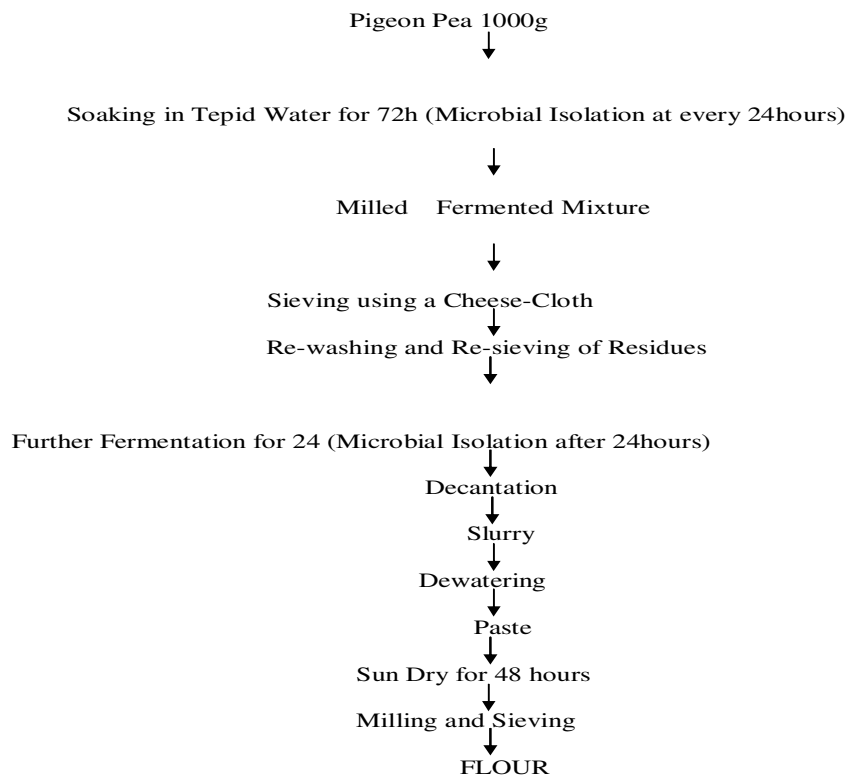
**Keywords:** Pigeon pea, fermentation, microbiological status, sensory qualities.

## INTRODUCTION

The use of increase consumption of food legumes such as *Vigna unguiculata* and *Glycine max* in campaign, had been advocated as substitutes for animal protein in Nigeria Oloyo (2004). Pigeon pea (*Cajanus cajan*) cultivated widely in Nigeria is known as 'otili' in south-west Nigeria, is one of the most drought –tolerant and widely grown legumes in tropical and sub-tropical regions. Its cultivation has been reported in more than seven countries including Nigeria (Aiyelaja and Bello, 2006, Odeny 2007). However, pigeon pea is still underutilized as food due to its tough texture, long cooking duration and lack of education on its nutritional potentials. (Fasoyiro *et al.*, 2009, Amaefule and Nwagbara, 2004, Odeny 2007) . Its consumption is occasional when the dried mature seed is cooked and consumed either alone or in combination with a starchy staple like yam.

Pigeon pea contains 20-22% protein, 1.2% fat, 65% carbohydrate and 3.8% ash(Onweluzo & Nwabugwu 2009) high levels of cysteine , methionine , and lysine (Osagie 1998) and smaller amount of oligosaccharides, than soybean (Singh 1988) .

The microorganisms in food products are diverse due to intrinsic and extrinsic factors which affect the functional properties, processing, storage and consumption (Oshodi and Ekperigin, 1989, Dullon 2004). Lactic acid spontaneous has been found to influence nutrient density and microbial and enzymatic activities of the raw material which can lead to improvement of the flavor, aroma and texture of the product. Fermentation can also increase the soluble phenolic content of legumes like pigeon pea and consequently enhance its antioxidant activities. Legume fermentation of legume in Nigeria is usually done to produce condiments like 'iru' or 'dawadawa' (Oboh *et*



**Figure 1.** Flow chart showing fermentation process of pigeon pea seeds

*al.*, 2009).

Although, remarkable improvement in the nutritive value and quality of legume seeds have been achieved through dehulling, heat treatment, germination, fermentation, Oloyo (2004). Torres *et al.*, (2006) Forster *et al.*,

(2011) Adebowale (2011) Onweluzo and Nwabugwu (2009). However not much has been done to explore the possible food products that can be derived from fermented pigeon pea. The present study was aimed at observing the effect of fermentation as it influenced the nutritional quality and microbiological status of pigeon pea and to explore the possibility of its use in the production of 'moinmoin'.

## MATERIALS AND METHODS

### Sample Preparation

Grains of pigeon pea were obtained from a local market in Ado-Ekiti, south-western Nigeria. One thousand grams (1 kilogram) of the seed were winnowed and weighed and soaked in tepid water for 3day 72h and at every 24h

of the fermentation microbial isolation was determined from the fermenting medium. After 72h the mixture was wet milled and wet sieved using a cheese cloth. The sieved mixture was rewashed and re-sieved and the shaft discarded. The sediment was left in the water for 24h for further fermentation. The supernatant was discarded, and the slurry was pressed to get water out and then dried at 60°C as shown in Figure 1. The dried mixture was milled and sieved (300mm sieve). The final flour was stored at 4°C for analyses.

### Enumeration of lactic acid bacteria

Enumeration was carried out according to the method of Abegaz (2007).

Ten grams of samples from every 24hours of fermentation were taken aseptically till end of 96 hours and homogenized in 90 ml of distilled water using Warring Blender (Philips Type HR 28151). Distilled sterile water was used for 10ml serial dilution. One ml was placed on De man, Rogosa and Sharpe (MRS) agar. The inoculated plates were incubated anaerobically for 48hours at 37°C and 48°C respectively for the enumeration of thermophillic and mesophillic Lactic Acid

Bacteria (LAB). Total viable counts of the LAB isolates were recorded as colony forming units per ml (cfu/ml) after 24 to 48 hr of incubation.

#### Enumeration of *Staphylococcus* spp.

Ten grams of samples from every 24 hours of fermentation were taken aseptically till end of 96 hours and homogenized in 90 ml of distilled water using Warring Blender (Philips Type HR 28151). Distilled sterile water was used for 10ml serial dilution. One ml was placed on Mannitol Salt Agar (MSA) (But grows best in Brain Heart Infusion Agar or Tryptone Soya Agar). The inoculated plates were incubated anaerobically for 24 hours at 30°C. Total viable counts of the isolates were recorded as colony forming units per ml (cfu/ml) after 24 hr of incubation.

#### Identification of Lactic Acid Bacteria

Isolates from plates with distinct colonies were sub cultured continuously on MRS agar till pure cultures were obtained. Pure culture colonies were examined for cell morphology, gram reaction, catalase test, nitrate reduction test, oxidase test and urease production. The suspensions of test organism were made using distilled sterile water until turbidity was obtained. Exactly 1 ml proportion of the suspension of the test organism was inoculated into vials containing the sugar powders of the various carbohydrates of the API 50 CHL kit. Growth at 10°C, 15°C and 45°C in MRS broth for 24 to 48 hours was determined. Growth in the presence of 6%, 8% and 10% NaCl was also observed in MRS broth incubated at 37°C for 24 to 48 hours. Identification by API 50 CHL (Bio Merieux, Marcy-l'Étoile, France) was carried out according to the instruction of the manufacturer.

Sterile normal saline was sprinkled on the tray on which the vials were placed to create a humid environment. Sterile liquid paraffin was used to seal the top of the vials to create an anaerobic environment and incubated at 37°C from 24 hours to 48 hours. A total of 50 representative gram positive, catalase negative, oxidase negative, urease positive, non-sporing, non-motile rods, *Coccobacillus*, cocci and tetrad were formed into clusters based on similarity in morphology, physiology and biochemical reactions.

#### Identification of *Staphylococcus* spp.

About 10 g of sample was added to 225 mL of sterile Buffered Peptone Water (BPW) and homogenized in a

(Warring Blender (Philips Type HR 28151). A 0.1 mL of the broth of the various dilutions were streaked aseptically onto sterile Blood Agar Plates (BAP) enriched with 7% heparinized sheep blood and incubated at 37°C for 24-48 h. The plates were examined for the presence of *Staphylococcus* colonies based on morphology: creamy, greyish, white or yellow colonies and haemolytic pattern on the surface of BAP. Presumed colonies were then sub-cultured on Nutrient Agar Plates (NAP) and incubated at 37°C for 48 h to get a pure culture. The pure isolates were then preserved and maintained for biochemical tests and characterization of the isolates. Biochemical tests were identified based on positive reaction to Grams staining, catalase by formation of bubbles on a drop of 3% H<sub>2</sub>O<sub>2</sub>.

*Staphylococcus* colonies identified by gram-staining and catalase tests were streaked on Mannitol Salt Agar plates, incubated at 37°C and examined after 24 h. Growth and change in the color from red to yellow colour; fermentation of mannitol by *S. aureus* causes yellow discoloration of the medium.

The tube coagulase test was performed in sterile tubes by adding 0.5 mL of selected isolates of *Staphylococcus* grown on BSA broth to 0.5 mL of citrated rabbit plasma. The tubes were incubated at 37°C along with a negative control tube containing a mixture of 0.5 ml of sterile BSA and 0.5 ml of rabbit plasma. Clotting was evaluated at 30 min intervals for the first 4 h of the test. The reaction of a loose clot to a solid clot was considered positive.

#### Sensitivity Test

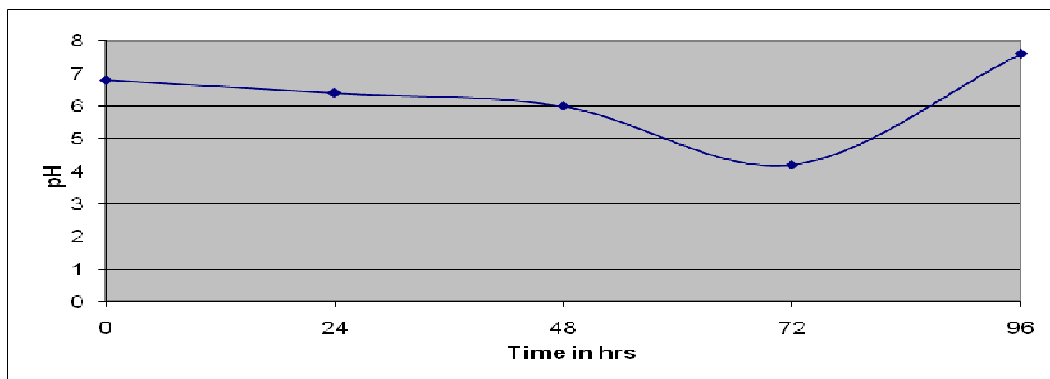
The disc diffusion method using the E-test AB (Biodisk Sona Sweden) on Mannitol Agar plus 2% NaCl was employed. The disk placed on the pure culture agar plates with inoculum densities of 0.5-1.0 ml were incubated at 35°C for 24h. Minimum Incubation concentration of ≤ 2-4mg/l was indicative of susceptibility (sensitivity)

#### Determination of pH, Total Titratable Acidity (TTA) during fermentation

pH and TTA were determined using standard methods of AOAC (2005a).

#### Proximate Analysis of fermented and unfermented pigeon pea

The chemical proximate composition in terms of crude protein, crude fibre, lipid content and calculated



**Figure 2.** The pH changes during fermentation of pigeon pea over 96 hours fermentation period

carbohydrate was carried out according to standard methods of AOAC (2005b).

#### Functional and Water absorption capacities

Water absorption capacity (WAC) was determined as described by Beuchat (1977), in which 1 gram of sample and 10 ml distilled water were added to 25 ml centrifuge tube, suspension was stirred on magnetic stirrer and allowed to stand for 30 minutes at 25°C. The volume of supernatant water on the suspension was measured. WAC was calculated as ml water.

#### Phytate and Trypsin Inhibition Activity (TIA)

Phytate content was determined using the method of Davies and Reid (1979); while TIA was determined as described by Kakade *et al.*, (1969).

#### 'Moin-moin' preparation from pigeon pea

About 25 g pepper, 25 g tomatoes, 25 g dry pepper and 10 g onion were finely blended with about 100 ml distilled water using Warring Blender (Kenwood BL335PK100/WowBL335001). The mixture was added to 500g of fermented pigeon pea (*Cajanus cajan*) flour and thoroughly homogenized with 250 ml distilled water using warring blender for 5 minutes. Fifty ml of red palm oil and 2.5 g of salt were added.

About 50 g each of the mixture was put in an aluminum tin with cover and cooked at 80°C for 45 minutes. The above procedure was repeated for unfermented pigeon pea flour.

#### Acceptability Trial

The trained panelists (25), all married women of between 35-40 years were randomly selected among the female's staff of the Federal Polytechnic, Ado-Ekiti Nigeria. They were made to abstain from eating or drinking one hour before the assessment, consisted of male and female adults that were familiar with the samples. Each panelist conducted an independent assessment in separate sensory booths of a fluorescent lit laboratory. Each panelist was provided with water to rinse his/her mouth before and after tasting each sample. A 9-point Hedonic scale was used to determine the overall acceptability of the 'moinmoin' products using: 1 = dislike extremely, 2 = dislike very much, 3 =dislike moderately, 4= dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much and 9 = like extremely as described by Ihekoronye and Ngoddy (1985).

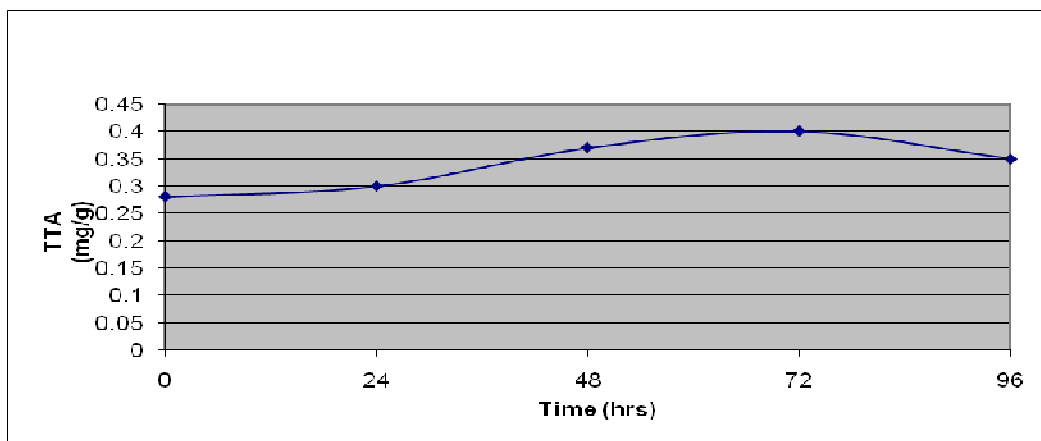
#### Statistical Analysis

The data from the chemical and sensory testing were subjected to analysis of variance (ANOVA) to determine statistical differences, while the least significant deviation (LSD) test was used to detect significant differences among the means.

## RESULTS AND DISCUSSION

#### pH and Total Titratable Acidity

There was a gradual decrease in the pH from 6.8 at 0



**Figure 3.** The total titratable acidity during fermentation of pigeon pea over 96 hours fermentation period

**Table 1.** Isolated microorganisms during 96 hours fermentation period of pigeon pea

Microorganism	Hours of Fermentation			
	0	24	48	96
<i>B. subtilis</i>	+	+	+	+
<i>B. megatarium</i>	+	+	+	+
<i>Staphylococcus Spp</i>	+	+	-	-
<i>Saccharomyces cerevisiae</i>	-	+	+	+

hour to 4.2 by 72 hours. However by the 96<sup>th</sup> hour there was an increase in pH to 7.6 (Figure.2) The Total Titrable Acidity (TTA), on the other hand increased slightly from an initial 0.21% to 0.40% by 72 hours however by the 96 hour the TTA (Figure. 2) decreased again to 0.35%. The increase in the pH at 96 hour of fermentation and decrease in the TTA might probably be due to the presence of some bacteria like *Bacillus megatarium* which have the ability to produce enzymes like proteases, that breakdown protein amino acid. Thus, the medium become alkaline due to the presence of alkaline amino acids.

### Microbial Isolation

In Table 1, the evolution of isolated microorganisms during the fermentation of pigeon pea is presented. As it can be observed *B. megatarium* disappeared after 48 hours. The disappearance may be due to its inability to survive the modified acidic environment after about 48 hour of fermentation. *Bacillus megatarium* is a proteolytic microbe that can utilize protein during its metabolic activities to release amino acids which might neutralize the acidic fermenting medium to become alkaline. The

presence of nitrogenous source from pigeon pea might have contributed to the survival of *B. megatarium* up to 48 hours of fermentation this is in agreement with the finding of Sabra and Abou-Zeid (2008) who reported that addition of nitrogenous source may enhance biomass yield of *B. megatarium*. The isolated bacteria species: *B. subtilis*, *B. megatarium*, *Staphylococcus spp.* and the yeast *Saccharomyces cerevisiae* were found to be associated with pigeon pea fermentation. This finding is in agreement with the findings of Okafor (1975, Moktam and Sarkar (2010). LAB which dominated the early stage of fermentation might have done so because of its rapid growth rate, dominance was followed by yeasts. The production of acetic acids by heterofermentative LAB during fermentation must have inhibited Gram-negative bacteria most of which are pathogenic. LAB dominance was followed by yeasts; this finding agrees with the report of Holzapfel (2002 and Moktam and Sarkar (2010). The presence of yeast strain (*S. cerevisiae*) might lead to degradation of starch rendering it available to the lactic acid bacteria for utilization thus enhancing the rapid growth of LAB throughout the fermentation period. As can be seen in Table 2, also some bacterial strains have the ability to hydrolyze starch.

Food-borne diseases are of major concern, worldwide.

**Table 2.** Colony morphology and biochemical characteristics of bacteria spp. isolated from fermented pigeon pea flour

CM	CC	M T	E S	C T	IT	M R	VP T	N R	O T	S H	G L	AR	F R	L A	MA	MT	S U	M N	Probable Identity
Large, flat, whitish Colony With entire edges	G+ve rods	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	-	+	<i>B. subtilis</i>
Dull, thick and wrinkled surface	G+ve rods	+	+	-	+	-	+	+	+	+	+	+	-	-	+	+	+	+	<i>B. megatarium</i>
Smooth growth with entire edges, whitish yellow in colour	G+ve Cocci (bunches)	-	-	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	<i>Staphylococcus</i> Spp
Cream colour and fluffy	Ovoid shape										+A & G	+	+	-	+A & G	+	+	+	<i>S.cerevisae</i>

Key: += positive, - =Negative, +A&G = positive to acid and gas, AR= Arabinose, GL= glucose, FR=Fructose, LA= lactose, MA = Maltose, MT= Mannitol, SU= sucrose, MN= Manose, CM= colony morphology, CC= cell characteristics, MT=Motility test, ES= Endospore staining, CT= Catalase test, IT= Indole test, MR= Methyl Red, VPT= Voges Prosteur test, NR= Nitrate Reduction, OT= Oxidase test, SH= Starch Hydrolysis

*Staphylococcus* species are prevalent food-borne bacterial pathogens that cause food poisoning in humans when ingested in contaminated foods. They cause Staphylococcus food poisoning by toxin production (Salandra et al., 2008). Even though, this species grow in large numbers in the environment and in nasal membrane and skin; the absence of *Staphylococcus* spp. in the first 24hour of the fermentation may be because it was initially present in insufficient numbers. On the other hand, the disappearance of a *Bacillus megatarium* may be due to its inability to survive the modified acidic environment after about 48 hour of fermentation. However, *Bacillus subtilis* was able to withstand the various acidic and alkaline conditions associated with this fermentation process. The isolation of *B. subtilis* in this study is in agreement with observations of Adebayo – Tayo et al., (2009) who reported the isolation of *B. subtilis* among other microorganism during fermentation of pigeon pea.

### Biochemical Characteristics of Isolates

This study showed that the two *Bacillus* species and one yeast isolate, *Saccharomyces cerevisiae* might have broken down lactose thus its negative reaction to lactose (Table 2); this might be due to the fact that *B.subtilis* utilizes nitrate during anaerobic fermentation to give lactate acetate as the major anaerobic fermentation products. Also *B. subtilis* which has two distinct nitrogen reductase also reduced nitrate this agrees with the finding of Cruz et al.,(2000). *B. megatarium* is said to contribute to flavor of fermented products (Dullon 2004).

Coagulase production was described as one of the most reliable criteria for the identification of pathogenic *Staphylococcus* species. The results in this study showed that coagulase positive Staphylococcus (CPS) species occurred in the fermenting medium. This may be due to lack of hygienic measures in the, preparation, of the fermented complementary food. Also, the method of their

**Table 4.** Chemical proximate composition of fermented and unfermented pigeon pea flour (g/100g).

Parameters (%)	Fermented Pigeon pea (%)	Unfermented Pigeon pea (%)
Moisture	10.0	10.8
Ash	3.4	3.6
Crude protein (N x 6.25)	19.43	20.65
Crude fiber	3.6	5.2
Crude fat	1.14	1.62
Carbohydrate (by difference)	62.15	58.13

**Table 5.** Antinutritional factors in fermented and unfermented pigeon pea (mg/100g).

Parameter	Unfermented pigeon pea	Fermented pigeon pea
Tannin	2.0	1.20
Phytate	0.013	0.006
CNmg/g	3.05	2.48
TIA	8.62	4.18

Key: CN = Cyanide

sale is entirely based on tradition. Because CPS species are a part of the normal teat skin flora and mucosa of humans and animals, some species are also found free-living in the environment.

#### Proximate Chemical Composition of fermented and unfermented pigeon pea

The proximate chemical composition of the samples showed that fermentation process helped to enhance the nutritive value of pigeon pea. The value of crude protein,

ash, crude fiber, crude fat, of the unfermented sample were 19.43%, 3.4%, 3.6%, 1.42% respectively, while the fermented sample had protein, ash crude fiber, crude fat value of 20.65%, 3.6%, 5.2% and 1.62% respectively.

The crude protein of fermented sample was comparable to the unfermented but crude fiber was significantly ( $p < 0.05$ ) reduced in fermented compared to unfermented samples. Ash content of both samples was comparable and these values were also comparable to that reported for unfermented seed by Akande *et al.*, (2010). The moisture content, crude protein and ash of fermented pigeon pea had comparable values with the finding of Onwelezo and Nwabugwu (2009), but this study recorded higher crude fiber, ash and crude fat values. For the unfermented the moisture content, crude fiber were comparable to the finding of Onwelezo and Nwabugwu (2009) but this study observed lower values of fat and

higher values of crude fiber while the ash was significantly higher ( $p < 0.05$ ) than that of Onwelezo and Nwabugwu (2009).

The slight decrease of crude protein in fermented sample might be due to possible metabolic utilization by microorganisms that hydrolyze protein to release amino acids needed for new synthesis. The crude protein in this study was however higher than that reported by Oboh *et al.*, (2006) and lower than the value reported by Adebawale and Maliki (2011) while fat, ash and crude fiber were significantly ( $p > 0.05$ ) lower. Decrease in fat content might be due to the presence of lipolytic enzymes that could have hydrolyzed fat to glycerol and fatty acids. The reduced fiber content in this study agrees with Torres *et al.*, (2006). The decrease in carbohydrate in this study might be as a result of breakdown of carbohydrate by fermenting microorganisms which might have also resulted to loss in dry matter and volatiles due to hydrolysis of carbohydrate to simple sugars. The moisture content was also higher than that reported by Akande *et al.*, (2010).

#### Antinutritional factors

The effect of fermentation on the antinutritional factors of pigeon pea is shown in Table 5; soaking during fermentation process activates endogenous phytases in legumes which decrease trypsin inhibitor activity (TIA) and this might be responsible for the decreased TIA

**Table 6.** Functional properties of fermented and unfermented pigeon pea

Parameter	Unfermented pigeon pea	Fermented pigeon pea
WAC	5.0	3.20
Least Gelation	4.2	8.0

**Table 7.** Showing The Average Scores of Sensory Evaluation of 'moin-moin' from unfermented and fermented of pigeon pea by 25 member panel

Sample	Colour	Texture	Taste	Aroma
A	2.69	2.82	2.86	2.69
B	0.628	2.82	1.60	2.00
STD	±0.4733	±0.467	±1.4198	±0.1069

**Key:** Sample A = 'Moin-moin' from fermented pigeon pea, Sample B = 'Moinmoin' from unfermented pigeon pea.

value in fermented sample. Increase towards alkaline condition between 72-96 hours might explain a decrease in ANF factors and breakdown in pigeon pea compounds (Holzapfel 2002).

### Functional Properties

The soaking involved during fermentation process must have contributed to the lower value of Water Absorption Activity (WAC) in fermented sample when compared to

unfermented sample this is contrary to the finding of Fasoyiro *et al.*, (2010) which stated that fermented pigeon pea had a higher WAC value compared to its unfermented analogue.

### Sensory Attributes of "moinmoin"

The hedonic mean scores of the fermented and unfermented samples are presented in Table 7. The mean score generally ranged between 0.6276 and 2.8632 for the sensory attributes tested, including aroma, texture, taste and color. The 'moinmoin' made from fermented pigeon pea was observed to have higher mean hedonic scores in terms of all the attributes while lower scores were recorded for the 'moinmoin' made from unfermented pigeon pea. *B. megaterium* and yeasts might have contributed to the flavor of fermented foods (Erlinda and Vigilio 2011).

The relative importance of fermentation in enhancing the nutritional value of legume is evident in this study. LAB influences flavor of fermented foods by production of volatile flavor components or by reducing activity or inactivating enzymes which might lead to production of

flavor. *B. megaterium* and *Saccharomyces cerevisiae* isolated in this study have been reported to contribute to flavor of fermented foods. Fasoyiro *et al.*, (2010) reported that pigeon pea 'moinmoin' was acceptable in terms of flavor, mouth-feel and overall acceptability compared with cowpea 'moinmoin'.

### CONCLUSION

Fermentation increased protein, and crude fiber and significantly ( $p > 0.5$ ) reduced crude fat of pigeon pea. The process also reduced anti nutritional factors of pigeon pea. *Bacillus* spp. and *Saccharomyces cerevisiae* were isolated at the end of 96 hours of fermentation. 'Moinmoin' prepared from fermented pigeon pea were favorably accepted compared to 'moinmoin' prepared from unfermented pigeon pea. The improvement of the protein value of the pigeon pea (*Cajanus cajan*) flour suggest it can be used as a potential source to fortify the protein content of some local staples like cereals and tuber flours. The consumption of products like 'moinmoin' derived from fermented pigeon pea should be encouraged to ameliorate the problem of protein energy malnutrition in developing countries like Nigeria.

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