MYCOLOGIA
https://doi.org/10.1080/00275514.2020.1804235

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Phylogenetic diversity and prevalence of mycoflora in ready-to-eat supermarket and roadside-vended peanuts

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ABSTRACT

Little is known of the mycobiota present in ready-to-eat peanuts consumed in Southern Africa. Knowledge of the mycobiota and aflatoxigenic species can elucidate potential health risks associated with consumption of ready-to-eat peanuts sold by supermarkets and roadside vendors. We investigated the culturable mycobiota diversity in supermarket and roadside-vended peanuts as well as the presence of five aflatoxin biosynthesis pathway–related genes (*aflR, aflJ, aflM, aflD*, and *aflP*) in 15 suspected aflatoxigenic isolates, with a focus on Mafikeng, South Africa. Mean colony-forming unit (CFU) counts of 288.7 and 619.7 CFU/g were observed in supermarket and roadside-vended peanuts, respectively. A total of 145 fungal isolates comprising 26 distinct taxa (based on 97% internal transcribed spacer region [ITS1-5.8S-ITS2] sequence similarity) were obtained, including strains representing *Aspergillus, Acremonium, Alternaria, Bipolaris, Chaetomium, Ectophoma, Epicoccum, Hamigera, Leancillium, Monascus, Penicillium, Periconia, Talaromyces*, and *Trichoderma*. Phylogenetic analyses of concatenated sequences of the ITS1-5.8S-ITS2, β-tubulin, and calmodulin genes delineated the species of A*spergillus*, which included *A. flavus, A. fumigatus, A. hiratsukae, A. niger*, and *A. parasiticus*. Higher species richness was obtained from supermarket peanuts compared with roadside-vended peanuts, with eight species common to both sources. Across supermarket or roadside-vended peanuts, *A. fumigatus, A. niger*, and *A. flavus* were prevalent (>40% incidence). In contrast, strains related to or representing *Ectophoma multirostata, Aspergillus hiratsukae, Bipolaris zeae, Chaetomium bostrychodes, Epicoccum nigrum, Hamigera paravellanea, Lecanicillium aphanocladii, Monascus ruber, Periconia macrospinosa, Periconia lateralis, Talaromyces funiculosus, Talaromyces minioluteus, Talaromyces wortmannii, Talaromyces* spp., and *Trichoderma* sp. were detected in either supermarket or roadside-vended peanuts. Among the five aflatoxin biosynthesis pathway–related genes, *aflD* and *aflM* were more prevalent (87%) and *aflR* was the least prevalent (40%). Findings suggest that roasted peanuts meant for human consumption and sold at supermarkets and by roadside vendors are contaminated with potential toxin-producing fungi. Hence, proper processing and packaging of peanuts before vending is recommended.

ARTICLE HISTORY
Received 28 May 2020
Accepted 29 July 2020

KEYWORDS
Aflatoxigenic species; *Aspergillus*; fungal diversity; phylogenetics; public health

INTRODUCTION

Cereals, nuts, and other agricultural produce stored as oil crops are usually accompanied by many microorganisms such as filamentous fungi, yeasts, and bacteria that can multiply under favorable conditions of their growth. The proliferation of microorganisms under such favorable conditions may induce chemical changes in the product and ultimately lead to lower product quality and damage to stored produce (Choudhary and Kumari 2010).

Amongst microorganisms that may be associated with stored agricultural produce, filamentous fungi pose the greatest threat to quality and safety. This is because many filamentous fungi produce toxins with significant economic effects in many crops, including wheat, maize, field pistachios, nuts, cotton seeds, and tea (Alshannaq and Yu 2017). It is estimated that 25% of the world's crop production is contaminated with fungal compounds (Eltariki et al. 2018). Such fungal contamination occurs during the different stages of production, transportation, or storage (Gustavsson et al. 2011; Salas et al. 2017).

Aspergillus species are among the frequent and widespread fungi that contaminate foods and agricultural produce (Reddy and Salleh 2011; Mahmoud et al. 2014). The relative dominance of *Aspergillus* species reflects their capacity to grow in otherwise unfavorable

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environmental conditions, including wide temperature ranges (5–45 C) and humidity conditions, as well as the ease of aerial dispersal of their spores.

Mycotoxins produced by *Aspergillus* and other moulds such as *Fusarium, Penicillium*, and *Alternaria* are especially important from an economic standpoint. At present, more than 200 types of fungal toxins (mycotoxins) are known to cause health risks to humans and animals (Zain 2011). The most common toxins are aflatoxins, ochratoxins, fumonisins, trichothecene, patulin, rubratoxin, citrinin, and zearalenone (Adetuniji et al. 2014). Large doses of aflatoxins can lead to aflatoxicosis and potentially fatal diseases, mainly through damage to the liver (WHO 2018). Aflatoxins also are genotoxic; they can damage DNA and cause cancer in some animal species (Bennett and Klich 2003). Aflatoxin B1 has been implicated as a potent liver carcinogen, causing hepatocellular carcinoma (HCC) in humans and a variety of animals (Smith 2003). It also has been classified as Group 1 human carcinogen by the International Agency for Research on Cancer, causing immune system disruption and stunted growth in children (Smith 2003; Adetunji et al. 2017).

Aflatoxins pose a major concern in the handling and consumption of peanuts in warm regions of the world (Adetunji et al. 2018). Peanuts are popular snacks in South Africa and are an important element in indigenous cuisines. The nutritional and organoleptic properties of peanuts, as well as some locally acclaimed advantages of peanut consumption, contribute to a robust demand for the nut in South Africa (Kamika et al. 2014).

Previous studies have evaluated the mycoflora in stored peanuts via methods such as traditional isolation, as well as enumeration and identification by phenotypic characteristics, including microscopic and whole-colony features (e.g., colony color, shape, pigmentation), and reproductive traits (Kozakiewicz 1989; Boland and Smith 1991; Klich 2002). However, these traditional identification characteristics may be unstable and can change under different environmental conditions and in some cases are uninformative (Gallo et al. 2012). Furthermore, polymorphic fungal strains within the same species may be misidentified especially if traditional methods of identification are not complemented with molecular markers such as the internal transcribed spacer (ITS) region. The ITS region has been utilized widely because it is a suitable phylogenetic marker for most fungal taxon assignments (Mello et al. 2011; Raja et al. 2017; see also Tedersoo et al. 2014; Oladipo et al. 2016; Ojuri et al. 2018; Ezeokoli et al. 2019). Insights from this marker can be congruent with those from other regions of the fungal genome (Mello et al. 2011; Arfi et al. 2012). However, for closely related species of the *Aspergillus* section *Flavi*, other genotypic markers such as the calmodulin (*caM*) and β-tubulin (*benA*) gene regions are often utilized in conjunction with morphological characteristics and phylogenetic analyses of the ITS region for further resolution (Samuels et al. 2002; Samson et al. 2006; Varga et al. 2011).

Although several authors have reported the incidence of fungi in peanuts in countries other than South Africa (Gachomo et al. 2004; Moore et al. 2018; Ojuri et al. 2018; Adetunji et al. 2018), to the best of our knowledge, no information is available on the cultivable mycobiota of peanuts consumed in South Africa (especially in the North West region, where this study is focused). Additionally, no previous studies have carried out the comparison of mycobiota present in roadside-vended peanuts and supermarket peanuts sold in North West Province and South Africa as a whole. Therefore, this study aimed to characterize the mycobiota diversity in peanuts sold in supermarkets and roadside-vended peanuts in South Africa. We used a combination of the ITS, *caM*, and/or *benA* gene sequences to identify fungal isolates obtained from peanut samples. Furthermore, the presence of the five most important genes in the aflatoxin biosynthesis pathway among *Aspergillus* isolates was explored.

MATERIALS AND METHODS

Sample collection and treatment.— Samples of roasted peanuts were collected from supermarkets and roadside vendors in Mafikeng City (25°51′S, 25°38′E), North West Province, South Africa. Sampling at the supermarkets was based on availability, with detailed information on the sampling location and methodology provided in an earlier paper (Adetunji et al. 2019b).

For supermarket and roadside-vended peanuts, three packs of roasted shelled peanut weighing approximately 100 g were purchased around the city where possible. Roasted peanuts from supermarkets were sold in laminated, vacuum-proof polypropylene pouches, whereas roadside-vended roasted peanuts were sold in transparent, manually tied, low-density polyethylene packs. In supermarkets where multiple peanut brands were sold, all available brands were purchased and considered distinct sample units.

The three packs of peanut collected from either supermarkets or roadside vendors were composited for each brand or vendor. In total, 52 composite peanut samples (30 from supermarkets and 22 from roadside vendors) were purchased. Samples were stored at room temperature (27 C) under dry condition prior to analysis.

For downstream analyses, each composite sample was divided into two equal portions. One portion was

pulverized in a Waring blender (model M20; IKA, Staufen im Breisgau, Germany) and stored in a zip-sealing bag at −20 C for fungal enumeration. The other portion was used for direct plating for fungal isolation.

Enumeration of mycobiota in peanut samples.— The population of fungi present in the peanuts were enumerated using the dilution plate techniques of Adetunji et al. (2019b). Appropriate 10-fold dilutions of pulverized peanut samples in sterile water were spread plated on potato dextrose agar (PDA) and incubated in the dark at 28 C for 3 d. Thereafter, the mycobiota present in each sample was enumerated and reported as colony-forming units per gram of peanut sample (CFU/g).

Evaluation of internal microbiota.— The direct seed plating method, as described by Adetunji et al. (2019b), was adopted for the isolation of the internal borne mycoflora in the peanuts. Six to eight peanut seeds (10–20 g) were surface-sterilized by 2% (*v*/*v*) sodium hypochlorite solution for 1 min and rinsed with three changes of sterile distilled water for 2 min. Six half-cotyledons were placed aseptically at equidistance in Petri dishes containing molten PDA containing 0.01% (*w*/*v*) chloramphenicol. Three replicates were made, and the plates were incubated in the dark at 25 C for 7 d. Each fungal colony was aseptically inoculated onto sterile PDA for final purification at 25 C for 5 d. Based on colony appearance (e.g., color, texture, size, and sclerotium formation), 61 distinct isolates and/or representatives were selected for DNA extraction.

Marker-based identification of fungal isolates.— Genomic DNA of fungal isolates was extracted via the ZR Fungal/Bacterial Soil Microbe DNA kit (Zymo Research, Irvine, California) following the manufacturer's protocol. For all isolates, the internal transcribed spacer region (ITS1-5.8S-ITS2 = ITS; approximately 550–600 bp) was amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The polymerase chain reaction (PCR) conditions were 95 C for 2 min, 35 cycles of 95 C for 30 s, 54 C for 30 s, and 72 C for 1 min, and an additional 72 C for 10 min (Adetunji et al. 2019b).

Species-level resolution of closely related filamentous *Aspergillus* species was determined by amplification and sequencing of the β-tubulin (*benA*) and calmodulin (*caM*) genes following Hong et al. (2008) and Glass and Donaldson (1995), respectively, with a few modifications. For *benA* (approximately 500 bp), the primer pair ben2f (5′-TCCAGACTGGTCAGTGTGTAA-3′) and bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass and Donaldson 1995; Hubka et al. 2013) was used, with initial denaturation of 94 C for 3 min, 32 cycles of 94 C for 45 s, 55 C for 45 s, and 72 C for 1 min, and a final extension at 72 C for 5 min. For *caM* (approximately 550 bp), the primer set CMD5 (5′-CCGAGTACAAGGAGGCCTTC-3′) and CMD6 (5′-CCGATAGAGGTCATAACGTGG-3′) designed by Hong et al. (2008) was used, with 94 C for 1 min, 35 cycles of 94 C for 1 min, 55 C for 30 s, and 72 C for 1 min, and a final extension of 72 C for 10 min. All PCRs were performed in 25 µL reaction volume containing 8.5 µL nuclease-free water, 12.5 µL 2× PCR Master Mix (New England Biolabs, Ipswich, Massachusetts), 0.4 µM of each forward and reverse oligonucleotide primer, and 2 µL of template DNA. All amplicons were verified after electrophoresis on a 1% (*w*/*v*) agarose gel. Sequencing was conducted on an ABI Prism 3500XL DNA Sequencer (Applied Biosystems, Foster City, California) at the Inqaba Biotechnical Industrial, Pretoria, South Africa.

Operational taxonomic units, taxonomic assignment, and phylogenetic relatedness.— All sequence electropherograms were manually inspected and edited using Chromas Lite 2.1 (Technelysium, South Brisbane, Australia). Thereafter, edited ITS sequences were clustered into operational taxonomic units (OTUs) at 97% ITS sequence similarity in mothur 1.37.2 (Schloss et al. 2009). Subsequently, OTU representatives were selected based on the sequence with the smallest maximum distance to the other sequences in each cluster. Representative sequences were then used for a taxonomic assignment against the UNITE database (Nilsson et al. 2019). Furthermore, ITS sequences in the database, as well as those of partial *benA* and *caM* genes of closely related type strains in the GenBank, were selected for phylogenetic analysis as previously described (Ojuri et al. 2018).

For closely related *Aspergillus* species, phylogenetic analysis was based on concatenated ITS, *benA*, and *caM* sequences. For the combined analysis, multiple sequence alignments generated separately for the ITS region (542 sites/142 variable), partial *benA* (444 sites/247 variable sites), and partial *caM* (550 sites/321 variable sites) in MUSCLE (Edgar 2004) were edited for gaps and thereafter concatenated to generate a total of 1536 sites (with 710 variable sites). Prior to phylogenetic tree construction, model selection for the tree was determined based on the lowest Bayesian information criterion in MEGA X (Kumar et al. 2018). MEGA X was further used for all phylogenetic inferences, by employing the Kimura 2-parameter model with rates among site based on a gamma distribution with invariant sites. Support for

tree branching was based on 1000 bootstrap replications. Sequences of isolates are available in the GenBank under the accession numbers MT079270–MT079329 (for ITS), MT427837–MT427865 (for *caM*), and MT445993–MT446022 (for *benA*).

PCR detection of aflatoxin-producing genes of *Aspergilli*.— End-point PCR was used to evaluate suspected *Aspergillus* isolates for the presence of five genes (*aflR, aflJ, aflM, aflD*, and *aflP*) that are part of the 25-gene cluster involved in the aflatoxin biosynthetic pathway (Yu et al. 2004). Among these, *aflD, aflM*, and *aflP* are biosynthesis genes that code for proteins involved in the early, middle, and last stages, respectively, of the aflatoxin biosynthetic pathway. In turn, *aflR* and a*flJ* are regulatory genes that coordinate the activities of the structural genes (Yu et al. 2004; Abdel-Hadi et al. 2010; Norlia et al. 2019).

For the evaluation, 15 *Aspergilli* isolates that previously tested positive for aflatoxin production using thin-layer chromatography (TLC) analysis (results not shown) were selected. The genes, primer sequences, and product sizes are shown in TABLE 1. PCR conditions were optimized separately for the target genes, as shown in TABLE 1. Except for the primer sets, PCR composition was the same as reported earlier for ITS, *benA*, and *caM* amplifications. The presence of genes was determined based on the presence or absence of bands on 1% (*w*/*v*) agarose gel following electrophoresis.

Statistical analysis.— The mean fungal colony-forming units (CFU)/g of peanut from the two different points of purchase (supermarkets and roadside vendors) were compared by one-way analysis of variance (ANOVA) in SPSS 20.0 (IBM, Armonk, New York). Statistical significance was set at probability (P) < 0.05.

RESULTS

Mycobiota load and diversity in peanut samples: Supermarket vs. roadside.— A total of 145 isolates was recovered from peanuts (supermarket, 89; roadside, 56). The mean fungal colony-forming units per gram was significantly (P < 0.05) higher in roadside-vended peanuts (619.70 CFU/g) than in supermarket-retailed peanuts (288.71 CFU/g). The cultivable mycobiota richness count (based on colonial appearance) ranged from 0 to 8 and 0 to 18 for the supermarket and roadside-vended peanut samples, respectively (data not shown).

Based on morphological characteristics, 61 representative isolates were selected for further analysis. These represented 26 OTUs (TABLE 2), with OTU7 most abundant (SUPPLEMENTARY FIG. 1). The total

Table 1. PCR primers and conditions used for detecting aflatoxin-related genes in selected *Aspergillus* isolates.

Target gene	Product size	Primer information (5′→3′)	Optimized conditions	Reference
aflR	1032 bp	AflR1 (F): TATCTCCCCCGGGCATCTCCGG AflR2 (R): CCGTCAGACAGCCACTGGACACGG	95 C for 4 min; 30 cycles of 95 C for 1 min, 60 C for 1 min, and 72 C for 30 s; and 72 C for 10 min	Shapira et al. 1996
aflJ (*estA*)	684 bp	AflS (F): TGAATCCGTACCCTTTGAGG AflS (R): GGAATGGGATGGAGATGAGA	95 C for 10 min; 30 cycles of 95 C for 50 s, 58 C for 50 s, and 72 C for 2 min; and 72 C for 10 min	Meyers et al. 1998
aflM (*Ver*)	537 bp	Ver1 (F): GCCGCAGGCCGCGGAGAAAGTGGT Ver2 (R): GGGGATATACTCCCGCGACACAGCC	95 C for 4 min; 30 cycles of 95 C for 1 min, 58 C for 1 min, and 72 C for 30 s; and 72 C for 10 min	Geisen 1996
aflD (*Nor*)	400 bp	Nor1 (F): ACCGCTACGCCGGCACTCTCGGCAC Nor2 (R): ACCGCTACGCCGGCACTCTCGGCAC	94 C for 10 min; 33 cycles of 94 C for 1 min, 65 C for 1 min, and 72 C for 2 min; and 72 C for 5 min	Geisen 1996
aflP (*omt-A*)	797 bp	omt1 (F): GTGGACGGACCTAGTCCGACATCAC omt2 (R): GTCGGCGCCACGCACTGGGTTGGGG	94 C for 5 min; 33 cycles of 94 C for 1 min, 75 C for 1 min, and 72 C for 2 min; and 72 C 10 min	Geisen 1996

Table 2. Operational taxonomic unit (OTU) abundance and taxonomic diversity of mycobiota based on ITS (ITS1-5.8S-ITS2) sequences.

		UNITE ITS database match	
OTU no. (Strain ID)	Accession number	Closest match	% Similarity
OTU1 (NWU8)	MT079276	*Acremonium persicinum*	100
OTU2 (NWU63)	MT079329	*Alternaria alternata*	100
OTU3 (NWU62)	MT079328	*Ectophoma multirostrata*	100
OTU4 (NWU1)	MT079270	*Aspergillus flavus*	100
OTU5 (NWU21)	MT079289	*Aspergillus fumigatus*	100
OTU6 (NWU58)	MT079325	*Aspergillus hiratsukae*	100
OTU7 (NWU28)	MT079296	*Aspergillus niger*	100
OTU8 (NWU54)	MT079321	*Aspergillus parasiticus*	100
OTU9 (NWU27)	MT079295	*Bipolaris zeae*	100
OTU10 (NWU59)	MT079326	*Chaetomium bostrychodes*	100
OTU11 (NWU46)	MT079314	*Epicoccum nigrum*	100
OTU12 (NWU56)	MT079323	*Epicoccum sorghinum*	100
OTU13 (NWU6)	MT079275	*Epicoccum sorghinum*	100
OTU14 (NWU17)	MT079285	*Hamigera paravellanea*	100
OTU15 (NWU12)	MT079280	*Lecanicillium aphanocladii*	100
OTU16 (NWU15)	MT079283	*Monascus ruber*	100
OTU17 (NWU26)	MT079294	*Penicillium rubens*	100
OTU18(NWU25)	MT079293	*Talaromyces funiculosus*	100
OTU19 (NWU47)	MT079315	*Periconia macrospinosa*	100
OTU20 (NWU13)	MT079281	*Periconia lateralis*	100
OTU21 (NWU40)	MT079308	*Talaromyces islandicus*	100
OTU22 (NWU45)	MT079313	*Talaromyces minioluteus*	100
OTU23 (NWU14)	MT079282	*Talaromyces* sp.	100
OTU24 (NWU42)	MT079310	*Talaromyces wortmannii*	100
OTU25 (NWU20)	MT079288	*Talaromyces* sp.	100
OTU26 (NWU11)	MT079279	*Trichoderma* sp.	100

richness in supermarket samples was higher than in roadside-vended peanuts (25 vs. 9 OTUs, respectively). Only 8 OTUs were common to both supermarket and roadside-vended peanuts.

The fungi identified in this study all belong to the Ascomycota and are closely related to or represent species of *Aspergillus* (five species), *Acremonium, Alternaria, Bipolaris, Chaetomium Ectophoma, Epicoccum* (two species), *Hamigera, Lecanicillium, Monascus, Penicillium, Periconia* (two species), *Talaromyces* (five species), and *Trichoderma* (TABLE 2). The taxonomic delineation of isolates in TABLE 2 is supported by ITS sequence–based maximum likelihood phylogenetic trees (data not shown). However, phylogenetic reconstruction using concatenated ITS-*benA*-*caM* sequences suggests that isolate strain NWU21 is distinct from type cultures of *Aspergillus fumigatus* (FIG. 1).

Aspergillus species were the most prevalent in both supermarket and roadside-vended peanuts in the study area (FIG. 2), particularly *Aspergillus fumigatus*. Other species with high incidence (>40% in at least supermarket or roadside-vended peanuts) included *A. flavus* and *A. niger* (FIG. 2). *Ectophoma multirostata* was detected in roadside-vended peanuts (incidence of 9.1%, two samples), but it was not detected in supermarket samples. In contrast, species closely related to or representing *Aspergillus hiratsukae, Bipolaris zeae, Chaetomium bostrychodes, Epicoccum nigrum, Hamigera paravellanea, Lecanicillium aphanocladii, Monascus ruber, Periconia macrospinosa, P. lateralis, Talaromyces funiculosus, Talaromyces miioluteus, Talaromyces wortmannii, Talaromyces* spp., and *Trichoderma* sp. were detected in supermarket peanuts but not in roadside-vended peanuts (FIG. 2).

Prevalence of aflatoxin-related genes.—The presence of at least one of the five genes involved in the aflatoxins biosynthetic pathway was detected in each of the 15 suspected aflatoxin positive isolates (FIG. 3). The overall incidence of these genes varied from 40% (6/15) to 87% (13/15), with *aflD* and *aflM* genes having the highest incidence (87%) and *aflR* being the least prevalent gene in the isolates (FIG. 3).

DISCUSSION

Handling practices, mode of transportation, and storage facilities for foods and agricultural produce influence the microbial load and diversity in such foods or produce (Adetunji et al. 2019b). Microbial proliferation in foods could occur at different stages of the food chain, including at the point of sale (Waliyar et al. 2015). This study investigated whether the fungal community in peanut samples sold in Mafikeng, South Africa, differs when peanuts are sourced from supermarkets as opposed to roadside vendors. We further investigated the

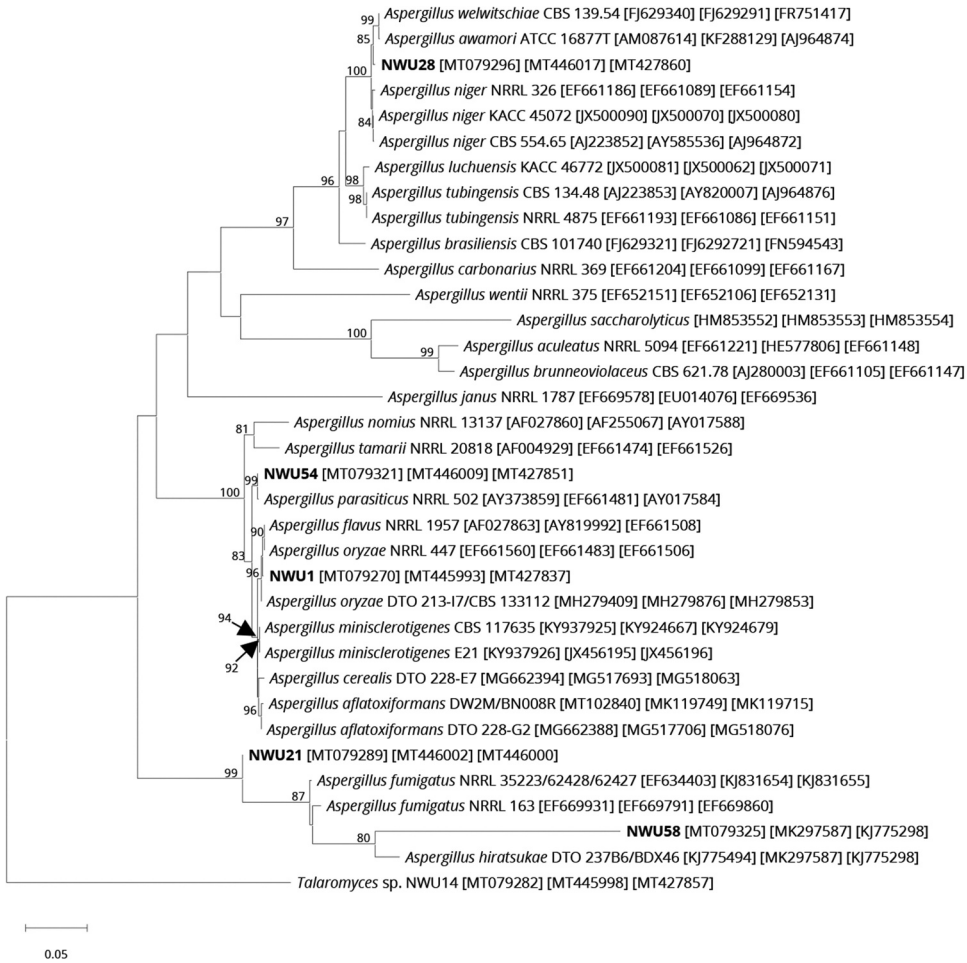

Figure 1. Maximum likelihood phylogenetic tree showing evolutionary relatedness of *Aspergillus* isolates with those of type cultures in the GenBank. The phylogenetic tree was constructed using concatenated ITS-*benA*-*caM* sequences. Support for tree branches was based on 1000 bootstrap replications. Bootstrap values less than 70 are not shown. Accession numbers of sequences are in square brackets in the order of ITS, *benA*, and *caM* genes. Tree is rooted on *Talaromyces* sp.

prevalence of five genes related to the aflatoxin biosynthesis pathway in *Aspergillus* isolates.

The prevalence of fungi in peanuts reported here corroborates the findings of Kamika et al. (2014) who recorded fungal loads ranging from 40 to 21 000 CFU/g in peanut samples from Pretoria, South Africa. Peanuts and oilseeds are highly vulnerable to fungal attack at both pre- and postharvest stages either due to a variety of factors, including certain agricultural practices, storage systems/techniques, and/or transportation systems through the distribution chain. Because we do not know the origin of the peanuts prior to arrival at the supermarket or roadside vendor, it is unclear whether the high fungal load observed here and by Kamika et al. (2014) reflects processing, handling, or storage techniques.

The relatively higher phylotype richness in supermarket samples compared with roadside-vended peanuts may be as a result of prolonged storage periods and other extrinsic factors such as storage conditions and quality of packaging material. The effect of quality and type of packaging material on the quality attributes of peanut during storage has been reported (Adebiyi et al. 2002; Fu et al. 2018). The type of packaging material has a significant influence on peanut quality index. The most commonly used packaging material for roadside-vended peanuts is low-density polyethylene bags, which are favored because they are inexpensive. However, polyethylene provides a low barrier quality to oxygen and relative humidity, potentially encouraging fungal growth (Fu et al. 2018). In contrast, the supermarket peanuts were packaged in polypropylene materials ranging from laminated to nonlaminated, transparent, or opaque polypropylene pouches. Polypropylene bags possess excellent chemical resistance and strength. Moreover, unlike low-density polyethylene bags, polypropylene bags are not breathable, ultimately promoting longer product shelf-life. Most of the polypropylene pouches for peanuts were laminated or tinted to prevent

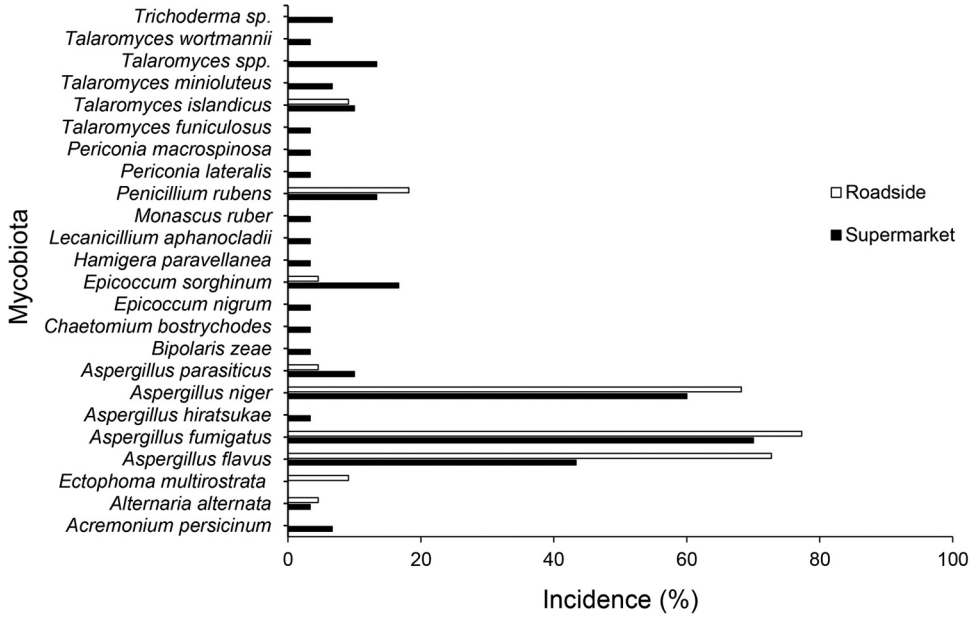

Figure 2. Incidence of mycobiota in the supermarket and roadside-vended peanuts.

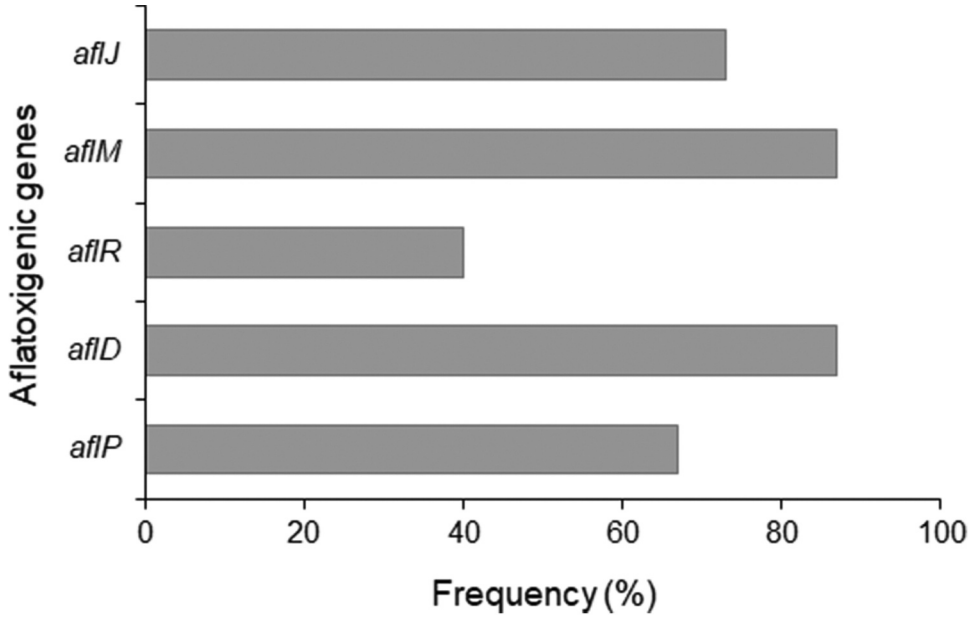

Figure 3. Incidence of aflatoxigenic genes in *Aspergilli*. Incidence was expressed as the percentage of the quotient of the number of *Aspergillus* isolates in which the respective genes were detected and the number of *Aspergillus* (15) isolates investigated.

direct exposure to light, which could lead to darkening of nuts but might also support fungal proliferation (Fu et al. 2018).

Most of the roadside vendors sell freshly roasted peanuts in small quantities. In contrast, roasted peanuts sold in the supermarket undergo a long distribution chain, starting from the processor through to the distributors and retailers. The high richness of fungi in supermarket peanuts may reflect the display of peanuts on shelves for a more extended period than expected due to low patronage and/or fluctuations in storage temperature due to the use of air conditioner during the day (cool temperature) and room temperature (warmer temperature) after work hours. A study conducted in China by Xing et al. (2016) on the influence of storage conditions and time on mycobiota of stored shell and unshelled peanuts revealed that the population of *Aspergillus* fungi was directly proportional to the length

of storage whereas the population of *Rhizopus* fungi decreased with increase in storage duration.

Contrary to the findings of Gachomo et al. (2004), who observed that *R. stolonifer* was the most predominant (80% occurrence) mould, followed by *Fusarium* sp. (45%) and *Aspergillus* species (24%), in peanut samples from Nairobi, Kenya, species of *Rhizopus* and *Fusarium* were not found in the peanut samples investigated in this study. However, the prevalence of *Aspergillus* species in the South African peanut corroborates previous reports on fungal biodiversity in peanuts from different parts of the world (Gachomo et al. 2004; Rossetto et al. 2005; Nakai et al. 2008). *Aspergillus* species are economically important fungal species, especially the mycotoxin-producing *Aspergillus* such as *A. flavus, A. niger*, and *A. parasiticus*. The prevalence of these potentially toxigenic species in peanuts is of serious concern. The incidence of *Aspergillus* spp. and other toxigenic fungi in raw peanuts and peanut-based products has been noted previously, particularly in tropical and subtropical climate regions (Gachomo et al. 2004). These climatic conditions naturally support the growth of species belonging to the *Aspergillus* section *Flavi*, especially toxigenic *A. flavus, A. parasiticus, A. nomius*, and *A. minisclerotigenus*, amongst others. These species produce aflatoxins that can cause aflatoxicosis and, at times, death in humans (Lewis et al. 2005; Wild and Gong 2010).

In the present study, the prevalence of a fungus representing or related to *Hamigera paravellania* in the supermarket and roadside-vended peanuts is worthy of investigation. *Hamigera paravellania* is a lesser-known species within order Eurotiales, which also contains genera such as *Penicillium, Aspergillus, Talaromyces, Monascus*, and *Paecilomyces*, all commonly found in soil. The organism had been isolated from soil samples in different countries, including the USA, Spain, and India (Igarashi et al. 2014). One of the major features of many species of the Eurotiales in the broad sense is their ability to produce secondary metabolites (Inglis et al. 2013). At least five metabolite families have been extracted and characterized from *Hamigera paravellania*, including the anthraquinone pigments emodin, ω-hydroxyemodin, and emodic acid (Natori et al. 1965; Cehulová et al. 1996; Isaka et al. 2010). Similarly, hamigerone and dihydrohamigerone are polyketide antifungal metabolites obtained from *Hamigera* species (Breinholt et al. 1997). In a study by Igarashi et al. (2014), avellanins A and B were identified as the most commonly produced metabolites by strains of *Hamigera*. The effect of these metabolites on human and animals is not yet known. To the best of our knowledge, this study reports for the first time the incidence of *H. paravellanea* in peanut.

Penicillium spp. are among the agriculturally important fungi whose presence in food should raise concern. With the exception of the *Aspergillus* spp., *Penicillium* spp. were the most abundant phylotype in the peanut samples. They have been implicated as the producers of citrinin, penicillic acid, ochratoxin A, and patulin (Perrone and Susca 2017). El-Banna et al. (1987) reported 18 different mycotoxins produced by various species of *Penicillium*. These mycotoxins include brevianamid A, citreoviridin citrinin, cyclopiazonic acid, ochratoxin A, patulin, penicillic acid, among others. Ochratoxin A and patulin are the most important of all toxins produced by *Penicillium* spp., and regulations for the toxins have been imposed in many countries (Perrone and Susca 2017). Ochratoxin A is a nephrotoxin, and its negative effect on fetal development and immune function had been reported (Pitt 2002). Indications are that ochratoxin A is also responsible for kidney degeneration in humans, where in extreme cases may lead to death (Pitt 2002). On the other hand, patulin is reported to be genotoxic and causes nausea, gastrointestinal disturbances, and vomiting in human (WHO 2018). The economics and health impact of these mycotoxins on humans and animals cannot be overemphasized; hence, the richness of *Penicillium* species in the peanut sample could indicate that the consumers of peanut might be at risk of exposure to acute or chronic toxicity when sufficient amounts of contaminated peanut are consumed (Adetunji et al. 2017). A call for mitigation strategies by relevant agencies in South Africa is needful, especially because peanut is a favorite snack consumed among the populace.

The findings of this report revealed the presence of *aflD* and *aflM* genes in larger percentage (87%; 13/15) of the *A. flavus* isolates examined and *aflR* to be the least frequent gene (40%) in the isolates. The *aflP* gene (*omt-A*) mediates the conversion of sterigmatocystin (ST) to *O*-methylsterigmatocystin in the aflatoxin biosynthetic pathway, *aflD* mediates the conversion of norsoloniric acid, the first stable product in the aflatoxin biosynthesis pathway, to averufanin through the activity of the ketoreductase enzyme, whereas *aflM* mediates the conversion of averatin to versicolorin A (Yu et al. 2004; Ryan et al. 2009). The high incidence of *aflD* and *aflM* genes amongst the selected isolates corroborates findings from previous studies by Adetunji et al. (2019b) and Norlia et al. (2019) who reported high percentages of these genes in *A. flavus* isolated from cashew nut and peanut, respectively. The presence of all aflatoxin biosynthesis pathways genes in the isolates indicates that the isolates have the potential to produce aflatoxin under favorable physiological and environmental conditions (Abdel-Hadi et al. 2010; Adetunji et al. 2019a, 2019b). However, it is important to note that the detection of such genes is not the sole factor in evaluating the

potential of these isolates to produce aflatoxins. Indeed, factors such as frameshift (insertion/deletions) mutations and environmental cues may affect the transcription and expression of the functional gene products (i.e., aflatoxins). Nevertheless, the detection of these genes to a large extent confirms our preliminary TLC analysis data that showed that the 15 isolates are aflatoxin producers.

In conclusion, roasted peanuts meant for human consumption bought at the supermarket and from roadside vendors are contaminated with potential toxin-producing fungi whose toxins when released into the peanut are capable of imposing threat to consumers. Contrary to our prestudy expectation, the phylotype richness, including potential mycotoxin-producing species, was higher in supermarket sold peanuts compared with the roadside-vended peanuts. On the other hand, the use of the low-quality, low-density polyethylene nylon, which has poor resistance to oxygen and excessive light penetration, for packaging of roadside-vended peanuts as opposed to the laminated, vacuum-proof polypropylene pouches of supermarket peanuts likely predisposes high fungal load in roadside-vended peanuts and consequently negative health impact on consumers of roadside-vended peanuts. Hence, indications are that consumers of roasted stored ready-to-eat peanuts might be at risk of mycotoxicosis. Poor quality control practices, inadequate storage conditions, and prolonged duration of shelf storage are potential predisposing factors for peanut contamination by toxigenic fungi. We recommended that peanut should be processed appropriately and packaged and dried to the best acceptable limit prior to circulation via the value distribution chain. In addition, the choice of packaging material should be informed by the intended length of the storage period.

Further characterization studies are required to investigate isolate strain NWU21 to determine whether it does constitute a new strain distinct from type cultures of *Aspergillus fumigatus*. In addition, further quantification studies are required to investigate the presence and levels of aflatoxins and other mycotoxins in the ready-to-eat peanuts.

FUNDING

This study was funded by National Research Foundation South Africa (grant UID: 105882) and supported by the Food Safety Niche Area, North-West University, South Africa.

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