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Fungal species isolated from peanuts in major Kenyan markets: Emphasis on *Aspergillus* section *Flavi*

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ABSTRACT

A survey was conducted in Nairobi, Nyanza and Western provinces in Kenya between March and July 2009 with 1263 peanut products sampled out of which 705 samples underwent microbial analysis. The study aimed at determining the incidence of fungal species – emphasis on *Aspergillus* section *Flavi* – associated with peanut products. A 0.5 kg representative sample was obtained from each surveyed vendor and the colony forming units (CFU) of fungal species determined. The samples were also analyzed for total aflatoxin level while isolates of *Aspergillus flavus* and *Aspergillus parasiticus* were screened for production of aflatoxin B1, B2, G1 and G2. Eight fungal species were detected in the samples and were in decreasing order of CFU/g of sample: *A. flavus* S-strain (467), *A. flavus* L-strain (341), *Penicillium* spp. (326), *Aspergillus niger* (156), *Aspergillus tamari* (27), *Aspergillus alliaceus* (21), *A. parasiticus* (10), and *Aspergillus caelatus* (5). The overall incidence of *A. flavus* S-strain in samples from Nairobi was 92 and 1425% higher than samples from Nyanza and Western regions, respectively. The combined incidence of *A. flavus* and *A. parasiticus* was varied significantly ($p \leq 0.05$) with peanut product: peanut flour (69%), shelled raw peanuts (53%), spoilt peanuts (49%), boiled podded peanuts (45%), podded peanuts (39%), peanut butter (31%), fried peanuts (22%) and roasted peanuts (20%). Seventy three percent of *A. flavus* and *A. parasiticus* isolates produced at least one of the aflatoxin types, with 66% producing aflatoxin B1. The total aflatoxin level among peanut products ranged from 0 to 1629 μg/g; and there was a positive correlation ($r = 0.2711$) between the incidence of *A. flavus* and *A. parasiticus*, and total aflatoxin level. The high incidence of aflatoxin producing fungi in peanuts traded in Kenyan markets implies a risk of aflatoxin contamination, highlighting the need for stakeholders to promote sound practices at all stages of the peanut value chain in order to minimize market access by non-complying products.

1. Introduction

Crops often become contaminated with aflatoxins, which are toxic fungal metabolites, in tropical production areas throughout the world (Cotty and Jaime-Garcia, 2007). Maize, peanuts, oilseeds, spices and tree nuts are common substrates for aflatoxin contamination (Lisker et al., 1993; Hedayati et al., 2007; Richard and Abbas, 2008; Rigo et al., 2002; Shephard, 2008). Maize and peanuts are the main sources of human exposure to aflatoxin especially in regions with weak regulatory systems because they are highly consumed besides being the most susceptible crops to aflatoxin contamination (Wu and Khlangwiset, 2010). Aflatoxins can be produced at pre- and post-harvest stages (Waliyar et al., 2008), and consumption of aflatoxin contaminated foods affects human health (Godet and Munaut, 2010) causing aflatoxicosis (Lewis et al., 2005), cancer (IARC, 2002; Wu, 2010), stunted growth in children and/or immune suppression (Wu, 2010) among others. It has been estimated that more than five billion people in developing countries are at risk of chronic exposure to aflatoxins through contaminated foods (Strosnider et al., 2006).

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The fungi responsible for production of aflatoxins are mainly *Aspergillus flavus* and *Aspergillus parasiticus* which belong to *Aspergillus* section *Flavi* (Cotty et al., 1994), and to a lesser extent *Aspergillus nomius* (Dorner, 2002; Vaamonde et al., 2003). However, fungi in *Aspergillus* section *Flavi* exist in complex communities composed of individuals or strains that vary widely in aflatoxin-producing ability (Cotty, 2006). Based on morphological, genetic and physiological criteria, *A. flavus* can be divided into two morphotypes (Cotty, 1994). The S-strain produces numerous, small sclerotia and high levels of B-aflatoxins (Garber and Cotty, 1997; Samson et al., 2000), with some S-strains producing both B- and G-aflatoxins (Barros et al., 2006; Cardwell and Cotty, 2002; Okoth et al., 2012). The L-strain produces fewer, larger sclerotia, and typically less B-aflatoxins or no aflatoxins at all (Barros et al., 2006; Garber and Cotty, 1997; Probst et al., 2011; Samson et al., 2000). *A. parasiticus* and *A. nomius* produce both B- and G-aflatoxins (Cardwell and Cotty, 2002; Ehrlich et al., 2003). Many authors have shown evidence that *A. flavus sensu lato* may consist of several species (Geiser et al., 2000, 1998; Pildain et al., 2008). The second group of section *Flavi* comprises the aflatoxin non-producing species, *Aspergillus oryzae* and *Aspergillus sojae*. The ability to produce aflatoxins is not universal in some *Aspergillus* species such as *Aspergillus tamari* where certain strains are aflatoxin producers while others are non-aflatoxigenic (Goto et al., 1996; Ito et al., 2001).

Peanuts (*Arachis hypogaea* L.) are a profitable and reliable crop in the western region of Kenya – Nyanza and Western provinces – and are planted during the short and long rainy seasons (Mutegi et al., 2009). Local varieties are susceptible to diseases and insect pests that result in plant stress, predisposing the peanuts to aflatoxin contamination (Chapin et al., 2004; Hell et al., 2000). Contamination of peanuts by aflatoxin can occur at all points in the value chain (Mehan et al., 1991) including production, acquisition and handling of raw materials, processing, storage and distribution (Bastianelli and Le Bas, 2002; Waliyar et al., 2003; Wu et al., 2011). More than eight fungal pathogens (Gachomo et al., 2004) and high aflatoxin levels (up to 7525 μg/kg; Mutegi et al., 2009) have previously been reported in peanuts in Kenya.

Few studies have been conducted on the prevalence of *Aspergillus* section *Flavi* on peanuts in Kenya (Gachomo et al., 2004; Mutegi et al., 2012). The current findings form part of a study that was undertaken to assess the prevalence of aflatoxin in marketed peanuts in Kenya. Results on aflatoxin levels and market attributes that contribute to the reported levels are published elsewhere (Mutegi et al., 2013). The objectives of this study were to (i) determine the incidence of fungal species – with emphasis on *Aspergillus* section *Flavi* – associated with peanuts and peanut products in three provinces in Kenya; (ii) screen *A. flavus* and *A. parasiticus* isolates for production of aflatoxin B1, B2, G1 and G2 and; (iii) determine whether the incidence of *A. flavus* and *A. parasiticus* is associated with the level of aflatoxin in peanuts.

2. Materials and methods

2.1. Study sites

Raw peanuts and peanut products were collected from three provinces in Kenya namely Nairobi, Western (Busia District) and Nyanza (Homa Bay, Kisii Central, Rachuonyo and Kisumu East Districts). The sites were chosen based on their importance in serving as major market outlets for peanuts that are produced locally or imported from neighbouring countries, as well as their significance as regions of peanut production (Mutegi et al., 2013).

2.2. Survey and collection of peanut samples

Samples and information relating to each sample were gathered through a survey conducted in Nairobi, Nyanza and Western provinces in Kenya between March and July 2009 (Mutegi et al., 2013). A pre-testing exercise was carried out in Nairobi region involving 50 vendors; 28 and 22 from Nairobi North and Nairobi South districts, respectively. Information gathered was used to design a structured questionnaire to collect data on market traits and practices that were related to either mould or aflatoxin contamination of peanuts (data not shown). Participants in the survey were identified through purposeful sampling, focussing on vendors who were trading in peanuts. Data on market traits and practices collected included information on demographics of the vendor; type of peanut products traded in the market; type of packaging material used for peanut products in the market; sources of peanuts traded; post-harvest crop protection method used by peanut vendors; type of peanut market outlet. Other data recorded were the state of the marketing structures (describing the condition of the roofing material, walls and floors, if any, and ventilation). The major peanut products sampled included podded raw kernels, shelled raw kernels, roasted kernels, peanut butter, boiled kernels, fried kernels, peanut flour, and spoilt kernels.[1] A 0.5 kg representative sample was obtained from each surveyed vendor, and stored in a cold room until processed. In instances where the peanuts were already packaged and sealed, at least 400 g of the product, either as a single or several packets depending on the quantity in each packet, was purchased for analysis. In cases where podded samples were collected, shelling was done manually. Out of 1263 samples collected (Mutegi et al., 2013), 705 representative samples based on the type of product and the sampling location were picked for microbial assay.

2.3. Sample preparation

The 0.5 kg sample from each vendor was thoroughly mixed and two subsamples (100 g each) blended in a kitchen grinder (Kanchan Multipurpose Kitchen Machine, Kanchan International Limited, Mumbai, India). In cases where podded samples were collected, shelling was done manually. In cases where packaged peanut products collected from vendors were less than 0.5 kg, at least 400 g of the product was purchased for analysis. For peanut butter paste, a 200 g sub-sample was drawn from each 400 g sample purchased from the vendors and divided into two equal portions with no further grinding. Grinders were cleaned with 70% ethanol between sample grinding to minimize cross-contamination.

2.4. Microbiological assays

Isolation of fungal species was carried out on modified dichloran rose bengal (MDRB) agar (Horn and Dorner, 1998; 10 g glucose, 2.5 g peptone, 0.5 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 20 g agar, 25 mg rose Bengal; pH was adjusted to 5.6 using 0.01 M HCl). To inhibit bacterial growth and ensure the medium was semi-selective for *Aspergillus* section *Flavi*, 5 mL of 4 mg/L dichloran (in acetone), 40 mg/L streptomycin and 1 mg/L chlortetracycline were added to the medium through a sterile 0.25 μm syringe filter.

From each of the 100 g ground sub-samples, 2 replicates of 2.5 g each were placed in calibrated centrifuge tubes, into which 10 mL of 2% water agar solution was added and mixed thoroughly. A 0.2 mL

[1] This category defines nuts considered unwholesome after sorting is done, and sold in the market at a lower price. They are characterized by broken, mouldy, discoloured and shrivelled kernels.

aliquot of the suspension was pipetted, spread onto MDRB plates, and incubated for 7 days at 30 °C, after which the colonies were identified and classified. Colony counts of *A. flavus* L-strain, *A. flavus* S-strain, *A. parasiticus*, *A. alliaceus*, *Aspergillus caelatus*, *A. tamarii*, *Aspergillus niger* and *Penicillium* spp. were recorded. The presence or absence of *Rhizopus* spp. in the samples was also recorded.

Pure fungal colonies on MDRB agar medium were sub-cultured onto Czapek yeast extract agar (CYA; 1 g K_2HPO_4, 10 mL Czapek concentrate, 5 g powdered yeast extract, 30 g sucrose, 15 g agar), whose pH was adjusted to 7.2 and incubated at 30 °C for 5–7 days. Species of *Aspergillus* section *Flavi* were distinguished based on colony colour, shape, margins, elevation, pigmentation, texture, pattern of growth and conidial morphology characteristics (Klich, 2002), and by comparison with reference strains obtained from Dr. Bruce Horn (USDA National Peanut Research Laboratory, Dawson, Georgia, USA). Identification of the other fungal species (besides *Aspergillus* spp.) was also based on cultural and morphological characteristics.

2.5. Screening isolates of A. flavus and A. parasiticus for aflatoxin production

A total of 617 isolates of *A. flavus* and *A. parasiticus* (193, 251 and 261 from Nairobi, Nyanza and Western provinces, respectively) were screened for production of aflatoxin B1, B2, G1 and G2. The screening was done in high sucrose yeast extract (YES) liquid medium (Horn and Dorner, 1998). The YES medium was prepared by dissolving 150 g sucrose, 20 g yeast extract (Difco, Franklin Lakes, New Jersey, USA), 10 g soystone (BD Bacto, Franklin Lakes, New Jersey, USA) and 40 g glucose in 1 L distilled water, and the pH adjusted to 5.9 with 0.01 m HCl. Conidia from single spore colonies of *A. flavus* (S- and L-strains) and *A. parasiticus* were inoculated into 6 mL vials containing 2 mL YES medium. Inoculated vials were incubated in the dark at 30 °C for 7 days, with intermittent shaking using a Stuart® vortex shaker (Bibby Scientific Limited, Staffordshire, UK). After incubation, 2 mL of chloroform was pipetted into each vial, the mixture vortexed for 60 s and left to stand overnight in a fume hood. Using a micro-pipette, 5 μL of the chloroform extract was spotted on silica gel 60 thin layer chromatography (TLC) plates (EMD Chemicals Inc., Gibbstown, New Jersey, USA), along with analytical grade standards of aflatoxin B1, B2, G1 and G2 (ICRISAT, Patancheru, India). Previously selected toxigenic strains were used as positive controls. The plates were developed in chloroform, acetone and distilled water, in a ratio of 88:12:1.5 respectively, until the solvent covered about 90% of the plate length. The plates were transferred to a darkroom and scored for the presence or absence of the four aflatoxin types under UV-light.

2.6. Aflatoxin analysis

The samples were mixed thoroughly and ground in the laboratory using a dry mill kitchen grinder (Kanchan Multipurpose Kitchen Machine, Kanchan International Limited, Mumbai, India). In cases where peanut butter paste was sampled, no grinding was needed. A 200 g sub-sample was drawn from each 500 (or 400) g sample and divided into two equal portions. The powder (or peanut paste) was triturated in a blender in 70% methanol (70 mL absolute methanol in 30 mL distilled water, v/v) containing 0.5% potassium chloride (w/v) until thoroughly mixed. The extract was transferred to a conical flask and shaken for 30 min at 300 rpm. The extract was then filtered through Whatman No. 41 filter paper and diluted 1:10 in phosphate buffered saline containing 500 μl/l Tween-20 (PBS-Tween) and analyzed for aflatoxin levels using indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA) as described by Waliyar et al. (2005). This method has a detection limit of 0.5 μg/kg. Aflatoxin data reported in this study relates to the 705 (out of 1263) samples used for microbial analysis.

2.7. Determining correlation between the population of Aspergillus species and aflatoxin levels

Pearson correlation coefficient (SPSS version 16) was used to establish the correlation between the population of *A. flavus* and *A. parasiticus* and the total aflatoxin detected in the peanut products.

2.8. Data analyses

Data were subjected to analysis of variance (ANOVA) using PROC ANOVA procedure of Genstat Discovery 2 statistical software (Version 13, Lawes Agricultural Trust, Rothamsted Experimental Station, 2006) and means were compared using Fisher's protected LSD test at 5% significance level. For the isolation data, infection frequency from each Petri plate was pooled and mean frequency determined. Skewed percentage data were transformed using arcsine $\sqrt{p/100}$ while other skewed data were transformed to $\log_{10}$ for data analysis and separation of means. Pearson correlation coefficient (SPSS version 16) was used to establish the correlation between the population of *A. flavus* and *A. parasiticus* to total aflatoxin level.

For aflatoxin data, samples were grouped into three categories based on their aflatoxin content: samples with: ≤4 μg/kg, >4 μg/kg to ≤10 μg/kg, and >10 μg/kg. The ≤4 μg/kg category represents the European Union (EU) regulatory limit for total aflatoxin (EC, 2006); peanuts in the second category would be rejected under the European Union regulations but would be accepted under the Kenya Bureau of Standards (KEBS) regulatory limit of 10 μg/kg (KEBS, 2007), while nuts in the third category would be rejected under both the KEBS and EU standards.

3. Results

3.1. Incidence of fungal species associated with peanuts

Eight fungal species were isolated from raw peanuts and peanut products sampled from Nairobi, Nyanza and Western provinces (Table 1). Incidence of the fungal species was significantly ($p \leq 0.05$) different and occurred as follows in decreasing order of CFU/g: *A. flavus* S-strain (467), *A. flavus* L-strain (341), *A. niger* (156), *A. tamari* (27), *A. alliaceus* (21), *A. parasiticus* (10), and *A. caelatus* (5). *Rhizopus* spp. were isolated from 8.5% of the samples while the incidence of *Penicillium* spp. was 326 CFU/g. Other frequently isolated fungi included *Fusarium* and *Trichoderma* species. There was a significantly ($p \leq 0.05$) higher incidence of fungal species in peanuts sampled from Western (mean = 214 CFU/g) than from Nairobi (mean = 175 CFU/g) and Nyanza (mean = 70 CFU/g) provinces. The overall infection rate of peanuts and peanut products by *A. flavus* S-strain regardless of the region where they were sampled from was higher by a factor of 1.4 compared to infection by *A. flavus* L-strain. However, the incidence of *A. flavus* S and L strains in peanuts sampled from Nairobi was not consistent with the corresponding incidence of the two strains in samples from Nyanza and Western provinces. In samples from Nairobi, the incidence of *A. flavus* S-strain was 347% higher than of *A. flavus* L-strain. This was in comparison to Western and Nyanza provinces where the incidence of *A. flavus* L-strain was 30% and 293%, respectively higher than of *A. flavus* S-strain.

The incidence of *Aspergillus* spp. in different peanut products varied significantly ($p \leq 0.05$). Overall, spoilt peanuts, peanut flour and shelled raw nuts were the most infected with *Aspergillus* spp.

Table 1
Colony forming units [CFU/g peanuts] of fungal species isolated from peanuts and peanut products sampled from Nairobi, Nyanza and Western provinces of Kenya.

Peanut product	AF[S]	AF[L]	AP	AA	AC	AN	AT	PEN	Others[a]	Total [*n*]
A. Nairobi										
Shelled raw peanuts	562.1	488.8	11.5	0.0	0.0	164.8	5.2	447.2	43.2	33
Peanut butter	4.7	11.3	0.0	0.0	0.0	1.4	0.1	20.7	0.7	33
Roasted peanuts	63.1	12.9	0.1	0.0	0.0	0.3	1.0	34.7	2.2	75
Fried peanuts	0.2	6.4	0.0	0.0	0.0	1.5	0.0	6.1	1.7	22
Peanut flour	257.8	706.7	4.8	0.0	8.5	280.0	5.6	215.2	48.1	9
Spoilt peanuts[b]	4535.5	466.6	50.6	0.0	0.0	1221.5	301.2	421.2	36.1	11
Others[c]	193.0	35.3	0.0	0.0	0.0	35.7	0.0	130.7	24.0	10
Mean	882.9	197.7	10.6	0.0	0.4	245.7	51.6	172.0	17.3	193
A. Nyanza										
Shelled raw peanuts	200.9	200.5	8.4	0.5	13.4	256.1	5.1	403.7	25.5	103
Podded peanuts	20.7	47.2	0.3	0.0	1.2	13.1	1.0	336.7	67.8	51
Peanut butter	106.1	151.1	1.7	0.1	17.2	65.4	0.8	250.9	14.7	34
Roasted peanuts	43.6	39.0	1.6	0.7	2.2	38.5	0.7	119.6	8.4	28
Fried peanuts	10.8	190.8	0.0	0.0	0.0	274.2	0.0	142.5	25.8	4
Spoilt peanuts[b]	32.7	979.7	0.0	0.0	63.0	157.7	0.0	107.3	14.3	10
Boiled podded nuts	42.9	93.8	0.7	0.0	0.0	2.4	0.0	94.4	27.8	15
Others[c]	5.6	118.3	16.7	0.0	8.9	37.8	0.0	237.2	23.9	6
Mean	57.9	227.6	3.5	0.1	13.2	102.4	0.2	196.7	25.1	251
A. Western										
Shelled raw peanuts	406.4	662.8	3.1	57.6	0.0	329.2	118.4	1375.1	98.1	137
Podded peanuts	1158.8	141.7	12.5	0.0	0.0	10.8	8.3	1764.2	45.0	8
Peanut butter	131.3	136.4	59.2	0.0	0.0	3.8	0.0	319.2	10.0	13
Roasted peanuts	29.7	217.3	0.6	32.6	0.0	39.0	0.6	114.1	7.9	56
Peanut flour	1640.2	2046.9	53.2	16.5	0.0	552.1	149.0	511.5	52.3	28
Fried peanuts	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2
Spoilt peanuts[b]	589.6	1960.8	0.0	0.0	0.0	147.1	0.0	206.3	6.3	8
Boiled podded nuts	17.8	18.9	0.0	0.0	0.0	6.7	0.0	671.0	6.7	3
Others[c]	111.7	266.1	0.0	57.2	0.0	17.8	0.6	92.2	18.3	6
Mean	459.9	597.8	14.6	63.7	0.0	121.3	30.4	608.5	27.2	261
LSD[d]	330.0	241.1	ns	ns	ns	254.6	70.9	468.4	ns	

AF[S] – *Aspergillus flavus* S-strain, AF[L] – *A. flavus* L-strain, AP – *A. parasiticus*, AA – *A. alliaceaus*, AC - *A. caelatus*, AN – *A. niger*, AT – *A. tamarii*, PEN – *Penicillium* spp.
[a] Other fungal pathogens – *Fusarium* spp. *Trichoderma* spp., unidentified fungal species.
[b] Spoilt peanuts: nuts that are separated from visually good kernels. They normally are comprised of broken, mouldy, discoloured, shrinkled kernels. They are sold in the markets but at a lower price.
[c] Other peanut products: Fried and roasted powdered nuts, nuts fried with masala, peanut cake, podded roasted nuts, roasted decoated peanuts and peanuts soaked before roasting.
[d] Least significant difference (Independent multivariate analysis, Fisher's protected LSD test, $p \leq 0.05$); ns – not significant.

while peanuts subjected to heat treatment – fried, roasted and boiled – were significantly less infected. A similar trend was observed in the isolation rate of *Penicillium* spp.

3.2. Incidence of *A. flavus* and *A. parasiticus* in peanuts

There were significant ($p \leq 0.05$) differences in isolation frequency of *A. flavus* (S- and L-strains) and *A. parasiticus* from different peanut products sampled from the three study provinces (Table 2). The incidence of the three pathogens ranged from 0 to 100%. Overall, peanut products from Western province were the most infected (mean = 43%), with no significant ($p \geq 0.05$) difference in infection levels of products sampled from Nairobi (mean = 36%) and Nyanza (mean = 36%) provinces. However, the highest incidence for individual products was recorded in Nairobi. *A. flavus* S-strain and *A. parasiticus* were isolated in the highest frequencies from spoilt peanuts (91% and 46%, respectively) sampled from Nairobi, while *A. flavus* L-strain was isolated from 100% of peanut flour samples from the same province.

Only samples from Nyanza were significantly ($p \leq 0.05$) more infected by *A. flavus* L-strain than by the S-strain. There was no significant ($p \geq 0.05$) difference in incidence of *A parasiticus* for samples from the three regions. Overall, the mean infection levels of the products by the three fungal pathogens were in decreasing order: peanut flour (69%), shelled raw peanuts (53%), spoilt peanuts (49%), boiled podded peanuts (45%), raw podded peanuts (39%), peanut butter (31%), fried peanuts (22%), roasted peanuts (20%) and other peanut products (47%).

3.3. Aflatoxin types associated with *A. flavus* and *A. parasiticus* isolates

Out of the 617 isolates of *A. flavus* S- and L-strains and *A. parasiticus* assayed, 73% produced at least one of the aflatoxin types, B1, B2, G1 or G2. However, 27% of the isolates did not produce any of the aflatoxin types with 18% of such isolates having been isolated from peanuts sampled from Western province. The most common aflatoxin type was B1, followed by B2, G1 and G2, with an incidence of 66%, 44%, 36% and 32%, respectively (Figs. 1 and 2).

There was no significant ($p \geq 0.05$) association between the incidence of specific aflatoxin types and the peanut product source of the isolate. A significantly ($p \leq 0.05$) higher proportion of isolates obtained from samples from Nyanza province (78%) were toxigenic for aflatoxin B1 compared to isolates from samples sourced from Western (58%) and Nairobi (59%) provinces. In contrast, a significantly ($p \leq 0.05$) higher proportion of isolates obtained from samples from Western (56%) and Nairobi (48%) were toxigenic for aflatoxin B2 compared to isolates from samples sourced from Nyanza province (30%). However, there was no significant ($p \geq 0.05$) association between the province of sample origin and aflatoxin G1 and G2 production.

3.4. Aflatoxin contamination levels of various peanut products

There was a significant ($p \leq 0.05$) variation in the total aflatoxin levels among peanut products ranging from 0 to 1629 μg/g (Table 3). The total aflatoxin levels varied among peanut products

Table 2
Incidence [%] of *Aspergillus flavus* and *A. parasiticus* from peanuts and peanut products sampled from Nairobi, Nyanza and Western provinces in Kenya.

Province	Peanut product	AF[S]		AF[L]		AP		Total [*n*]
Nairobi	Shelled raw peanuts	75.8	b-c	66.7	d-f	15.2	cd	33
	Peanut butter	21.2	j-l	21.2	jk	0.0	f	33
	Roasted peanuts	10.7	lm	13.3	kl	2.7	ef	75
	Fried peanuts	4.5	m	22.7	jk	0.0	f	22
	Peanut flour	88.9	a-c	100.0	a	22.2	bc	9
	Spoilt peanut	90.9	a	90.9	ab	45.5	a	11
	Others[a]	20.0	kl	40.0	hi	0.0	f	10
Nyanza	Shelled raw peanuts	58.3	e	74.8	c-e	13.6	d	103
	Podded peanuts	39.2	g-i	54.9	f-h	3.9	ef	51
	Peanut butter	44.1	f-h	55.9	f-h	14.7	cd	34
	Roasted peanuts	26.5	i-k	57.1	fg	14.3	cd	28
	Fried peanuts	50.0	e-g	75.0	b-e	0.0	f	4
	Spoilt peanut	50.0	e-g	60.0	e-g	0.0	f	10
	Boiled [podded] nuts	34.2	h-j	73.3	c-e	13.3	d	15
	Others[a]	5.6	m	33.3	ij	16.7	b-d	6
Western	Shelled raw peanuts	80.3	a-c	82.5	b-d	10.2	de	137
	Podded peanuts	62.5	de	62.5	e-g	12.5	d	8
	Peanut butter	53.8	ef	53.8	f-h	15.4	cd	13
	Roasted peanuts	25.0	jk	30.4	ij	1.8	f	56
	Peanut flour	89.3	ab	89.3	a-c	25.0	b	28
	Fried peanuts	50.0	e-g	0.0	l	0.0	f	2
	Spoilt peanut	50.0	e-g	50.0	gh	0.0	f	8
	Boiled [podded] nuts	75.0	c	75.0	b-e	0.0	f	3
	Others[a]	83.3	a-c	83.3	bc	0.0	f	6

AF[S] – *A. flavus* S-strain, AF[L] – *A. flavus* L-strain, AP – *A. parasiticus*, AA - *A. alliaceaus*, AC - *A. caelatus*, AT – *A. tamarii*.
Means followed by the same letter(s) within the column are not significantly different (Independent multivariate analysis, Fisher's protected LSD test, $p \leq 0.05$).
[a] Other peanut products: Fried and roasted powdered nuts, nuts fried with masala, peanut cake, podded roasted nuts, roasted decoated peanuts and peanuts soaked before roasting.

with spoilt peanuts (mean = 119,309 μg/kg), peanut flour (mean = 70,005 μg/kg) and peanut butter (mean = 19,502 μg/kg) being the most contaminated products while the corresponding products with the lowest average contamination levels were boiled podded peanuts (0.2 μg/kg), podded raw peanuts (405 μg/kg) and fried peanuts (1618 μg/kg). Aflatoxin contamination of 41% of the samples was above the acceptable 10 μg/kg limit based on KEBS standards.

3.5. Correlation between the population of Aspergillus species and aflatoxin contamination

There was a positive association ($r = 0.2711$) between the incidence of *A. flavus* and *A. parasiticus* and total aflatoxin level (Fig. 3).

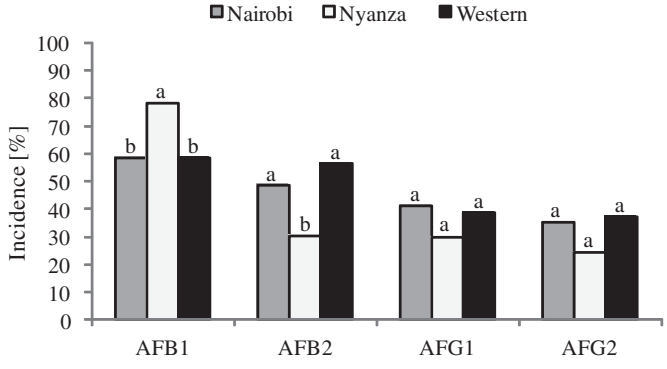

Fig. 1. Incidence of different aflatoxin types among cultures of *A. flavus* and *A. parasiticus* isolated from peanut samples obtained from traders in Nairobi, Nyanza and Western provinces, Kenya in 2009. AFB1 – Aflatoxin B1, AFB2 – Aflatoxin B2, AFG1 – Aflatoxin G1, AFG2 – Aflatoxin G2. Different letters indicate significantly ($p \leq 0.05$) different incidences of specific aflatoxin types among the provinces.

However, there was no significant ($p \geq 0.05$) association between sample type and aflatoxin level.

4. Discussion and conclusion

Since the 2004 aflatoxicosis outbreak in Kenya which resulted in 125 deaths (Lewis et al., 2005; Strosnider et al., 2006), greater global public attention has been drawn to aflatoxin and its associated health risk. Exposure of consumers in Kenya to aflatoxin remains a major health challenge that requires efforts aimed at documenting the exposure levels and subsequently implementing necessary intervention measures. Reviews by Hell and Mutegi (2011) and Wagacha and Muthomi (2008) highlight pre- and post-harvest intervention measures which can be adopted to reduce aflatoxin contamination of peanuts particularly in the tropics. Management of aflatoxin producing fungi in the field can be achieved through timely harvest and application of atoxigenic isolates of *A. flavus* which competitively exclude aflatoxin producers and, thereby, reduce aflatoxin levels in a crop (Cotty and Mellon, 2006; Dorner, 2004). Post harvest interventions that reduce aflatoxin levels include rapid and proper drying, proper transportation and packaging, sorting, cleaning, drying, smoking, post harvest insect control, and use of botanicals or synthetic pesticides as storage protectants (Hell and Mutegi, 2011; Wagacha and Muthomi, 2008). Maize and peanuts are the most susceptible crops to aflatoxin contamination (Lisker et al., 1993; Smith and Moss, 1985; Wu and Khlangwiset, 2010) and could be a significant pathway of introduction of aflatoxin to the human food chain. This study therefore focused on determining the incidence and diversity of aflatoxin-producing fungal species with a view to assessing the risk of aflatoxin contamination of raw peanuts and peanut products marketed in Kenya.

More than eight fungal species were isolated from raw peanuts and peanut products in the three study provinces with *A. flavus* (S- and L-strains) being the most commonly isolated. The mean fungal incidence for the different peanut products varied from 20% to 69%. Previous studies have reported isolation of diverse fungal pathogens from peanuts in eastern Africa (Gachomo et al., 2004; Ismail, 2001; Mutegi et al., 2012). High incidence (>65%) of *A. flavus*, *A. parasiticus* and *A. niger* in peanuts has been reported in Kenya (Gachomo et al., 2004; Mutegi et al., 2012) with *A. caelatus*, *A. alliaceus* and *A. tamarii* occurring in lower frequencies (Mutegi et al., 2012).

Several studies have quantified the relationship between the incidence of aflatoxin-producing fungi in peanuts and maize and levels of aflatoxin. A positive correlation has been reported between the incidence of toxigenic strains and aflatoxin B1 concentration in peanut cake in Nigeria (Ezekiel et al., 2013). A recent study by Mutegi et al. (2012) also reported a positive association between the incidence of *A. flavus* and *A. parasiticus* and levels of aflatoxin in peanuts sampled from three provinces in Kenya. *A. flavus* S-strain has been found to be the dominant strain in soil and maize within aflatoxicosis outbreak regions in Eastern Province of Kenya with the L-strain dominating in non-outbreak regions (Okoth et al., 2012; Probst et al., 2010). Okoth et al. (2012) further reported that *A. flavus* S-strain isolates produce relatively larger amounts of total aflatoxins, B toxins and lower levels of G toxins. In contrast, atoxigenic *A. flavus* L-strain isolates have been associated with lower aflatoxin content in maize when co-inoculated with a highly toxigenic *A. flavus* S-strain isolate (Probst et al., 2011). *A. flavus* is composed of phenotypically and genotypically diverse vegetative compatibility groups which vary in aflatoxin-producing abilities (Donner et al., 2010; Mehl et al., 2012; Probst et al., 2011). Under several biological control initiatives, application of atoxigenic *A. flavus* L-strains has successfully reduced aflatoxin

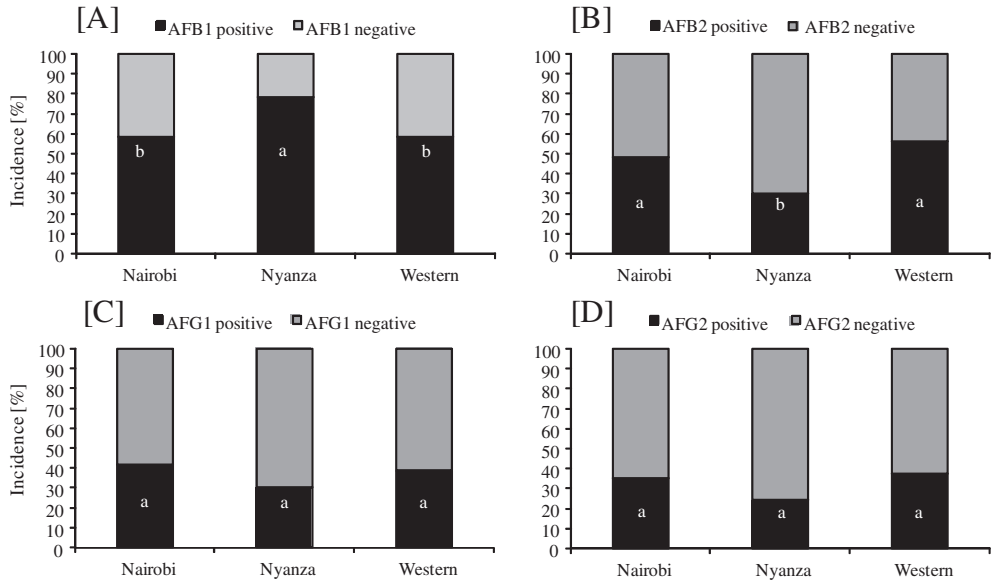

Fig. 2. Proportion [%] of *A. flavus* and *A. parasiticus* isolates producing different aflatoxin types: [A] Aflatoxin B1, [B] Aflatoxin B2, [C] Aflatoxin G1, [D] Aflatoxin G2. A total of 617 isolates obtained from 705 peanut and peanut products sampled from traders in Nairobi, Nyanza and Western provinces, Kenya in 2009 were screened for production of the four aflatoxin types with a thin-layer chromatography protocol. Different letters indicate significantly ($p \leq 0.05$) different incidences of toxigenic isolates among the provinces for specific aflatoxin types.

contamination of crops such as peanuts and maize through competitive exclusion of aflatoxin producers mainly the S strains (Cotty and Mellon, 2006; Mehl et al., 2012; Probst et al., 2011).

The high fungal diversity found in peanuts could be attributed to fluctuations in climate – erratic rainfall, high temperatures and high humidity – which expose the crop to conditions favourable for proliferation of aflatoxin-producers (Cotty and Jaime-Garcia, 2007; Wu and Khlangwiset, 2010). Additionally, smallholder production conditions in Kenya are conducive for fungal infection and high levels of aflatoxin production (Mutegi et al., 2009). Damage of pods and kernels during weeding, harvesting, drying, transportation and marketing favour colonization of kernels by aflatoxin-producing and other saprophytic fungi (Chapin et al., 2004), and subsequent aflatoxin contamination (Guo et al., 2009, 2003; Nigam et al., 2009). The high incidence of *A. flavus* strains in podded peanuts sampled from Western province confirmed the risk of pre-harvest infection of peanut kernels by aflatoxin-producing fungal species (Cotty, 1990; Waliyar et al., 2003). However, storage of raw peanuts in

Table 3
Proportion [%] of aflatoxin contamination level categories [μg/kg] for peanut products sampled from various market outlets in three provinces in Kenya.

Province	Peanut product	≤4	>4–10	>10	Range	Mean		Total [*n*]
Nairobi	Shelled raw peanut	28.1[a]	3.1	68.8	0.5–119,116	10,132	cd	32
	Roasted peanuts	46.7	8.0	45.3	0.0–85,531	2935	d	75
	Peanut butter	6.1	0.0	93.9	0.5–136,130	12,635	cd	33
	Peanut flour	0.0	11.1	88.9	9.0–120781	14,067	cd	9
	Fried peanuts	36.4	18.2	45.5	0.0–22,601	1839	d	22
	Spoilt peanut	0.0	9.1	90.9	5.6–56,572	14,328	cd	11
	Others[b]	22.2	11.1	66.7	0.0–2145	388	d	9
Nyanza	Podded raw nuts	100.0	0.0	0.0	0.0–0.5	0.0	d	49
	Shelled raw peanut	87.4	2.3	10.3	0.0–3044	63.9	d	87
	Roasted peanuts	85.0	10.0	5.0	0.0–20.9	2.1	d	20
	Peanut butter	58.6	10.3	31.0	0.0–58,647	2718	d	29
	Boiled podded nuts	100.0	0.0	0.0	0.0–2.2	0.2	d	15
	Fried peanuts	100.0	0.0	0.0	0.0–1.8	0.6	d	3
	Spoilt peanut	83.3	0.0	16.7	0.0–2152	359	d	6
	Others[c]	66.7	0.0	33.3	0.0–94,189	15,831	cd	6
Western	Podded raw nuts	50.0	0.0	50.0	0.0–20978	5362	d	4
	Shelled raw peanut	47.7	2.8	49.5	0.0–820,190	22,647	c	107
	Roasted peanuts	56.1	0.0	43.9	0.0–757949	24,488	c	41
	Peanut flour	10.0	0.0	90.0	0.0–820,190	97,973	b	18
	Peanut butter	22.2	0.0	77.8	0.0–582,013	98,761	b	9
	Spoilt peanut	25.0	0.0	75.0	2.2–1,628,692	586,431	a	4
	Others[d]	66.7	0.0	33.3	0.0–263	88.5	d	3

Means followed by the same letter(s) within the column are not significantly different (Independent multivariate analysis, Fisher's protected LSD test, $p \leq 0.05$).
[a] Limit of detection (LOD) = 0.5 μg/kg; 0 indicates below LOD.
[b] Peanut cake, nuts fried with masala and chicken mash.
[c] Peanut cake, nuts fried with masala and dehusked and flavoured nuts.
[d] Fried roasted peanuts, podded boiled peanuts, podded roasted peanuts.

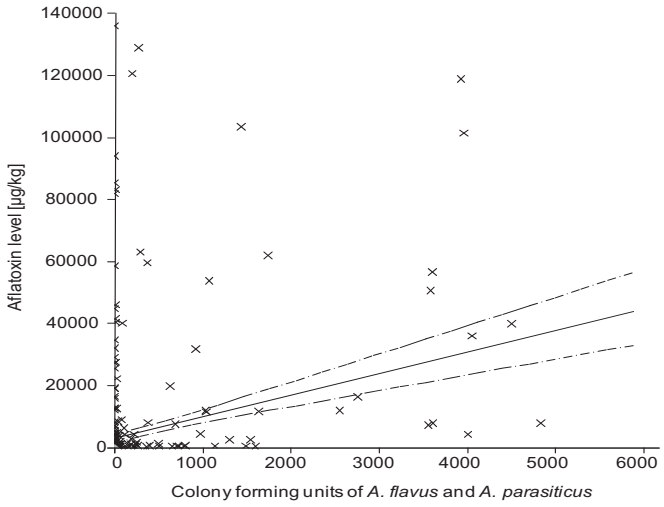

Fig. 3. Scatter plot of isolation incidence [CFU/g peanuts] of *A. flavus* and *A. parasiticus* against aflatoxin level. Fitted and observed relationship at $p \leq 0.05$. Outlier values were omitted in the scatter plot.

pods reduces fungal and aflatoxin contamination with the pods acting as a physical protection against fungal infection (Hell et al., 2000; Kaaya and Warren, 2005).

Peanuts sampled from Western province were significantly more infected by fungal pathogens than samples from Nairobi and Nyanza by a factor of 6.7 and 53.8, respectively. The higher infection rate in Western and Nairobi provinces could be attributed to peanut trade characteristics in the two provinces. Whereas majority of peanuts in Nyanza are locally produced, Western province is a vibrant trade corridor with neighbouring Uganda – a leading producer of peanuts in the region – through the Busia border. Similarly, there is no documented peanut production in Nairobi and therefore peanuts traded in the province are either sourced from other parts of Kenya or neighbouring countries with Uganda, Tanzania, Malawi and Zambia being the most important sources (data not shown). Environmental factors such as rainfall, humidity, temperature and respiration are likely to accelerate growth of fungal pathogens of peanuts during transportation by aflatoxigenic fungi. A recent study in Western Kenya by Mutegi et al. (2009) concluded that long trade chains pre-dispose peanut kernels to fungal infection and aflatoxin contamination with the mode and characteristics of transportation playing a significant role.

Among the eight fungal species identified in the current study, only *A. niger* and *Penicillium* spp. are not known producers of aflatoxin. Although *A. flavus* and *A. parasiticus* are the species most frequently implicated in aflatoxin contamination (Cotty, 2006), the wide fungal species diversity poses a risk of exposure of peanut consumers to other secondary metabolites associated with the species. Among the mycotoxins produced by *Penicillium* species are ochratoxins, patulin, citrinin, penicillic acid, cyclopiazonic acid, nephrotoxic glycopeptides and rubratoxin (Frisvad and Thrane, 2002; O'Brien et al., 2006; Weidenbörner, 2008), while *A. niger* produces, ochratoxin A and malformins among others (Frisvad et al., 2007; Noonim et al., 2009). With such diverse toxic metabolites associated with the fungal species isolated from peanut samples in the current study, the health risk posed to peanut consumers in Kenya requires greater research efforts for timely intervention.

Fungal communities resident in various locations differ widely in aflatoxin-producing potential (Donner et al., 2009; Garber and Cotty, 1997). Sixty six percent of the *A. flavus* and *A. parasiticus* isolates assayed in the current study tested positive for production of aflatoxin B1. In nature, aflatoxin B1 is the most prevalent aflatoxin type (Kumar et al., 2008). The high detection rate of aflatoxin B1 could also be attributed to inclusion of isolates of *A. flavus* and *A. parasiticus* in the metabolite's detection assays. *A. parasiticus* and some isolates of *A. flavus* produce both B- and G-aflatoxins (Barros et al., 2006; Cardwell and Cotty, 2002; Ehrlich et al., 2003). The inability of 27% of the isolates to produce any of the four assayed aflatoxin types is important as efforts for biological control using atoxigenic strains gain momentum in Kenya. The atoxigenic strains should be investigated further for their competitiveness and potential in biocontrol programs currently being undertaken in the country.

Eighty nine percent of peanut flour samples had aflatoxin levels above 10 µg/kg. Ease of colonization by fungal pathogens, increased surface area through milling and the hygroscopic nature of flour imply that peanut flour could be a preferred substrate for fungal proliferation and aflatoxin contamination compared to other peanut products. It is also possible that peanut flour was processed from low grade peanuts which could be difficult to sell as wholesome kernels and which were likely to be contaminated with aflatoxin. Due to its high nutritional value, peanut flour is used extensively as a weaning food in several developing nations (Plahar et al., 2005) including Kenya, posing a risk of aflatoxin exposure to the most vulnerable children (Bhat and Vasanthi, 2003; Gong et al., 2002). On the other hand, peanuts stored in pods were among the least contaminated with aflatoxin. This could be attributed to the protective role played by the pods against fungi that penetrate the kernels. Breaking of pods - through mechanical damage, by insects or during drought stress in the last stages of growth – increases chances of fungal infection and subsequent aflatoxin contamination of the kernels (Dorner et al., 1989; Hell et al., 2000; Kaaya and Kyamuhangire, 2006).

Worth noting, high aflatoxin levels were not necessarily correlated to high incidence of aflatoxin producing fungi in the peanut products especially heat processed products. Processing of peanuts using heat, e.g. roasting, reduces the population of fungal pathogens in peanuts without affecting aflatoxin levels (Mutegi et al., 2013). The high aflatoxin contamination level of spoilt nuts without a corresponding high incidence of *A. flavus* and *A. parasiticus* could be attributed to death of the aflatoxin-producing moulds after they had already infected the kernels and contaminated them with aflatoxin. This implies that testing – upon which rejection of peanuts and peanut products as unsafe should be based – is the only reliable method of confirming aflatoxin contamination in peanuts, but there are multiple methods to reduce fungal contamination which will also reduce subsequent toxin contamination.

The high incidence of aflatoxin producing fungal species in raw peanuts and peanut products from major markets in Kenya poses a health risk to consumers. This was supported by the positive correlation between the incidence of *A. flavus* and *A. parasiticus* and total aflatoxin level as well as the high percentage (41%) of peanuts which did not meet the KEBS standards (10 µg/kg for total aflatoxins). It is therefore imperative to continuously conduct microbial assays – particularly for raw peanuts – in order to determine the incidence and diversity of fungal species associated with peanuts in order to predict the risk of aflatoxin contamination and consequently develop appropriate intervention measures. Such strategies should also target non-aflatoxigenic fungal pathogens known to produce other mycotoxins. Stakeholders in the peanut trade in Kenya should also invest in sensitization of farmers, traders and consumers in order to promote sound practices at all stages of the peanut value chain in order to minimize access into markets by non-complying products.

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