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TRENDS IN TOTAL AFLATOXINS AND NUTRITIONAL IMPACT OF TRITICUM SPP DURING FERMENTATION OF KUNUN-ZAKI; A NIGERIA SORGHUM BICOLOR BASED NON-ALCOHOLIC BEVERAGE.

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ABSTRACT

Moulds secondary metabolites such as aflatoxins are carcinogenic when consumed in food and feed. The aim of this research was to assess total aflatoxin decontamination from contaminated wheat-sorghum composite used for ``kunun-zaki`` production. The cereal composite; wholesome *Triticum* spp and *Sorghum bicolor* were blended to a different percentage ratio (5, 7.5 and 10 %). Naturally aflatoxin contaminated cereals were also blended with wholesome *Sorghum bicolor* to 1,2 and 3%. The various blends were batch fermented at ambient temperature in open fermentors to produce Kunun-zaki. Enzyme Linked immunosorbent assay (ELISA) was used to detect and quantify total aflatoxins. Increasing the percentage of naturally aflatoxin contaminated cereal correlated well with increase in total aflatoxin contents. However, there was no significant difference (*P>0.05*) between the total aflatoxin levels in the different stages of kunun-zaki production. The mean total aflatoxin decontamination was 18.9% and 52.9% for kunun-zaki stored at 4 and 28±2^{o}C. The protein content increased significantly (2.72-4.90%) as the percentage wheat substitution increased from 5, 7.5, and 10%. From this research, aflatoxin decontamination through fermentation of the beverage is not significantly higher and the aflatoxin contaminated cereals could serve as an inoculum of its toxic content into kunun-zaki, consequently, it's likely health threats to consumers. There is a need for awareness on the quality of raw material for kunun-zaki production.

Key words:, *cereal, moulds, aflatoxins, fermentation, kunun-zaki.*

INTRODUCTION

Kunun-zaki is a Nigerian cereal-based fermented non-alcoholic beverage. There are various types of the processed kunun (Amusa et al. 2009; Essien et al. 2009). From North east and middle belt of Northern Nigeria, *Sorghum bicolor,* had mainly been used for kunun-zaki production and sometimes in composite with other cereals such as maize, millet and other *Sorghum* species, using crude arbitrary ratios for its production. Though wheat (*Triticum* spp) contains a higher percentage of protein 13-18% (Zuzana et al. 2009) than other cereals, most processors do not use it for kunun-zaki production, because it is very expensive, not commonly cultivated and often regarded as a rich man`s option. Kunun-zaki is a low viscosity beverage with a characteristic creamy appearance with sweet, sour taste (Odom et al. 2012). Its production protocols and packaging and distribution are not yet standardized (Elmahmood et

al. 2007) and mostly non-mechanized, thus left to the expertise of individual local processors. The beverage is one of the most consumed Nigeria, indigenous fermented non-alcoholic beverages. Consequently, it has been used for refreshment and widely consumed because of its ability to create some form of satiety. It is believed to be of immense social, economic and medicinal importance to its numerous consumers (Akoma et al. 2006). Naturally, cereals have been reported to be contaminated with aflatoxins (Cotty et al. 2006; Noelia et al., 2012) . Aflatoxins are mycotoxins produced as secondary metabolites that pose a serious health hazards to humans and animals. They are carcinogenic (Elmahmood and Doughari, 2007) and another study reported genetic defects at foetal stages due to the ability of aflatoxins to cross the Placental barrier (Odom et al. 2012). Animal mortally rate also increases when fed with aflatoxin contaminated meal (Amusa et al. 2009) Aflatoxins are difuranocoumarin derivatives produced via a polyketide pathway (Yu et al.2004) by two foundamental aflatoxigenic moulds strains; Aspergillus flavus and Aspergillus parasiticus (Klich et al. 2000). There are four main types of aflatoxins (B1, B2, G1and G2) documented (Bankole and Adebanjo, 2003) . The occurrence of these moulds in cereals causing lethal cases of aflatoxicosis had been reported in United Kingdom (Kelly , 2009) , India (Ananth and Farid, 2003) , Kenya, 2004 (Ananth and Farid, 2002) , Malawi (Charles,2007), in both human and animals who consumed aflatoxin contaminated cereal products in the form of food or poultry feed. Aflatoxigenic moulds and aflatoxins in cereals such as wheat have been published (Noelia, et al. 2011; Ananth and Farid, 2003) and also reported in fermented foods such as bread (Zuzana et al. 2009). It is for this reason that the objective of this research was carried out to assess the fate of aflatoxins during fermentation of aflatoxin contaminated wheat-sorghum composite and to assay for wheat probable improvement of the nutritional content of sorghum kunun-zaki.

MATERIALS AND METHODS

Sample collection and processing

Twenty kilograms (20 kg) of *Sorghum bicolor* and ten kilograms (10 kg) of Nigerian wheat cultivar (*Triticum* spp) were bought at Kaduna central market. Each consignment of the two cereals was sorted into visibly spoiled (discoloured, mouldy appearance, decayed, and chipped) and wholesome grains according to the methods of Adegoke et al. (2010) .

Preparation of composite cereal for fermentation

Wholesome wheat was blended 5, 7.5 and 10% with *Sorghum bicolor* to give a sample of (1kg) each for kunun-zaki production. Naturally spoiled, mouldy yellowish suspected aflatoxin contaminated cereals were sorted and also blended 1, 2, and 3% each with 100% S*orghum bicolor* to obtain another separate sample of (1kg) each for Kunun-zaki production. The first blend of the wholesome cereal was constituted in duplicate. The second blend was spiked with standard aflatoxin B to serve as control.

Kunun-zaki production

The method of Egbere et al. (2008) was adopted for kunun-zaki production from winnowing to slurry making. The subsequent steps were slightly modified. An aliquot of 200 mls (20%) portion of the slurry was removed and sterilized spices (ginger, alligator pepper) added to it and kept separately. Then 150 mls of aseptically dried sweet potato was wet ground into a paste and added to the separately kept 20% slurry. The remaining slurry (80%) portion was gelatinized by addition of boiling water while stirring until thick consistency was achieved. The liquefying agents were then added to the gel after cooling to approximately 30^{o}C. It is believed that this heat does not affect the activity of the alpha amylase in the malted cereal. The mixture was then stirred gently until liquefied or lost its consistency. Finally, the mixture was incubated at ambient temperature for 12hrs to ferment aerobically in an open stainless steel bioreactor. The finished product was then sieved using sterile muslin cloth. Sterilized sugar was added to taste and aseptically bottled and immediately used for physicochemical studies and aflatoxin determination.

Isolation of moulds from composite cereals and kunun-zaki

Isolation and identification of moulds associated with the composite cereals and kunun-zaki were carried out according to the method of Amusa and Odunbaku, (2009) with slight modifications. Briefly appropriate dilutions were made and 0.1ml was aseptically

inoculated on sterile gelled potato dextrose agar in duplicates and was spread with sterile bent glass rod. The inoculated petri dishes were incubated at ambient temperature (28±2°C) in a dark cupboard for 3-5days.

Identification of moulds associated with cereal and kunu-zaki

Macroscopic and microscopic identification of the moulds were carried out according to standard methods APHA, (1999) and as documented by Ellis, (2006). Macroscopically, colonies with dark-green morphology and rough conidia were characterized as *Aspergillus parasiticus*, while, isolates with characteristics yellowish-green colonies and smooth conidia were characterized as *Aspergillus flavus*. Microscopic Method was carried out by mounting a drop of lacto phenol cotton blue on a clean microscope slide. The mould cultures obtained were then teased out with sterile shape pointed needle and placed inside the stain and covered with clean cover slip. The slide was then mounted on the stage of the microscope and examined microscopically for identification using ×10 and × 40 objectives.

Polyphasic detection of aflatoxigenic moulds

Identification of aflatoxigenic moulds from the isolates was carried out using specific media: modified yeast extract sucrose agar (YES) supplemented with 0.3% methyl β cyclodextrin and 0.6% sodium Desoxycholate as described by other researchers (Ordaz et al. 2003; Gashgari et al. 2010; Alborch et al. 2012). All visible beige ring and blue fluorescent ring surrounding the colonies under UV light was recorded as aflatoxigenic moulds.

Detection and quantification of total aflatoxins using Helica Biosystem ELISA kits

Aliquots of 15g each of the dry wheat blends were sampled and ground using binatone blender 446 Nakai Japan to roughly 200 µm. A 10g each of the slurry, gel and the ready to drink kunun-zaki was also used for detection and quantification of total aflatoxins according to the method of Wartu et al. (2009) using enzyme link immunosorbent assay (ELISA). An ELISA kits was obtained from Helica Biosystems Inc.U.S.A. and was used according to manufacturers instruction. The Helica total aflatoxin assay is a solid phase direct competitive enzyme immunoassay. An aflatoxin specific antibody optimized to cross react with all four subtypes of aflatoxin coated to a polystyrene micro well. Toxins are extracted with 70% methanol from the samples. The extracted sample and HRP-conjugated aflatoxin B1 were mixed and added to the antibody-coated micro well. Aflatoxin from the extracted sample and the HRP conjugated aflatoxin B1 compete to bind with the antibody coated to the micro well. Micro well contents were decanted and non specific reactants were removed by washing. An enzyme substrate trimethylbenzane (TMB) was added and blue colour developed. The intensity of the colour was directly proportional to the amount of bound conjugate and inversely proportional to the concentration of aflatoxin in the sample or standard. The micro wells were measured optically by micro plate reader with an absorbance filter of 450nm. The optical densities of the samples were compared to the optical densities of the kit standards and interpretative result determined.

Analysis of physicochemical parameters

The pH and the percentage free fatty acid value were determined according to the methods adopted by Negedu et al. (2010) . The pH of 20ml of kunun-zaki was determined using pH meter (PYE UNICAM model 292 MK2) after standardization with Oxiod buffers of pH 4 and 7. The free fatty acid was determined using 2 ml of kunun-zaki. Then 25ml ethanol (95%) and 25ml diethyl ether neutralized with 0.1M ethanol-potassium hydroxide was added. Phenolphthalein indication was added to the solution and was titrated with 0.1M potassium hydroxide solution to a faint pink colour that persisted for 15 seconds. The percentage free fatty acid was calculated as follows:

$$\text{Acid value} = \frac{56.1 \times T \times V}{M}$$

M= volume in mls of test sample
V= volume of potassium hydroxide used

T= Molarity of ethanolic acid
% free fatty acid = acid valve x 2^{-1}
The percentage protein, moisture, titratable acidity and ash were carried out according to the methods of Amusa and Odunbaku, (2009) and from appropriate standards (AOAC,1990) .

RESULTS

Different genus of fungi was isolated from the cereals and the kunun-zaki. Table 1 presents moulds that were isolated from the raw materials and kunun-zaki obtained after fermentation. Eight fungal species were isolated from the raw material blends; *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Fusarium moniliforme, Penicillium crysogenum, Fusarium oxysporum, Saccharomyces cerevisiae, Rhizopus stolinifer*. Five fungal species were also isolated from the fermented product; *Aspergillus flavus, Fusarium oxysporum,Saccharomyces cerevisiae, Candida* spp, and *Aspergillus niger*. Four fungal species: *Aspergillus parasiticus, penicillium crysogenum, Fusarium moniliforme,* and *Rhizopus stolinifer* were isolated from the raw materials, but were however not detected in the finished product. *Candida* spp was not isolated from the cereals but was detected in the finished product. Aflatoxigenic moulds detected by polyphasic method from the raw materials, intermediate and kunun-zaki showed that the aflatoxigenic moulds were positive for some of the samples (data not shown) Table 2 shows the level of natural total aflatoxins along the various processing steps of kunun-zaki production and the total aflatoxin percentage loss shown in parenthesis. The slurry obtained after wet milling had more total aflatoxins compared to the other step total aflatoxin level. Conversely, the final product had lower total aflatoxin levels compared to the other stages. There was no aflatoxin detected from wholesome cereal blended. There was a correlation between the total aflatoxin levels of the spoilt-wholesome cereal and the various blends; as the percentage of the spoilt cereal increased from 1-3%, the level of total aflatoxins also increased. The slurry had higher total aflatoxin levels than the raw material flour. However, there was no significant difference in the level of aflatoxins in the different stages of kunun-zaki production steps. The mean aflatoxin decontaminations after 48hrs were 18.9 (data not shown) and 52.90% from refrigerated and ambient temperature stored kunun-zaki respectively. Table 3 presents the nutritional impact of wheat-sorghum composite on kunun-zaki. The percentage moisture was relatively the same for all the samples. The percentage ash decreased as the percentage substitution of sorghum with wheat increased from 5, 7.5, and 10%. While the pH increased slightly from 5.61-6.20, the titratable acidity reduced as the percentage substitution of wheat to sorghum also increased.

Table1
Moulds associated with the production of kunun-zaki.

Moulds	wholesome wheat and Sorghum raw material composite	wet ground cereal blend (slurry)	slurry + hot water (gel)	kunun-zaki
Aspergillus flavus	+	+	+	+
Aspergillus parasiticus	+	+	+	-
Aspergillus niger	+	+	+	+
Penicllium crysogenum	+	+	+	-
Fusarium moniliforme	+	+	-	-
Fusarium oxysporum	+	+	+	+
Saccharomyces cerevisae	+	+	+	+
Rhizopus stolinifer	+	-	-	-
Candida mycoderma	-	-	-	+

Results obtained are from duplicate samples
Key: +, detected, - , not detected

Table 2
Level of total aflatoxin during kunun-zaki production steps at 28±2°C

	Total Aflatoxin (ppb) in Spoiled grain - wholesome sorghum blend			
Sample	1	2	3	wws composite
Flour blend	7.08	26.95	36.85	Nil
Slurry	8.00 *(2.50*)*	27.70*(2.71*)*	37.80 *(2.50*)*	Nil
Gel	6.90 *(13.75**)*	24.00 *(13.35**)*	33.20 *(12.17**)*	Nil
Fermented kunun-zaki	3.76 *(53.00**)*	13.50*(52.00**)*	17.5 *(53.70**)*	Nil

Key, values are mean of duplicate samples
****, percentage aflatoxin content higher than raw material aflatoxin***
*****, percentage aflatoxin loss.***
1, 2, and 3, represents percentage of mouldy cereal to 100% wholesome sorghum
wws, wholesome wheat sorghum composite

Table 3
Nutritional impact of composite Triticum spp on sorghum bicolor kunun-zaki.

		Triticum spp - *sorghum bicolor* composite (%)		
Parameter	100 % wholesome sorghum	5	7.5	10
% Moisture	86.50	86.40	86.50	86.45
% ash	1.25	1.23	1.18	1.11
% crude protein	2.72	2.74	3.85	4.90
p^H	5.61	5.68	5.99	6.20
Titratable acidity	0.46	0.45	0.40	0.37
% Carbohydrate	82.80	82.25	80.30	75.10

Key, the values are mean of duplicate samples
5, 7.5 and 10 represents ratio (percentage) of wholesome Triticum spp blended with sorghum bicolor

DISCUSSION

Kunun-zaki is a nutritious and an anti thirst beverage commonly sold in communities from northern Nigeria. Its processing methods are however not mechanized. *Sorghum bicolor* and other cereals such as wheat, maize are chiefly produced within the northern states hence their prompt use of kunun-zaki production. Since cereals are prone to natural contamination by moulds (Noelia et al. 2011), kunun-zaki could be prone to contamination by toxin inoculum from the unwholesome raw material. This study was designed to assess the levels of aflatoxins retention in contaminated wheat-sorghum composite fermented kunun-zaki. Consequently, this mould`s secondary metabolites such as aflatoxins could pose the consumers to public health threats. This study revealed the occurrence of some moulds and aflatoxins from kunun-zaki, even though there was brief heating (100^0C) applied at the stage of gelatinization process, vegetative moulds might have been inactivated but, the heating process was probably not sufficient to sterilize out moulds spore. Such spore germinates thereafter to recontaminates the finished product. More so, Kunun-zaki fermentation is carried out in open bioreactors usually open drums and containers which subsequently expose the products to recontamination by ubiquitous moulds. Varying microbial levels has been reported in kunun-zaki as a result of arbitrary formulations and unstandardized practices during its production. The appearance of some of the isolated moulds in this

study such as *Rhizopus stolinifer* in food stuff includes an elevation of pH beyond the safety value of 4.6 making the environment more conducive for the growth of moulds (Efiuvwevwere and Akano.1997). The implication of the aflatoxigenic *Aspergillus flavus and Aspergillus parasiticus* in foods was also documented as an important tool in assessing the toxicological status of food (Chaelae et al. 2002). This is obvious from this research because of the level of aflatoxin detected. In addition, El Khoury et al. (2011) also reported that the two moulds are capable of producing aflatoxin B1, B2, G1, G2. Their presence in raw material and kunun-zaki therefore constitute a health hazard to the consumer. Though aflatoxins are very stable to heat in dry state up to their melting points, however, Ananth and Farid, (2003) documented that heating aflatoxin contaminated food in the presence of moisture, leads to destruction of aflatoxins over a period of time. This destruction leads to the opening of the aflatoxin lactone ring with the possibility of decarboxylation and loss of the methoxy group from the aromatic ring at elevated temperatures. Such destruction occurs either with aflatoxin in oils or aqueous solution as would have been the case for kunun-zaki. But kunun-zaki processing involves very brief heating by pouring boiled water into the large volume of ground wet milled cereal slurry with continuous stirring which immediately drops the temperature below 100^{o}C. This drop in temperature and lack of continuous heating along kunun-zaki production protocol could probably be the reason why a reduction of the natural total aflatoxin in aqueous kunu-zaki was not appreciable. The higher values of total aflatoxins obtained from the slurry than the raw material flour could be due to the effect of the release of the toxin from the matrices of the cereal slurry particles because of its high surface area. The better aflatoxin absorption by room temperature fermented product was due to the most likely reaction of the acid produced during the fermentation process on the aflatoxins, which probably catalyzed addition of water across the double bound in the aflatoxin furan ring leading to conversions of the toxin to various adducts, such as acetoxy derivative as documented by Chaelae et al. (2002). The detection of total aflatoxins across all the samples along the Kunun-zaki production steps depicts that aflatoxin contaminated raw materials will only serve as a source of aflatoxin inoculum into the finished product and hence its likely consequence of aflatoxicosis in consumers. There was no aflatoxin extracted from the liquefying agent and sweet potato. The mean 52.9% total aflatoxin absorption obtained from this research was a clear indication that kunun-zaki fermentation process partially eliminated the aflatoxins. Earlier report Oluwafemi and Ikeowa, (2005) showed 50% reduction of aflatoxin in contaminated maize used for pap (ogi) production. Another study by El-Tawila et al. (2003) reported 41.17% total aflatoxin absorption after dough baking into bread. These are relatively solid cereal fermented products compared to liquid fermented kunun-zaki beverage. All the same, decontamination of aflatoxin from either solid or liquid cereal based product such as kunun-zaki by fermentation is not efficient. The percentage crude protein significantly improved from 2.72 - 4.90% because of the dilution of wheat containing high protein compared to sorghum with low protein content. The higher the percentage wheat substitution, the higher percentage protein released into the Kunun-zaki matrices. The high fibre obtained from the composite wheat-sorghum could be beneficial to consumers as this could stimulate the secretion of digestive juices and increase the viscosity of stomach contents, thereby retarding gastric emptying (Leclere et al. 1994) . The titratable acidity, the percentage ash and percentage carbohydrate showed some slight decrease as the level of wheat substitution increased in the composite because of the substitution of *Sorghum* containing high carbohydrate with wheat containing a very high percentage of protein.

CONCLUSION

It was obvious from this research work that wheat-sorghum fermentation into kunun-zaki reduced 52.9% total aflatoxin. This implies that aerobic fermentation of aflatoxin contaminated cereal is not a technique that will efficiently remove aflatoxins and individuals consuming food products produced through fermentation where the raw material is contaminated with aflatoxins could be a victim of

health problems associated with aflatoxins. Nigeria wheat cultivar contains a high percentage of proteins and when blended with *sorghum bicolor* could serve as a cheap and available source of plant protein and thus improved the nutritional content of Kunu-zaki. The establishment of mechanized kunun-zaki processing unit that could produce safe, packaged product to its consumers is advocated.

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