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Mycobiota of ground red pepper and their aflatoxigenic potential

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To investigate contamination of ground red pepper with fungi and mycotoxin, we obtained 30 ground red pepper samples from 15 manufacturers in the main chili-pepper-producing areas in Korea. Fungal contamination was evaluated by spreading diluted samples on potato dextrose agar plates. The total fungi counts ranged from 0 to 7.3×10^3 CFU/g. In the samples, the genus *Aspergillus* had the highest incidence, while *Paecilomyces* was isolated most frequently. The next most frequent genera were *Rhizopus*, *Penicillium*, *Cladosporium*, and *Alternaria*. Within *Aspergillus*, *A. ruber* was predominant, followed by *A. niger*, *A. amstelodami*, *A. ochraceus*, *A. terreus*, *A. versicolor*, *A. flavus*, and *A. fumigatus*. The samples were analyzed for aflatoxins, ochratoxin A, and citrinin by ultra-perfomance liquid chromatography (UPLC) with a fluorescence detector. Ochratoxin A was detected from three samples at 1.03–2.08 μg/kg, whereas no aflatoxins or citrinin were detected. To test the potential of fungal isolates to produce aflatoxin, we performed a PCR assay that screened for the *norB-cypA* gene for 64 *Aspergillus* isolates. As a result, a single 800-bp band was amplified from 10 *A. flavus* isolates, and one *Aspergillus* sp. isolate. UPLC analyses confirmed aflatoxin production by nine *A. flavus* isolates and one *Aspergillus* sp. isolate, which produced total aflatoxins at 146.88–909.53 μg/kg. This indicates that continuous monitoring of ground red pepper for toxigenic fungi is necessary to minimize mycotoxin contamination.

***Keywords*:** ground red pepper, fungi, *Aspergillus*, aflatoxin

Introduction

The global production of dried red pepper is increasing annually (Kim, 2014). Red pepper (*Capsicum annuum* L.) is widely used in Korean food as a spice, especially in Kimchi and red pepper paste. Annually, 160,000 tons of red pepper are produced in Korea and Koreans consume 2–3.5 kg of red pepper power per person every year (Kim *et al*., 2011).

During storage and processing, spices and herbs, including ground red pepper (e.g. chili powder, red pepper powder, and red chili pepper flakes) are easily contaminated by molds producing mycotoxins such as aflatoxin (AF) and ochratoxin A (OTA) (Fazekas *et al*., 2005; Salari *et al*., 2012). Mycotoxins are secondary metabolites produced by fungi and are harmful to humans and animals. There are more than 400 known mycotoxins, of which AF, produced mainly by *Aspergillus flavus* and *A. paraciticus*, is the most potent toxin found in nature. AF is also a group 1A carcinogen classified, as classified by the International Agency for Research on Cancer (IARC). OTA, which is produced by *Penicillium verrucosum*, *Aspergillus ochraceus*, and *A. niger* (Bellí *et al*., 2004), can cause chronic kidney disease (Pfohl-Leszkowicz and Manderville, 2007). OTA is classified as a group 2B carcinogen by IARC. Due to their high toxicity, the levels of AFs and OTA in contaminated food are regulated worldwide. In Korea, the maximum limit (ML) of total AFs in ground red pepper is 15 ppb (AFB_1, 10 ppb), while that of OTA is 7 ppb.

Several studies have examined fungal and mycotoxin contamination in red pepper/powder. Bokhari (2007) reported that *A. niger* was predominant in red pepper in Saudi Arabia, followed by *A. flavus*. In Turkey, *Penicillium* was found to be the dominant genus in ground red pepper, followed by *Rhizopus* and *A. niger* (Erdogan, 2004). In Korea, Ko *et al*. (2004) isolated 197 fungal species from discolored red pepper, including *Colletotrichum*, *Diaporthe*, and *Alternaria* as main contaminants. Regarding mycotoxins, Aydin *et al*. (2007) reported that of 100 samples of powdered red pepper collected in Istanbul, Turkey, AFB_1 was detected from all samples and 18 samples exceeded the ML (5 μg/kg). In Hungary, Fazekas *et al*. (2005) reported that 18 and 10 out of 70 samples were contaminated with AFB_1 and AFB_2, respectively, and 7 of them exceeded ML (5 μg/kg); OTA was simultaneously found in 32 samples and 8 of them exceeded the ML (10 μg/kg). There are several reports of mycotoxin contamination in Korea. Lee *et al*. (2013) found that 15 of 28 ground red pepper samples were contaminated with AFs, but were under the ML, and OTA was detected in 19 samples, but only one exceeded the ML (17.16 μg/kg). In another study of 192 samples, 2 and 42 were contaminated with AFs and OTA, respectively, and six of them exceeded the ML for OTA (Kim *et al*., 2009).

Several factors affect the microbial diversity of red pepper powder, such as pepper variety, climate conditions, and the cultivation environment. Few studies have examined the fungal and mycotoxin contamination of red pepper powder in Korea. To determine the current status of ground red pepper contamination by mycotoxigenic fungi, we investigated the mycobiota and mycotoxin contamination of ground red pepper obtained from the main chili pepper manufacturers in Korea. We also tested the AF production by some *Aspergillus* isolates, revealing their aflatoxigenic potential and

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Table 1. Number of ground red pepper samples used in this study

Province	Number
Gangwon	2
Chungnam	4
Chungbuk	2
Gyeongbuk	10
Gyeongnam	2
Jeonnam	4
Jeonbuk	6
Total	30

possible contamination.

Materials and Methods

Sampling and fungal occurrence

In 2015, we purchased 30 ground red pepper samples directly from 15 chili pepper powder manufacturers located in the main chili-pepper-producing areas in Korea (Table 1). All samples were stored at 4°C after purchase.

To investigate fungal occurrence in the samples, 1 g of each sample was mixed with 9 ml of sterile water. Each mixture was vortexed vigorously for 1 min. and then 200 µl of the mixture was spread onto potato dextrose agar (PDA, Difco) plates. After incubation at 25°C for 5 d, the growing fungal colonies were counted from 10 plates. The frequency of fungal occurrence was calculated as colony forming units (CFU) per gram of sample.

Fungal characterization

Fungal DNA was extracted using a described method (Lee *et al*., 2015). Fungal isolates were identified using β-tubulin or ITS genes. PCR was performed with primers Bt2a/Bt2b (β-tubulin) and ITS4/ITS5 (ITS), as reported (White *et al*., 1990; Glass and Donaldson, 1995). To screen for the AF biosynthesis gene, PCR was used to amplify the *norB-cypA* region using primers AP1729/AP3551, as described (Ehrlich *et al*., 2004; Hong *et al*., 2013). To screen for the ochratoxin/citrinin biosynthesis gene, the polyketide gene was amplified using AoLC35-12L/R primer pairs (Dao *et al*., 2005). To screen for the patulin biosynthesis gene, the *idh* gene was amplified using specific primer pairs as described (Dombrink-Kurtzman *et al*., 2007).

Mycotoxin analysis

Four grams of each ground red pepper sample were extracted with 70% methanol (with 1% NaCl) for AFs, 60% acetonitrile for OTA, and 70% methanol for CIT. The extracts were cleaned using immuno-affinity columns (AflaTest WB, OchraTest WB, CitriTest HPLC for AF, OTA, and CIT, respectively; VICAM), according to the manufacturer's instructions. Samples were dried under nitrogen gas, reconstituted in 1 ml of mobile phase solution, and then filtered (0.2 µm, SiliCycle) before injection into UPLC.

To investigate aflatoxin producibility, fungal isolates were cultured in Czapex-Dox broth (MB Cell) with 5 g of yeast extract added per L (Bacto yeast extract; Difco) at 250 rpm, 25°C for 2 weeks and each culture was filtered through Miracloth (Calbiochem) to remove fungal cells. Then, 5 ml of the culture filtrate was mixed with 15 ml of water and cleaned using AflaTest WB and filtered as described above.

Analysis was performed with UPLC (ACQUITY UPLC H-Class; Waters). Ten microliters of each sample was injected and a reverse-phase C18 column (ACQUITY UPLC BEH C18 1.7µm, 2.1 mm × 100 mm; Waters) was used. The mobile phase used to separate the AFs, OTA, and CIT were as follows: AFs, water-methanol-acetonitrile (62:22:16); OTA, water-acetonitrile-acetic acid (49.5:49.5:1); and CIT, 0.1% phosphoric acid-acetonitrile (50:50). Samples were separated isocratically with a flow rate of 0.3 ml/min for AFs and OTA and 0.3 ml/min for CIT. Quantitative analysis was carried out using a fluorescence detector (FLR Detector; Waters) with excitation and emission at 360 and 440 nm, respectively, for AFs, 333 and 460 nm for OTA, and 333 and 500 nm for CIT. The limit of quantification was 0.5 ppb for AFs and OTA and 1 ppm for CIT. The analysis was repeated three times.

Results

Mycobiota of ground red pepper

The sample contamination levels ranged from 0 to 7,275 CFU/g (Fig. 1). The average fungal count of the 29 contaminated samples was 343.7 CFU/g. One sample (No. 10, Fig. 1) showed uniquely heavy fungal contamination. When this sample was excluded, the average count of the remaining 28 samples dropped to 104.7 CFU/g.

Six fungal genera each occurred in more than 5% of the 30 ground red pepper samples (Fig. 2). The genus showing highest incidence was *Aspergillus* (80%), followed by *Rhizopus*

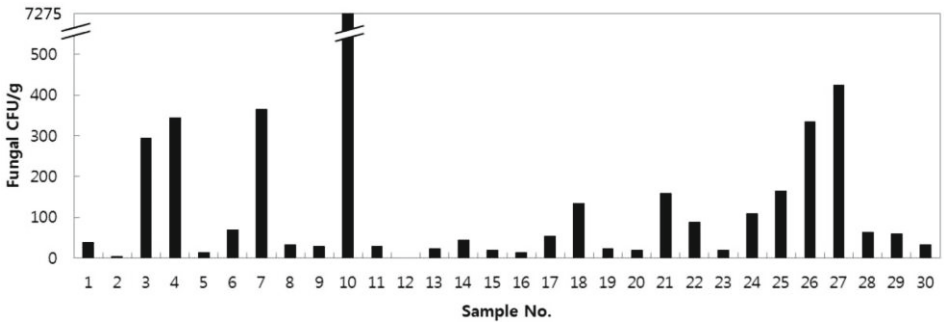

Fig. 1. Average mold count of the ground red pepper samples.

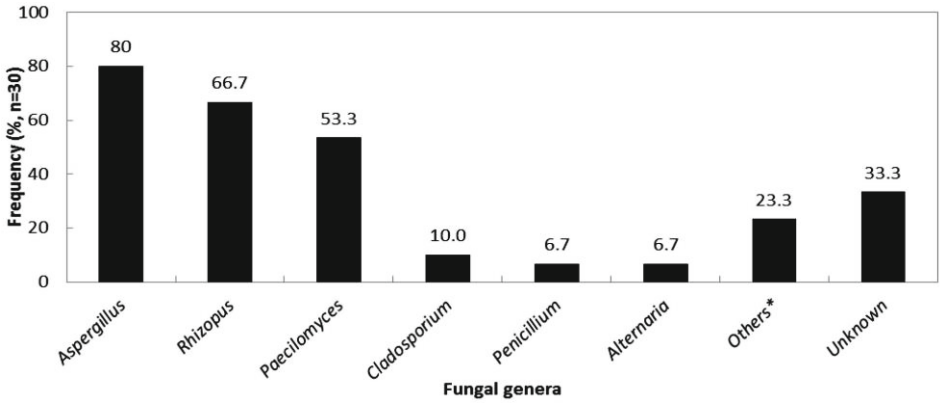

Fig. 2. Frequency of fungal genera detected in the ground red pepper samples. *Others include *Theilavia, Mucor, Cephalothea, Lichetheimia, Phaeosphaeria, Nigrospora*, and *Chaetomium*.

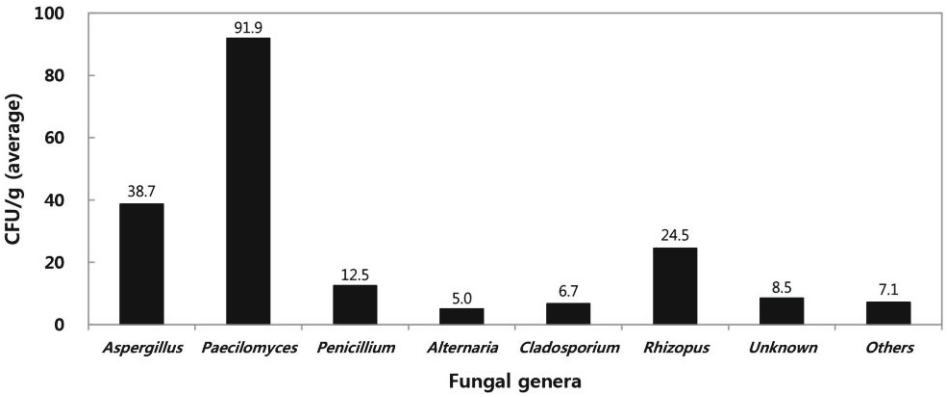

Fig. 3. Occurrence of fungi in the contaminated ground red peppers.

(67%), and *Paecilomyces* (53%). *Cladosporium*, *Penicillium*, and *Alternaria* were detected at frequencies of 5 to 10%. Minor genera detected (all < 3.3%) included *Thielavia*, *Mucor*, *Cephalothea*, *Lichtheimia*, *Phaeosphaeria*, *Nigrospora*, and *Chaetomium*.

Among the 29 contaminated samples, *Paecilomyces* was found most frequently, followed by *Aspergillus*, *Rhizopus*, *Penicillium*, *Cladosporium*, and *Alternaria* (Fig. 3). While the *Paecilomyces* isolates were not identified to the species level, several *Aspergillus* species were detected. When toxigenic genera were counted, *Aspergillus* was isolated the most frequently. The most frequent species was *A. ruber* (30 CFU/g, average), followed by *A. niger*, *A. amstelodami*, *A. ochraceus*, and *A. terreus*, *A. versicolor*, *A. flavus*, and *A. fumigatus* were detected at ≤ 10 CFU/g (Fig. 4).

Natural occurrence of mycotoxins

In the 30 ground red pepper samples, no AFs (B_1, B_2, G_1, G_2) or CIT were detected above the limit of quantification, which was 0.5 ppb for AF and 1 ppm for CIT. In comparison, OTA was detected from three samples at a range of 1.03–2.08 μg/kg, which was much lower than the ML (7 μg/kg).

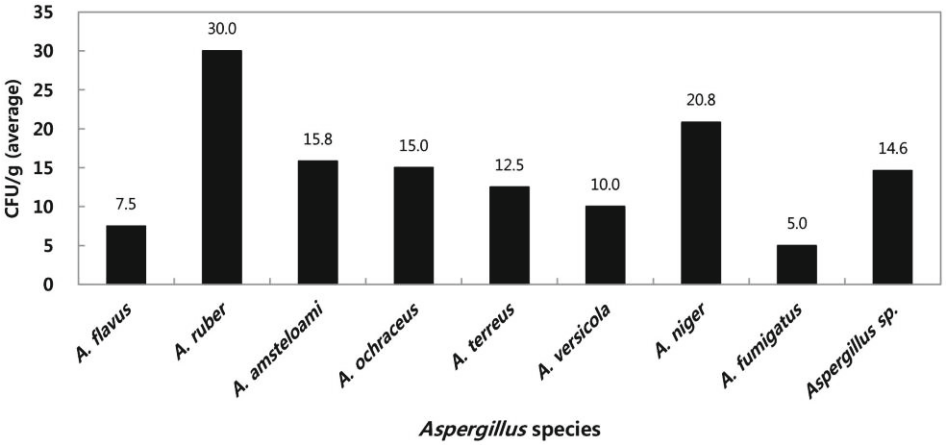

Fig. 4. Occurrence of *Aspergillus* species in the contaminated ground red peppers.

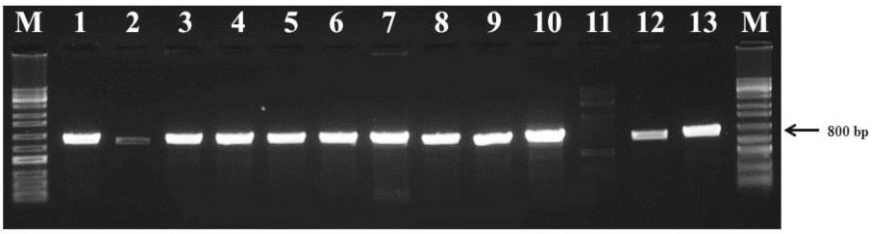

Fig. 5. Amplification of *norB-cypA* in *Aspergillus* spp. M, 1 kb marker; Lanes 1-10, *A. flavus* isolates (No.12, 1~9 in order in Table 2); 11, *A. ruber* (No.11); 12, *Aspergillus* sp. (No.10); 13, positive control of *A. flavus* (13SHCb04-?1).

Fungal potential for mycotoxin production

Sixty-four isolates, including all of the *A. flavus* and *A. ruber* isolates, were selected for an analysis of aflatoxin production. A single 800-bp band corresponding to the *norB-cypA* gene, which is amplified in AF producers, was found in 10 of 16 *A. flavus* isolates, and one *Aspergillus* sp. isolate (Fig. 5). The UPLC AF analyses indicated that 9 of the 10 *A. flavus* isolates and the one *Aspergillus* sp. from the sample #10 produced total AFs in the range of 146.88–909.53 μg/kg (Table 2). It is notable that the other *A. flavus* isolate from different sample (#27) produced only 2.4 μg/kg of AF B_1. PCR screening for the ochratoxin or patulin biosynthesis genes produced no expected band from the isolates (2 *A. ochraceus* and 1 *A. niger* for ochratoxin, 7 *Paecilomyces* for patulin) tested.

Discussion

Fungal contamination of chili/powder is reported frequently. Santos *et al.* (2011) reported fungal counts ranging between $< 1.0 \times 10^2$ and 4.6×10^4 CFU/g in *Capsicum* powder collected in Spain. In Saudi Arabia, Bokhari (2007) reported that total counts of fungi isolated from red pepper ranged between 1.8 and 1.5×10^3 CFU/g. In Iran, Salari *et al.* (2012) reported that mold and yeast contamination ranged from 2.4×10^4 to 4.6×10^6 CFU/g in Iranian red pepper spice. In Korea, Jeong *et al.* (2013) reported that fungi and yeasts counts in commercial ground red pepper ranged from 4.95×10^2 to 3.19×10^6 CFU/g in 10 samples produced in Korea or imported from China. In the current study, the level of fungal contamination in ground red pepper reached 7.3×10^3 CFU/g, indicating that fungal contamination of red pepper power produced in Korea in 2015 does not exceed the reported levels.

Our study of the mycobiota of ground red pepper is consistent with reports that *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Alternaria*, *Cladosporium*, and *Fusarium* are commonly isolated fungi from red pepper (Erdogan, 2004; Bokhari, 2007; Santos *et al.*, 2011; Jeswal and Kumar, 2015). Jeswal and Kumar (2015) reported that *Aspergillus* was the fungal genus most frequently isolated from red chili collected from local markets in Bihar State, India and the next most frequent ones were *Penicillium*, *Mucor*, *Fusarium*, and *Rhizopus*. In Turkey, *Aspergillus* (84.6%), *Penicillium* (80.1%), *Rhizopus* (69.2%), and *Geotrichum* (50%) were isolated from red pepper powders (Erdogan, 2004). Since *Rhizopus*, *Mucor*, *Alternaria*, *Cladosporium*, and *Fusarium* are known to be field fungi, while *Aspergillus* and *Penicillium* are storage fungi (Mandeel, 2005; Santos *et al.*, 2011), fungal contamination and proliferation on red pepper can occur at any time from field cultivation through to storage. This is the first report of *Paecilomyces* sp. in ground red pepper. Aziz *et al.* (1998) isolated *Paecilomyces variotii* from medicinal plants and spices. It is commonly found on decaying chili fruits stored in humid areas (Vrabcheva, 2000). There are reports that *Paecilomyces* can produce patulin in silage or airospores (Hacking and Rosser, 1981; Bokhari *et al.*, 2009). However, the *Paecilomyces* isolates in our study did not yield the expected band when screened using PCR, suggesting that these isolates cannot produce patulin.

The occurrence of toxigenic *Aspergillus* sp. in red pepper/powder has been reported often. Bokhari (2007) detected *A. flavus* at 780.2 CFU/g (average) in red pepper, along with

Table 2. Aflatoxins produced by *Aspergillus* isolates from the ground red peppers

No.	Sample	Isolate	Species	Aflatoxin (μg/kg)[a]				
				B_1	B_2	G_1	G_2	Total
1	#10	Gb02-4-A	*A. flavus*	230.28	1.47	16.4	0.87	249.01
2	#10	Gb02-5-A	*A. flavus*	889.32	14.53	4.4	1.28	909.53
3	#10	Gb02-8-8-A	*A. flavus*	274.88	2.27	5.8	1.01	283.96
4	#10	Gb02-#1	*A. flavus*	540.53	3.2	8.6	1.85	554.19
5	#10	Gb02-#2	*A. flavus*	433.17	3.6	8.2	1.37	446.35
6	#10	Gb02-#6	*A. flavus*	133.2	1.33	11.2	1.15	146.88
7	#10	Gb02-#7	*A. flavus*	693.07	6.8	10.8	1.77	712.44
8	#10	Gb02-#9	*A. flavus*	388.69	3.2	8.6	2.07	402.56
9	#10	Gb02-#10	*A. flavus*	151.27	1.6	4.2	0.74	157.81
10	#10	Gb02-2-2-a	*A.* sp.	242.73	2.67	7.8	0.91	254.11
11	#18	Gb10-9-A-2	*A. ruber*	nd	nd	nd	nd	nd
12	#27	Jb03-7-A5?	*A. flavus*	2.4	nd	nd	nd	2.4

[a] Average value of 3 replicates; nd, not detected

A. niger (1460.2 CFU/g), *A. ochraceus* (220.6 CFU/g), and *A. fumigatus* (4.6 CFU/g). Santos *et al.* (2011) reported that *Aspergillus* section *Nigri* was isolated the most frequently from chili powder samples. Erdogan (2004) isolated *A. niger* (57.7%) most often, followed by *A. flavus* (38.5%), *A. ochraceus* (30.8%), and *A. versicolor* (23.1%) from the red pepper powders. In this study, *A. ruber* was isolated the most frequently. Although *A. ruber* is not known to produce aflatoxin in general (Pitt and Hocking, 2009), there is a report that *A. ruber* (strain B38C) from food stuffs produced aflatoxin (Leitao *et al.*, 1989). In this study, the *A. ruber* isolate (Gb10-9-A-2, Table 2) had no expected band from PCR, or produced any AF. Further tests with more *A. ruber* strains are needed to confirm whether *A. ruber* can produce AFs. *A. niger* has also been reported to produce OTA (Abarca *et al.*, 1994). Therefore, it is important not to underestimate the potential of *A. ruber* or *A. niger* to produce AFs or OTA in ground red pepper.

Although *A. flavus* and several fungal species that can produce mycotoxins were found in our samples, no AFs were detected, not even from the heavily contaminated sample. We speculate that this might be because little amount of AFs was produced at the time of analysis or there was uneven sample collection associated with spot contamination for the analyses. All the aflatoxigenic strains from a single sample (sample #10) could be clones but their origin was not tested. Although they are speculated as clones, the capacity of aflatoxin production by each strain was various (Table 2). Production of by *A. flavus* isolates in this study is not unusual as some *A. flavus* S strains were reported to produce AF G_1 and G_2 along with AF B_1 and B_2 (Geiser *et al.*, 2000; Amaike and Keller, 2011). AFs are usually found in tropical and subtropical regions (Rustom, 1997) or in similar environments. The climate of southern Korea does not favor AF production, although AFs can be produced in hot and humid environments. Park *et al.* (2007) failed to detect AFs from eight samples of ground red pepper collected from Gyeonggi Province, Korea. Kim *et al.* (2009) detected AFB_1 from 2 of 192 red pepper powder samples at a range of 0.86–4.03 μg/kg, which did not exceed the ML of 10 μg/kg. In Gangwon Province, 15 out of 28 ground red pepper samples were found to be contaminated with AFs, but all were under the ML (Lee *et al.*, 2013).

OTA was detected from three samples at levels not exceeding the ML for red pepper powder in Korea. Two of the three samples contaminated with OTA were contaminated with *A. ruber*, *A. ochraceus*, *A. versicolor*, *A. niger*, *Paecilomyces*, and *Rhizopus*, some of which are known to produce OTA. However, the *A. ochraceus* and *A. niger* isolates in our study did not yield the expected band from PCR screening, suggesting that these isolates cannot produce ochratoxin. In the other sample, only *Paecilomyces* was detected. Since PDA was used to screen fungal contamination, we might have failed to detect all fungal contaminants. DG18 medium would be better for the enumeration of xerophilic molds such as *Aspergillus*, *Basipetospora*, and *Eurotium* because of their low a_w preference (Beuchat, 1992; Santos *et al.*, 2011). Another possible explanation might be the uneven distribution of the causal fungi in the samples.

In conclusion, our investigation of toxigenic mycobiota and mycotoxins in ground red pepper revealed that *Aspergillus* and *Paecilomyces* were predominant in the samples and some of the *Aspergillus* had toxigenic potential. Toxigenic fungal contaminants can produce mycotoxins in a suitable environment (Kim *et al.*, 2011). Although no AFs were detected in these samples, safety management to minimize mycotoxins is needed because fungal isolates from the contaminated ground red pepper (even a single sample) were able to produce significant amounts of AFs under the test conditions.

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