Studies on Bio-Deterioration, Aflatoxin Contamination and Nutritive Values of Processed Cashew (*Anacardium occidentale* L) Nuts during Storage

Opeyemi Uwangbaoje Lawal [1], Emmanuel Dayo Fagbohun[2]

[1]Department of Biological Science, Evangel University, Akaeze, P. M. B. 129, Abakaliki, Ebonyi State, Nigeria
[2]Department of Microbiology, Ekiti State University, P.M.B 5363, Ado Ekiti, Ekiti State, Nigeria
opeyemil@ymail.com

Abstract: The bio-deterioration of processed cashew nuts by aflatoxin producing mycobiota was investigated during twenty weeks of storage. Eight fungal species belonging to five genera were isolated using direct and washing method of isolation. They include *Absidia corymbifera*, *Aspergillus flavus, A. glaucus, A. niger, Fusarium* sp., *Mucor* sp., *Neurospora* sp. and *Penicillium* sp. The fugal species was observed to increase with increased storage time. The proximate analysis results showed that ash content, moisture, fat and fibre were found to decrease while the crude protein and carbohydrate content was found to increase during the storage period. The mineral analysis results showed that all the minerals analysed were depleted during the period of storage. Out of the three *Aspergillus* spp. that was isolated, only *Aspergillus flavus* was able to produce Aflatoxins B1 and B2 (200µg/L culture medium). This study however showed that the mycobiota of stored cashew nuts depleted the nutritive value of the nuts and also made it unsafe for human consumption as a result of the aflatoxins produced which is beyond the permissive level. Moreover, this can be prevented if there is adequate monitoring of fungal contaminations and mycotoxins in processed, packaged and stored cashew nuts which will help to establish a standard and also serve as quality control check.

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1. Introduction

Cashew (*Anacardium occidentale* L) is a small to medium sized tree belonging to the family Anacardiaceae (Suleiman, 2010) widely grown in tropical climates for the nutritional values of its nuts and apples (Morton, 2006). The seed which is a source of cashew nuts is a kidney shaped achene about 3cm long with a hard green pericarp which are removed by a process that involves burning off the shell oil and cook the seeds (Adebajo and Diyaolu, 2003). The plant is a native to northeastern Brazil. Its English name was derived from the Portuguese name for the fruit of the cashew tree, caju, which in turn was derived from the indigenous Tupi name, acaju (Morton, 2006).

Globally, cashew nuts are well appreciated and highly priced food delicacy because of their pleasant taste and flavour. The post-harvest processing and packaging have been commercialized and modern technology adopted in major producing countries like India and Tanzania (Adebajo and Diyaolu. 2003). In recent times, Nigeria has emerged as one of the leading producer of cashew nuts in Africa and among the ten leading producer in the world (FAO, 2007).

Cashew nuts are good source of proteins, carbohydrates and fats (Bhattacharjee *et al.,* 2003a). In addition, they are rich source of minerals such as manganese, potassium, copper, iron, magnesium, zinc and selenium which reduces the risk of heart related diseases, diabetes and obesity (Khan *et al.,* 2005; FAO, 2007). Also, they are rich in many essential vitamins such as pantothenic acid, pyridoxime, riboflavin and thiamin which the body needs to replenish nutrients and carry our normal body metabolisms (Suleiman, 2010).

Due to these high nutritional values, cashew nuts are subjected to microbial contamination during post harvest and processing. In Nigeria however, despite the establishment of cashew processing factories, peasant processing and packaging methods are still commonly used especially by the small scale producers. (Adebajo and Diyaolu, 2003; Suleiman, 2010). Several fungal species have been reported to attack cashew nuts being able to feed and multiply on the product during storage (Bhattacharjee *et al.,* 2003b) while some of these fungal species have been reported to produce aflatoxins (Suleiman, 2010). Aflatoxins are a group of secondary metabolites produced mainly by Aspergilli. They are carcinogenic and are capable of interfering with the proper functioning of the central nervous system (Jonathan *et al.,* 2012).

With regards to the total aflatoxins, the lowest European Union limit other than food for infants is 4.0µg/kg for products such as groundnuts, cashew nuts and tree nuts, dried fruit and its processed products, cereals and products derived from cereals, including processed cereal products (EU, 2006; Alhussaini 2012). While the highest limit is set for groundnuts, cashew nuts, almonds and pistachios at 15.0µg/kg. The fungal species that produce aflatoxin can infiltrate the hard outer shell to infest the nut without surface mouldness. The only way of ensuring that aflatoxin cannot be produced is to strictly adhere to the proper measures for collecting, storage and transport, as well as adopting a careful and hygienic method of processing (EU, 2006; Alhussaini 2012).

However, this paper is a report on the bio-deterioration of stored processed cashew nut samples and the role of the fungal isolates in production of aflatoxins and nutrient depletion.

2. Materials and Method

Collection of Samples

Six hundred pieces of raw cashew nuts were obtained from Ado Ekiti and were processed. The cashew nuts were spread in a tray and sundried for three months. They were roasted, put aseptically in a sterile glass bottle to prevent cross contamination and were stored for five months. Monthly examination for changes in the mycoflora, nutrient composition and aflatoxins production were carried out.

Isolation of Fungi from the Stored Cashew Nuts

The two methods used for the isolation of fungal species in this study were direct plating and washing method.

Direct Plating Method

10 pieces of the cashew nuts were randomly picked and examined for external mouldness. A sterile dissecting forceps was used to scrap the surface of the stored cashew nuts plated aseptically on Potato Dextrose Agar (PDA) plate and incubated at 28^{o}C for 7 days as described by Arotupin and Akinyosoye (2001). The pure cultures obtained were examined under the microscope for fruiting bodies, hyphae to determine the fungi present.

Washing Method

This was carried out by weighing 10g of randomly picked stored cashew nuts into 100 ml of sterile distilled water in a beaker. This was shaken thoroughly and drops of suspension of contaminated water were introduced into Potato Dextrose Agar plates. Sterile glass spreader was used to evenly spread the suspension on agar plate. The plates were incubated at 28^{o}C for 7 days and were observe for visible fungi growth.

Identification of Fungal Species

The associated fungi were identified by their macroscopic and microscopic features. The isolates were examined under bright daylight for the colour of the culture and further examination was carried out (Alexopolous *et al.,* 1996; Dungan, 2006).

Needle Mount Preparation Method

The method described by Fagbohun *et al.,* (2011) was used whereby fragment of the sporing surface of the initial culture was taken between the centre and the edge of the colony. This was teased out in drop of alcohol on a sterilized glass slide using a botany needle. The fragments were stained by adding a drop of lactophenol blue. A cover slip was applied and the preparation was examined under X10 and X40 objective lens of the microscope.

Slide Culture Technique

From a plate approximately 2 mm deep, 1 cm^2 PDA was cut and placed on a sterile glass slide. Fungus was inoculated into the four vertical sides using a sterile needle. A sterile cover slip was placed on it so that it over lapped the medium on all sides. The preparation was placed on a suitable support in a Petri dish containing blotting paper soaked in 20% glycerol in water. The preparation was kept moist at 28°C until adequate growth was observed. After removing the medium with scalpel, the fungus adhering to both cover slip and slide was examined (Crowley *et al.,* 1969). A drop of alcohol was added followed by a drop of lactophenol blue and the preparation was covered and examined under the low power objective of microscope.

Extraction of Aflatoxins

This was carried out according to the methods recommended by AOAC (1984) and described by Alhussaini (2012). The fungal isolates belonging to the genus *Aspergillus* (*A. flavus, A. glaucus* and *A. niger*) were cultivated on Potato dextrose broth at 28^{o}C for 7days under stationary conditions. For each fungal isolate, the mycelium suspension was homogenized using liquid nitrogen and remixed with the broth medium in the flask. Mycotoxin extraction was done using chloroform: water (10:1 v/v) mixture. The obtained crude extracts were purified by column chromatography containing anhydrous sodium sulphate (15g) and silica gel (10g). Extracts were air dried and kept in dark vials till chromatographic analysis.

Qualitative Estimation of Aflatoxins

This was carried out according to the methods recommended by AOAC (1984) and described by Alhussaini (2012). Precoated silica gel plates were used. Rectangular glass jar was used for developing chromatoplates. A suitable volume of solvent mixture (chloroform: methanol, 97:3 v/v) was placed in the bottom of the jar so that the starting spots on the plates would be 1 cm above the upper surface of the

solvent mixture. The chromatographic plates were activated by heating 1hr at 120°C in a hot air oven, and removed immediately to a desiccator to cool. Parallel starting spots, 2 cm from each side of the plate and 1.5 cm apart, were made with micropipets from chloroform extracts with reference aflatoxins. Spots were left to air dry. Prepared plates were then transferred to the chromatographic jar, developed to a suitable distance (10 cm), and removed. The solvent front was marked and the plates were air dry. Spots were viewed under UV light (366 nm) and the outline of each fluorescent spots was marked by sharp pin. Retention factor (Rf) values, colors, and intensities of the spots were compared with reference mycotoxins (El-Bazza *et al.,* 1982).

Quantitative Determination of Aflatoxins

The dilution-to-extinction (Coomes *et al.,* 1965) and comparison of standards (AOAC, 1984) techniques were used for estimation of aflatoxins concentrations (Alhussaini, 2012).

Proximate Analysis

The proximate analysis of the samples for moisture, ash, fibre and fat were done by the method of AOAC (2005). The nitrogen was determined by micro-Kjeldahl method as described by Pearson (1976) and the percentage nitrogen was converted to crude protein by multiplying 6.25. All determinations were performed in triplicates.

Mineral Analysis

The mineral was analyzed by dry ashing the samples at 550°C to constant weight and dissolving the ash in volumetric flask using distilled water, deionized water with a few drop of concentrated HCl. Sodium and potassium were determined by using a flame photometer (Model 405 Corning, UK) with NaCl and KCl standards. Phosphorus was determined colometrically using Spectronic 20 (Gallenkap, UK) as described by Pearson (1976) with KH_2PO4 as standard. All other metals were determined by atomic absorption spectrophotometer (Pekin-Elmar Model 403, Norwalk CT, USA). All determinations were done in triplicates. All chemicals used were analytical grade (BDH, London). Earlier, the detection limit of the metals has been determined according to Tuite (1961). The optimum analytical range was 0.1 to 0.5 absorbance unit with a coefficient of variation of 0.87 to 2.20%. All the proximate values were reported as percentage while the minerals were reported as mg/100g.

Statistical Analysis

Statistical analysis was carried out according to the method of Oloyo (2001) to determine the mean, standard deviation and coefficient of variation.

3. Results and Discussion

Mycobiota of processed cashew nuts during storage

The mycological analysis of the stored cashew nuts using the two methods of isolation revealed the isolation of 8 fungal species belonging to 5 genera as shown on tables 1, 2 and 3.

The fungal species include *Absidia corymbifera, Aspergillus flavus, Aspergillus glaucus, Aspergillus niger, Mucor* sp., *Neurospora crassa* and *Penicillium* sp. The direct plating method was used to isolate the fungal contaminant on the surface while the washing method was meant to isolate both the external and internal fungal contaminants (Amusa *et al.,* 2002). The index week of storage, it was observed that three fungal species belonging to three genera were isolated from the two methods used in this study. These fungal species (*Absidia corymbifera, Mucor* sp., and *Penicillium* sp) may have been introduced from the environment during the processing stage before storage. Moreso, at the fourth week of storage, the fungal species increased to five species (*Absidia corymbifera, A. flavus, A. niger*, *Mucor* sp. and *Penicillium* sp.) while at eighth and twelfth week the fungal species increased to six with the appearance of *Aspergillus glaucus* in addition to the previously occurred species.

At sixteenth week, the fungal species increased to seven with the appearance of *Fusarium* sp. in addition to others while at twentieth week, the fungal species increased to eight with the appearance of *Neurospora crassa* with direct plating method. The washing method on the other hand had eight fungal species at sixteenth and twentieth week with the appearance of *Fusarium* sp. and *Neurospora crassa.* Summarily, the occurrence of fungal species increased with increase in the storage period.

The result of this study is similar to the findings of Adebajo and Diyaolu (2003) who investigated the mycology and spoilage of retail cashew nuts in Lagos, Nigeria and reported that *A. niger, A. flavus, A. fumigatus, A. restrictus, A.* spp., *Penicillium* sp., *Mucor* sp. and *Rhizopus* sp. are associated with the biodeterioration of cashew nuts. Moreover, Freire and Kozakiewicz (2005) also investigated the mycobiota of cashew kernels and found that members of *Aspergillus* and *Penicillium* were dominant. He further reported the isolation of *Aspergillus clavatus*, *A.flavus, A. parasiticus, A. ochraceus, A. ustus, Penicillium citrinum* and *P. oxalicum. Similarly,* Suleiman (2010) investigated the occurrence and distribution of fungi associated with biodeterioration of cashew in storehouse located in Kogi state, Nigeria and reported the isolation *Aspergillus niger* as one of the fungal species causing bio-deterioration. Also, Alhussaini (2012) also reported the isolation of *A. flavus, A. niger, A. fumigatus, Penicillium* sp. from cashew nuts in Saudi Arabia.

Nutritional Changes of Cashew Nuts during Storage

Proximate analysis

The results of the proximate analysis of the cashew nuts during the twenty weeks of storage in g/100g are shown on table 4.

The ash content was observed to be decreased from 3.34 before storage to 2.51 at twentieth week of storage. The ash content is an indication of the mineral content of the sample (Pearson, 1976). This finding is similar to the work of Fagbohun and Faleye (2012) evaluated the nutritional and mycoflora changes during storage of groundnut (*Arachis hypogea*) and found that the fat content decreased from 2.78 in freshly processed to 2.64 at twentieth week of storage. The decrease in this ash content may be due to the infecting fungi that have used part of the mineral content of the cashew nuts for growth and metabolism (Onifade and Agboola, 2003).

The moisture content of the cashew nuts was observed to decrease during the period of storage from 5.44 before storage to 4.59 at twentieth week of storage. The shelf life of a product is influenced by the amount of water present in it. The decrease in the moisture content observed in this study could be attributed to the infecting fungi that utilize moisture for survival.

The fat content of the cashew nuts decreased from 48.19 in freshly processed cashew nuts to 42.67 in cashew nuts stored for twenty weeks. The decrease in fat content of cashew nuts may be due to the lipolytic activities of the storage fungi as a result of the production of lipolytic enzymes to metabolise and deplete the fat content of the stored cashew nuts (Onifade and Jeff- Agboola, 2003).

The crude fibre content also decreased from 1.00 in freshly prepared cashew nuts to 0.59 in samples at twentieth week of storage. This is similar to the findings of Fagbohun and Faleye (2012) that investigated the nutritional changes and mycoflora of groundnut (*Arachis hypogea*) during storage and reported the decrease in crude fibre from 9.82 in freshly processed groundnuts to 8.92 in samples at twentieth week of storage. Crude fibre have been reported by Amadioha (1998) to provide a distinction between the most digestible and least digestible carbohydrate and its importance in aiding peristalsis and reduce the risk of intestinal disorder. The crude protein of cashew nuts increased during the storage period from 21.15 in freshly processed to 22.65 in samples at twentieth week of storage. Protein is second largest chemical component of cashew nuts (Suleiman, 2010). The carbohydrate content was found to increase from 20.89 in freshly prepared to 26.94 in samples at twentieth week of storage. This is also in agreement to the findings of Fagbohun and Faleye (2012) that reported an increase in the carbohydrate content of groundnuts from 5.0 in freshly prepared to 5.53 in samples stored for twenty weeks. However, this is in contrast to the findings of Jonathan *et al.,* (2012) who investigated the nutritive value of sweet potato chips over nine months of storage and reported that the carbohydrates content decreased from 91.0 in freshly prepared to 9.9 after storage. Carbohydrates are the main source of energy and provide ideal fuel for the body to function properly.

Mineral analysis

The results of the mineral analysis of the cashew nuts during the twenty weeks of storage in mg/100g are shown on table 5.

It was observed from this study that all the minerals analysed (Sodium, Potassium, Calcium, Magnesium, Zinc, Iron, Copper, Manganese, Lead and Phosphorus) were found to be depleted with increase in storage period. This however is in agreement to the work of Fagbohun and Faleye (2012) who reported the depletion of minerals in groundnut during twenty weeks of storage. It is noteworthy that Lead (Pb) was detected in freshly processed cashew nuts though at minute quantity (0.01) which is within the permissible level for consumption.

Aflatoxins produced by fungi isolated from cashew nuts during storage

The results of the aflatoxin production by *Aspergillus flavus, Aspergillus glaucus* and *Aspergillus niger* are shown on table 6.

It was observed that only *Aspergillus flavus* produce aflatoxins B1 and Aflatoxin B2. Alhussaini (2012) studied the mycobiota and mycotoxins of nuts and some dried fruits from Saudi Arabia and reported the detection of ochratoxin A produced by *Aspergillus aculeatus* in cashew nuts. He also reported the detection of Aflatoxins B1, B2, G1, and G2 in other nuts and dried fruit investigated.

Conclusion

This study showed that cashew nuts are susceptible to fungal deterioration and possibly aflatoxin contamination especially during storage. This contamination could be due to processing and packaging under non-hygienic conditions, long-term storage in poor environmental conditions including high moisture and temperature. This can be prevented if there is adequate monitoring of fungal contaminations and mycotoxins in processed, packaged and stored cashew nuts which will help to establish a standard and also serve as quality control check.

Table 1: Fungi isolated from cashew nuts during twenty weeks of storage using direct plating method

Fungal species	Storage period (weeks)					
	0	4	8	12	16	20
Absidia corymbifera	+	+	+	+	+	+
Aspergillus flavus	-	+	+	+	+	+
Aspergillus glaucus	-	-	+	+	+	+
Aspergillus niger	-	+	+	+	+	+
Fusarium sp.	-	-	-	-	+	+
Mucor sp.	+	+	+	+	+	+
Neurospora crassa	-	-	-	-	-	+
Penicillium sp.	+	+	+	+	+	+

Legend: + = Present, - = Absent

Table 2: Fungi isolated from cashew nuts during twenty weeks of storage using washing method

Fungal species	Storage period (weeks)					
	0	4	8	12	16	20
Absidia corymbifera	+	+	+	+	+	+
Aspergillus flavus	-	+	+	+	+	+
Aspergillus glaucus	-	-	+	+	+	+
Aspergillus niger	-	+	+	+	+	+
Fusarium sp.	-	-	-	-	+	+
Mucor sp.	+	+	+	+	+	+
Neurospora crassa	-	-	-	-	+	+
Penicillium sp.	+	+	+	+	+	+

Legend: + = Present, - = Absent

Table 3: Summary of the fungi isolated from cashew nuts during twenty weeks of storage using different methods

Fungal species	Storage period (weeks)											
	0		4		8		12		16		20	
	A	B	A	B	A	B	A	B	A	B	A	B
Absidia corymbifera	+	+	+	+	+	+	+	+	+	+	+	+
Aspergillus flavus	-	-	+	+	+	+	+	+	+	+	+	+
Aspergillus glaucus	-	-	-	-	+	+	+	+	+	+	+	+
Aspergillus niger	-	-	+	+	+	+	+	+	+	+	+	+
Fusarium sp.	-	-	-	-	-	-	-	-	+	+	+	+
Mucor sp.	+	+	+	+	+	+	+	+	+	+	+	+
Neurospora crassa	-	-	-	-	-	-	-	-	-	+	+	-
Penicillium sp.	+	+	+	+	+	+	+	+	+	+	+	+

Legend: A = Direct plating method, B = Washing method, + = Present, - = Absent

Table 4: Summary of the results of proximate analysis of cashew nuts during twenty weeks of storage in g/100g

Storage period (weeks)	Ash	MC	CP	Fat	Fibre	CHO
Freshly prepared	3.34	5.44	21.15	48.19	1.00	20.89
4	3.92	6.09	22.15	47.70	0.77	19.38
8	3.05	7.11	22.35	45.94	0.91	20.65
12	2.86	4.81	22.76	43.16	0.84	25.59
16	2.81	4.66	22.79	43.03	0.81	25.91
20	2.51	4.59	22.65	42.67	0.59	26.94
OM	3.08	5.45	22.30	45.11	0.82	23.22
SD	0.49	0.99	0.61	2.48	0.13	3.27
CV	15.90	18.16	2.73	5.49	15.85	14.08

Legend: MC = Moisture content, CP = Crude protein, CHO = Carbohydrate, OM = Overall mean, SD = Standard deviation, CV = Coefficient of variation

Table 5: Summary of the results of mineral analysis of cashew nuts during twenty weeks of storage in mg/100g

Storage period (weeks)	Na	K	Ca	Mg	Zn	Fe	Cu	Mn	Pb	P
Freshly prepared	33.48	14.95	37.54	28.65	31.48	21.43	0.05	21.54	0.01	13.55
4	28.45	15.33	35.41	29.43	27.40	20.34	0.03	21.22	ND	12.11
8	29.58	13.63	35.84	27.54	30.12	20.41	0.02	20.35	ND	11.63
12	25.67	12.58	33.49	28.21	27.84	19.50	0.01	21.22	ND	10.42
16	25.59	12.46	33.25	28.50	26.75	19.45	0.01	20.76	ND	10.28
20	25.45	12.16	32.68	27.75	25.48	18.58	ND	19.55	ND	10.22
OM	28.03	13.51	34.70	28.34	28.17	19.95	0.02	20.77	0.00	11.36
SD	3.17	1.35	1.87	0.68	2.22	0.98	0.01	0.72	0.00	1.32
CV	11.30	9.99	5.38	2.39	7.88	4.91	50.00	3.46	0.00	11.61

Legend: Na = Sodium, K = Potassium, Ca = Calcium, Mg = Magnesium, Zn = Zinc, Fe = Iron, Cu = Copper, Mn = Manganese, Pb = Lead, P = Phosphorus, ND = Not detected, OM = Overall mean, SD = Standard deviation, CV = Coefficient of variation

Table 6: Aflatoxins produced by fungi isolated from cashew nuts during storage

Fungal species	Aflatoxin detected	Approximate concentration (µg/L) medium
A. flavus	Aflatoxin B1	200
	Aflatoxin B2	200
A. glaucus	-	-
A. niger	-	-

Corresponding Author:
Opeyemi Uwangbaoje LAWAL
Department of Biological Science
Evangel University, Akaeze.
Ebonyi State, Nigeria.
E-mail: opeyemil@ymail.com

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