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Natural Occurrence of Aflatoxigenic *Aspergillus* Species and Aflatoxins in Traditional Korean Fermentation Starters, *Meju* and *Nuruk*

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ABSTRACT - *Meju* and *nuruk* (respectively soybean and malt) are traditional Korean fermentation starters that are vulnerable to contamination by harmful microorganisms such as aflatoxigenic fungi and their associated aflatoxins (AFs). In this study, *Aspergillus* spp. were isolated and identified from a total of 57 *meju* and 18 *nuruk* samples collected from Korean markets. Their potential aflatoxigenicity was investigated by examining the presence of three aflatoxin biosynthetic genes (*aflO*, *aflP*, and *aflR*) using multiplex polymerase chain reaction (mPCR) assays. Thereafter, aflatoxin production of isolates and the natural occurrence of AFs in *meju* and *nuruk* samples were analyzed by high-performance liquid chromatography (HPLC). A total of 177 *Aspergillus* isolates were identified and 130 isolates were obtained from *meju* samples. Of these, 25 isolates (19.2%) contained all three aflatoxin biosynthetic genes, and five (20%) of these isolates produced aflatoxins. Forty-seven of the *Aspergillus* isolates were obtained from *nuruk* samples, five of which (10.6%) expressed all three AF biosynthetic genes; however, none of these strains produced AFs. HPLC analysis showed that 88% (51/58) of the *meju* samples and 39% (7/18) of *nuruk* samples were not contaminated with AFs (below limit of detection). Among the isolates isolated from *meju* and *nuruk*, there were aflatoxigenic strains containing all three aflatoxin biosynthetic genes or producing aflatoxin in medium, but the frequency of aflatoxin contamination was low in the *meju* and *nuruk* samples.

Key words : Aflatoxin, Fungal isolation, Aflatoxigenicity, *Meju*, *Nuruk*

Fermentation is the process of chemically degrading organic compounds using microorganisms. Fermented foods such as fermented milk, cheese, soybean paste, and fermented alcohol have been consumed since about 3,000 BC[1]. In Korea, to overcome nutritional deficiencies that may have resulted from the consumption of cereals as staple foods, ancient people naturally began eating fermented foods. Thus, fermented alcoholic beverages and *jang* (manufactured by fermenting and aging animal or plant materials that are then cultivated using fungi or bacteria after mixing with salt) derived from agricultural products were developed and played important roles in Korean diet[2].

Meju and *Nuruk* are the most widely used fermentation starters in the preparation of many traditional Korean fermented foods and beverages, including soy sauce (*Ganjang*), soybean paste (*Doenjang*), red pepper soy paste (*Gochujang*), *Takju*, and *Yakju*[3,4]. *Meju* can be divided into two groups based on the production processes by which it is made-traditional Korean-style *Meju* and commercialized modified *Meju*[5]. Traditional *Meju* is prepared using steamed or boiled soybeans and subsequent fermentation. During fermentation, fungi present in the surrounding environment are naturally inoculated and participate in the fermentation. In commercialized refined *Meju* production, soybeans are also steamed or boiled, but there is a difference in the strains that are selected for the fermentation process. *Nuruk* is made with food-grade raw grain by fermentation with various microorganisms, including bacteria, yeasts, and fungi of the *Aspergillus* and *Rhizopus* genus inoculated from the natural environment[6]. These two fermentation starters break down food proteins, produce unique flavor components, and give each fermented food and beverage their inherent characteristics. However, this process is also vulnerable to contamination by other microorganisms.

Aspergillus species (spp.) are major contributors to food contamination, along with *Fusarium* and *Penicillium*[7]. These fungi can grow at relatively high temperature and

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with low water activity[8]. Among them, the *Aspergillus* genus (especially *Aspergillus* section Flavi) produces various mycotoxins, including aflatoxins, sterigmatocystin, ochratoxin, patulin, cyclopiazonic acid, and fumonisins[9]. The production of aflatoxins is influenced by biotic factors (e.g., the microbial community in the surrounding environment) and abiotic factors (e.g., temperature, humidity). *Aspergillus* fungi can infect field crops and produce aflatoxins, and both fungi and aflatoxins remain in the plant until it is consumed after being stored and distributed[10]. *A. oryzae*, a nonpathogenic strain, is the major species involved in the production of *Nuruk* and *Meju*[11]. However, during the long aging and fermentation period, *A. oryzae* may inadvertently become contaminated with aflatoxigenic strains that were present among them[12]. *A. oryzae* is widely used in the production of traditional fermented Asian foods. It belongs to the *Aspergillus* section Flavi, along with *A. flavus* and *A. parasiticus*, and *A. nomius* that has similar genetic characteristics to *A. flavus*. Both *A. oryzae* and *A. flavus* have eight chromosomes, and the sizes of their genomes are about 38 Mb. Only 129 and 43 genes out of about 12,000 genes were found to be unique, respectively[13]. Surprisingly, although they have considerable genetic similarities, *A. flavus* represents one of the strains that can produce aflatoxins, whereas *A. oryzae* is generally regarded as safe. It has been reported that *A. oryzae* is unable to produce aflatoxins because the regulator gene in its aflatoxin biosynthesis pathway, *aflR*, is not expressed or functioning[14].

Aflatoxin is a carcinogen that induces hepatocellular carcinoma and was classified as a group 1 human carcinogen by the International Agency for Research on Cancer in 1993[15]. Aflatoxin levels in foods and feeds are regulated by legislation in more than 100 countries[16]. In Korea, the allowable total aflatoxin level (sum of aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2)) in foods is no more than 15.0 μg/kg, and the allowable AFB1 level is no more than 10 μg/kg[17]. However, there is no regulation regarding aflatoxin contamination levels in *Nuruk* because it is currently classified as a food additive.

Current studies of aflatoxins in *Meju* and *Nuruk* have investigated their fungal communities[18-21], the toxigenicity of the isolated strains[22-26], and the occurrence of mycotoxins in *Meju* and *Nuruk* samples[22]. However, studies simultaneously investigating all three aspects are rare, so it has been difficult to figure out whether strains isolated from samples contribute to aflatoxin contamination in the actual foods. Furthermore, microbial population studies in *Meju* and *Nuruk* have mostly focused on bacteria and yeast (especially in *Nuruk*), ignoring the fact that fungi are also an important part of the microbial community in these fermentation starters. In addition, current published information regarding the aflatoxin contamination of aflatoxins in *Nuruk* is scarce. Considering that *Aspergillus* spp. fungi are frequently detected in *Nuruk*, it is also important to confirm that their aflatoxin levels are below the legal limit for foodstuffs.

Therefore, in the present study, we isolated and identified *Aspergillus* spp. from *Nuruk* and *Meju* and investigated their potential aflatoxigenicity using multiplex polymerase chain reaction (mPCR) assays. We also analyzed the natural occurrence of aflatoxins in practical samples to gain a better understanding of the relationship between aflatoxigenicity of isolates and the contamination level of aflatoxins in marketed *Nuruk* and *Meju*.

Materials and Methods

Sample collection

A total of 57 *Meju* and 18 *Nuruk* samples were collected from online retailers and local markets in Korea in February and March, 2017. Each sample (about 1 kg) was homogenized by grinding into powder using a food-grade mixer (Shinil Co. Ltd, Seongnam, Korea) and stored in a -18°C refrigerator (Labogene, Lynge, Denmark) then equilibrated at room temperature before analysis.

Isolation and identification of *Aspergillus* species from *Meju* and *Nuruk* samples

Preparation of fungal isolates from food samples was conducted according to the Food Code of Korea with slight modification[27]. First, 10 g of each sample was mixed with 0.85% (w/v) saline solution to make tenfold serial dilutions. Next, each dilution was spread onto dichloran rose Bengal chloramphenicol (DRBC) (Becton, Dickinson and Company, Le Pont de Claix, France) agar plates in triplicate and incubated for 5-7 days at 25°C in incubators (Sejong Scientific Co., Bucheon, Korea). Next, plates with 30-300 colonies were selected and used for fungal isolation. All strains that formed colonies on the DRBC medium were transferred to potato dextrose agar (PDA) (Becton) for subculture until a pure culture was obtained. Among these isolates, green, yellow, or black colonies were screened first for molecular identification. Analysis of 18S rRNA sequences for identification was performed by Macrogen, Inc. (Seoul, Korea). All isolates were preserved in 12% glycerol/water (v/v) at -80°C in a Fisher Isotemp deep-freezer (Fisher Scientific, Hampton, NH, USA).

Aflatoxigenicity analysis using mPCR

Multiplex PCR was conducted to investigate the presence of aflatoxin biosynthetic genes. Fungal DNA was extracted from the pure culture of each isolate using the Qiagen DNeasy Ultraclean Microbial Kit (Qiagen, Hilden, Germany)

according to the manufacturer's instructions with some modifications. The concentration and purity of DNA were determined by the A260/A280 ratios using an Epoch™ microplate spectrophotometer (BioTek, Winooski, VT, USA). Primers targeting the two structural genes (*aflO* and *aflP*) and one regulatory gene (*aflR*) in the aflatoxin biosynthesis pathway were used[23-25]. The forward and reverse primers for *aflO* were 5'-GCC TTG ACA TGG AAA CCA TC-3' and 5'-CCA AGA TGG CCT GCT CTT TA-3', respectively, and the amplicon size was 1,333 bp[28]. For *aflP*, the forward and reverse primers were 5'-GTG GAC GGA CCT AGT CCG ACA TCA C-3' and 5'-GTC GGC GCC ACG CAC TGG GTT GGG G-3, respectively, and the amplicon size was 797 bp[29]. For *aflR*, the forward and reverse primers were 5'-TAT CTC CCC CCG GGC ATC TCC CGG-3' and 5'-CCG TCA GAC AGC CAC TGG ACA CGG-3', respectively, and the amplicon size was 1,032 bp[30]. PCR amplification was performed using an AccuPower® PCR PreMix kit (Bioneer, Daejeon, Korea) with a 20 μL reaction volume that contained 10-30 ng of template DNA, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 250 μM of each dNTP, 1-10 μM of each primer, and 1 U of *Taq* polymerase. PCR was carried out in a BIO-RAD T-100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following procedure. Pre-denaturation at 94°C for 4 min and then 30 cycles of three steps: (1) denaturation at 95°C for 1 min; (2) annealing at 64°C for 2 min, and; (3) extension at 72°C for 2 min. A final extension step was then carried out at 72°C for 10 min. PCR products were determined using electrophoresis with 2.0% agarose/1× Tris acetate-EDTA buffer (w/v) gels at 100 V for 50 min using BIO-RAD PowerPac HC (Bio-Rad Laboratories). After electrophoresis, gel images were visualized using a gel documentation system, Gel Doc™ EZ imager (Bio-Rad Laboratories).

Aflatoxin extraction from fungal culture

To analyze aflatoxin production by *Aspergillus* isolates, all isolates were incubated on PDA for 7 days at 25°C in triplicate. Agar plugs (5 mm diameter) from three different plates were taken from the center to the edge within the colony and collected in one 50 mL conical tube (SPL Life Science, Pocheon, Korea). Three agar plugs (0.5 g) were randomly selected and transferred to a test tube containing 1 mL of HPLC-grade methanol (Burdick and Jackson, Muskegon, MI, USA) and then sonicated with JAC Ultrasonic 3010 (Kodo Technical Research Co., Hwaseong, Korea) for 1 h to extract aflatoxins. After sonication, the extract was transferred to another test tube, dried under N_2 gas, and redissolved in 1 mL of 10% methanol (v/v). These procedures were repeated three times for efficient extraction. Next, 3 mL of the redissolved solution was loaded onto an AflaTest WB immunoaffinity column (IAC) (VICAM, Milford, MA, USA) at a flow rate of 1 drop per second, and the column was washed with 15 mL of HPLC-grade water (Burdick and Jackson). After removing residual water in the column using a syringe, aflatoxins were eluted from the column with 3 mL of HPLC-grade acetonitrile (Burdick and Jackson). The eluate was dried under N_2 gas and reconstituted in 0.5 mL of 50% methanol (v/v) and filtered through a 0.2 μm PVDF syringe filter (Whatman, Maidstone, UK), then stored at -20°C until analysis. The accuracy (i.e., recovery) of this method was tested by adding AFB1, AFB2, AFG1 and AFG2 standards to the agar plugs taken from blank PDA mediums with the spiking level of 20 μg/kg for AFB1 and AFG1, 6 μg/kg for AFB2 and AFG2. The method showed recoveries in the range of 74-92% with the linearity of R^2>0.999.

Aflatoxin extraction from *Meju* and *Nuruk* samples

To extract aflatoxins from *Meju*, 10 g of ground *Meju* sample and 1 g of sodium chloride (NaCl) (Junsei Chemical Co., Tokyo, Japan) were mixed with 50 mL of 80% methanol (v/v) and 25 mL of hexane (Burdick and Jackson, HPLC grade), homogenized using a high-speed mixer, Ultra Turrax, (IKA, Staufen, Germany) at 6,200 rpm for 3 min, and then filtered with Whatman No.4 filter paper. After filtration, 5 mL of filtrate was diluted with 35 mL of water containing 0.1% Tween-20 (v/v) and filtered again through Whatman GF/B filter paper (Whatman). Then, 20 mL of filtrate was loaded onto an IAC that was prewashed with 10 mL of water with a flow rate of 1–2 drops per second. After that, the IAC was washed with 15 mL of water and dried by rapidly passing air through it using a plastic syringe piston. Aflatoxins were then eluted twice with 2 mL of methanol containing 0.1% acetic acid (v/v) (4 mL of total eluate). The eluate was dried under N_2 gas and reconstituted in 0.5 mL of 50% methanol (v/v), filtered through a 0.2 μm syringe filter, then stored at -20°C until analysis. Aflatoxins in *Nuruk* samples were isolated and purified using the same procedure with slight differences (i.e., 0.5 g of NaCl instead of 1 g; phosphate-buffered saline instead of water containing 0.1% Tween-20 (v/v) as diluent). Recovery of this method was analyzed by spiking AFB1, AFB2, AFG1, and AFG2 standards in blank *Meju* and *Nuruk* samples the spiking level of 2.0 μg/kg for AFB1 and AFG1, 0.6 μg/kg for AFB2 and AFG2. The method showed a recovery in the range of 73-102% for *Meju* and 89-102% for *Nuruk* with the linearity of R^2>0.999 in both of *Meju* and *Nuruk* matrix.

Aflatoxin detection using HPLC-FLD

For aflatoxin detection, 50 μL of aflatoxin extracts was injected into the Agilent 1260 infinity series HPLC system

(Agilent, Santa Clara, CA, USA). Separation of analytes was achieved on a symmetry C18 column (4.6×150 mm, 3.5 μm; Waters, Wexford, Ireland). The mobile phases were methanol (solvent A), acetonitrile (solvent B) and water containing with 0.1% acetic acid (v/v, solvent C) with a constant flow rate of 1 mL/min. After isocratic step of 27% A/14% B for 10 min, the gradient was linearly changed to 10% A/44% B within 2 min, and then this condition was held constant for 16 min. The column was re-equilibrated to the initial condition (27% A/14% B) for 2 min. Aflatoxins were detected using a fluorescence detector with a post-column photochemical derivatization system (PHRED) (AURA Industries, New York, NY, USA) to enhance the fluorescence activity of aflatoxins. For the first 15 min the excitation wavelength (ex.) was 360 nm, and the emission wavelength (em.) was 455 nm; for the next 7 min the ex. was 276 nm and em. was 460 nm; for the last 13 min the ex. was 330 nm and em. was 460 nm.

Aflatoxin confirmation by LC-tandem mass spectrometry (LC-MS/MS)

Positive samples were further confirmed using an Accela UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Accela 600 quaternary pump and a Velos Pro mass spectrometer. Detection and determination were performed in positive electrospray ionization mode. Optimized MS parameters were: spray voltage, 5.0 kV; source heater temperature, 250°C; capillary temperature, 275°C; nitrogen sheath gas, 35; nitrogen auxiliary gas, 5; nitrogen sweep gas, 5; *m/z* range, 50–2000; collision-induced dissociation energy, 35%; isolation width, 3.0 *m/z*. Helium was used as the collision gas. Chromatographic separation of targeted analytes was achieved on a Waters Xbridge C18 column (3.4 mm particle size, 2.1 mm × 100 mm, Dublin, Ireland) with gradient elution. The mobile phase was a mixture of solvent A (water with 0.1% formic acid, v/v) and solvent B (methanol with 0.1% formic acid, v/v). The initial gradient was 90% solvent A and 10% solvent B, which was equilibrated for 3 min. Subsequently, solvent A was changed linearly to 5% in 13 min and then held for 3 min. Solvent B was changed to 95% in 13 min and then equilibrated for 3.1 min. Solvent A was changed directly to 90% and solvent B was changed to 10% in 16.1 min. The total run time was 20 min, the flow rate was set at 0.2 mL/min, the column temperature was 40°C, and the sample injection volume was 10 μL. The precursor ions $[M+H]^+$ and product ions used to identify the four toxins were AFB1: *m/z* 313.2 → *m/z* 285, AFB2: *m/z* 315.2 → *m/z* 259, AFG1: *m/z* 329.2 → *m/z* 311, and AFG2: *m/z* 331.2 → *m/z* 189.

Results

Isolation and identification of *Aspergillus* isolates from *Meju* and *Nuruk*

A total of 177 *Aspergillus* strains were isolated from *Nuruk* and *Meju* samples and identified (Table 1). The most frequently isolated species in *Nuruk* was *A. flavus* (21/47), followed by *A. fumigatus* (7/47), *A. niger* (7/47), *A. clavatus* (4/47), *A. versicolor* (2/47), *A. oryzae* (1/47), *A. variecolor* (1/47), *A. westerdijkiae* (1/47), and an unidentified *Aspergillus* sp. (3/47). Likewise, *A. flavus* (56/130) was also the most frequently isolated fungal species from *Meju*, followed by *A. niger* (21/130), *A. clavatus* (14/130), *A. fumigatus* (12/130), *A. nomius* (5/130), *A. oryzae* (5/130), *A. candidus* (3/130), *A. amstelodami* (1/130), *A. lentulus* (1/130), *A. pseudoglaucus* (1/130), *A. tamarii* (1/130), *A. versicolor* (1/130), and an unidentified *Aspergillus* sp. (9/130). *A. fumigatus*, *A. niger*, and *A. clavatus* were frequently detected in both *Nuruk* and *Meju*. However, a greater variety of *Aspergillus* fungi was isolated from *Meju* than from *Nuruk* samples, suggesting a more complex fungal community. *Aspergillus* section Flavi (*A. flavus*, *A. oryzae*, *A. nomius*, and

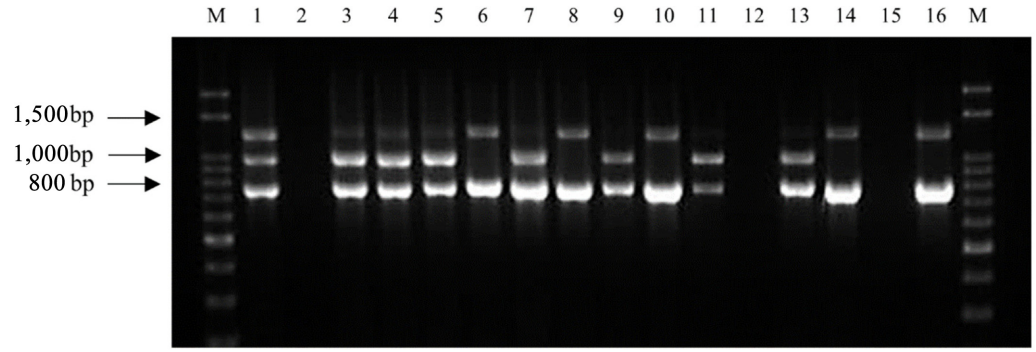

Fig. 1. Gel electrophoresis of multiplex PCR products (DNA fragments of each strain were isolated from *Meju* and *Nuruk*). M, Size marker; 1, Positive control (*Aspergillus parasiticus* KCCM 35078); 2, Negative control (no template); 3–10, *Aspergillus flavus*; 11, *Aspergillus clavatus*; 12, *Aspergillus niger*; 13, *Aspergillus nomius*; 14, *Aspergillus oryzae*; 15, *Aspergillus fumigatus*; 16, *Aspergillus* sp.

Table 1. Community of *Aspergillus* spp. in *Meju* and *Nuruk* samples

Sample	Species	Number of isolates	Relative density (%)[1)]	Relative frequency (%)[2)]
Meju	*Aspergillus flavus*	56	43.1	91.1
	Aspergillus niger	21	16.2	33.9
	Aspergillus clavatus	14	10.8	23.2
	Aspergillus fumigatus	12	9.2	21.4
	Aspergillus nomius	5	3.8	8.9
	Aspergillus oryzae	5	3.8	8.9
	Aspergillus candidus	3	2.3	3.6
	Aspergillus amstelodami	1	0.8	1.8
	Aspergillus lentulus	1	0.8	1.8
	Aspergillus pseudoglaucus	1	0.8	1.8
	Aspergillus tamarii	1	0.8	1.8
	Aspergillus versicolor	1	0.8	1.8
	Unidentified Aspergillus sp.	9	6.9	16.1
	Subtotal	130	100	
Nuruk	*Aspergillus flavus*	21	44.7	94.4
	Aspergillus fumigatus	7	14.9	27.8
	Aspergillus niger	7	14.9	27.8
	Aspergillus clavatus	4	8.5	22.2
	Aspergillus versicolor	2	4.3	11.1
	Aspergillus oryzae	1	2.1	5.6
	Aspergillus variecolor	1	2.1	5.6
	Aspergillus westerdijkiae	1	2.1	5.6
	Unidentified Aspergillus sp.	3	6.4	16.7
	Subtotal	47	100	

[1)] Relative density (%) = No. of identified fungi of corresponding species / No. of all *Aspergillus* spp. × 100.
[2)] Relative frequency (%) = No. of contaminated samples with corresponding fungi / No. of all analyzed samples × 100.

A. tamarii) was detected in 94.4% of *Nuruk* samples (17/18) and 91.1% of *Meju* samples (51/56).

Aflatoxigenicity analysis of *Aspergillus* isolates using mPCR

The presence of aflatoxin biosynthetic genes in the 177 isolates was investigated using mPCR (Fig. 1 and Table 2). As shown in Table 2, most (about 48.0%) of the isolates showed amplification of two tested genes or no amplification of any of the tested genes (about 26.0%). Only five isolates from *Nuruk* and 25 from *Meju* samples showed amplification of all three aflatoxin biosynthetic genes via mPCR. Among these 5 isolates from *Nuruk* samples, including four *A. flavus* strains and one unidentified *Aspergillus* sp., none produced aflatoxins. Among the 25 isolates from the *Meju* samples, 20 were *A. flavus*, three *A. oryzae*, and one an unidentified *Aspergillus* sp. Among these, only five *A. flavus* isolates produced aflatoxins. Quantitative HPLC analysis showed that the range of aflatoxin production was 23.92-9025.81 μg/kg for AFB1 and 0.68–93.12 μg/kg for AFB2.

Occurrence of aflatoxins in *Nuruk* and *Meju* samples

The natural occurrence of aflatoxins in *Meju* and *Nuruk* samples was determined by HPLC and further confirmed by LC-MS/MS. As shown in Table 3, AFB1, AFB2, AFG1, and AFG2 were detected in 12.5% (7/56), 5.4% (3/56), 1.8% (1/56), and 3.6% (2/56) of the *Meju* samples, respectively. The mPCR results showed that five *Meju* samples were contaminated with aflatoxigenic *A. flavus* strains. The average contamination levels of those five *Meju* samples were: 14.61 μg/kg for AFB1; 1.08 μg/kg for AFB2; 8.46 μg/kg for AFG1; and 0.49 μg/kg for AFG2. The chromatogram of the most highly contaminated samples is shown in Fig. 2. In *Nuruk* samples, 61% (11/18) and 9% (1/11) of the tested samples were contaminated with AFB1 and AFB2, respectively; however, no AFG1 or AFG2 was

Table 2. Distribution of aflatoxin biosynthetic genes and aflatoxin production

Food	Gene expression	Number of isolates	Aflatoxin production (μg/kg)			
			B1	B2	G1	G2
Meju	Three bands	25	23.92–9025.81	0.68–93.12	N.D. [2)]	N.D.
	Two bands	60	N.D.	N.D.	N.D.	N.D.
	One band	9	N.D.	N.D.	N.D.	N.D.
	No bands [1)]	36	N.D.	N.D.	N.D.	N.D.
	Total	130				
Nuruk	Three bands	5	N.D.	N.D.	N.D.	N.D.
	Two bands	26	N.D.	N.D.	N.D.	N.D.
	One band	6	N.D.	N.D.	N.D.	N.D.
	No bands	10	N.D.	N.D.	N.D.	N.D.
	Total	47				

[1)]Not detected in mPCR.
[2)]Not detected (LOD: 0.04 μg/kg for AFB1, 0.01 μg/kg for AFB2, 0.05 μg/kg for AFG1, 0.02 μg/kg for AFG2).

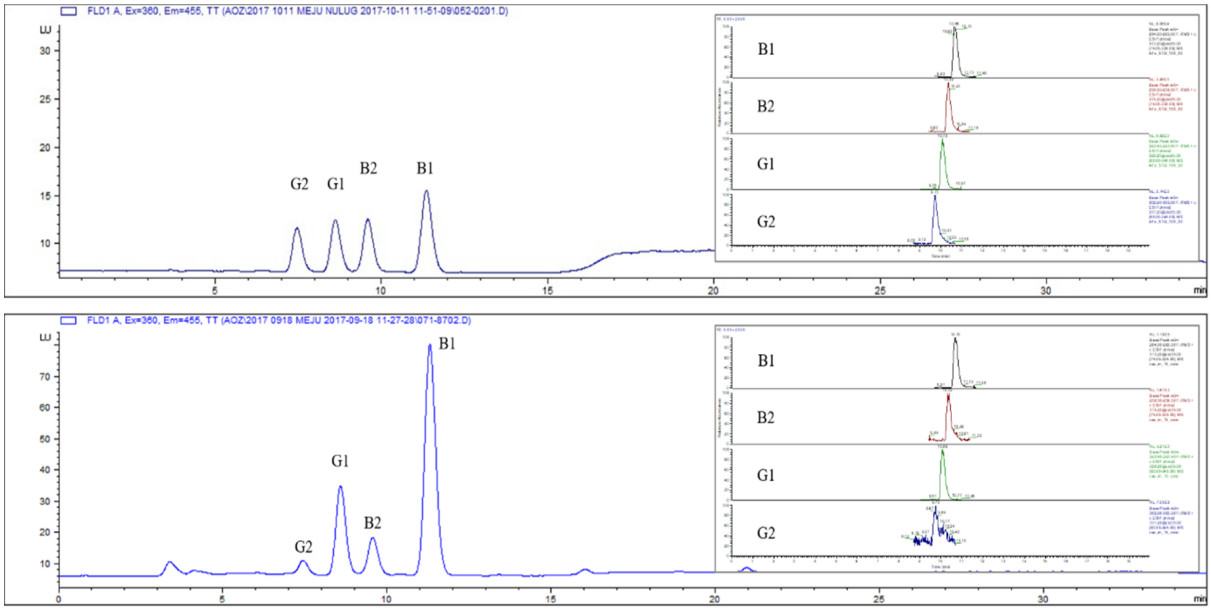

Fig. 2. Chromatogram using LC-MS/MS of standard aflatoxins (upper panel: 10 μg/kg of AFB1, AFG1; 3 μg/kg of AFB2, AFG2) and identical aflatoxins in a contaminated *Meju* sample (lower panel).

detected. Meanwhile, all of the positive samples were below the limit of total aflatoxins and AFB1 permitted by the Korean Ministry of Food and Drug Safety (MFDS). Aflatoxins in the positive samples were all confirmed by LC-MS/MS.

Discussion

Herein, a total of 177 strains of *Aspergillus* spp. were isolated from *Nuruk* and *Meju* samples. Their aflatoxigenicity was investigated using both mPCR and HPLC analysis, and their correlation with aflatoxin contamination in *Nuruk* and *Meju* samples was investigated. We found that *A. flavus* was the predominant strain in both starters. This result is inconsistent with previous research in which *A. oryzae*[31-33], *A. tritici*[19], and *A. candidus*[34] were the most frequently isolated fungal strains in *Nuruk* and *A. oryzae* was the most dominant fungal strain in *Meju*[21,25,35].

Both *A. flavus* and *A. oryzae* belong to the *Aspergillus* section Flavi. These two fungi have many phenotypic similarities. In fact, several studies have suggested that they may be ecotypes of the same species. Some researchers even suggest that *A. oryzae* resulted from the domestication of *A.*

Table 3. Occurrence of aflatoxins in *Nuruk* and *Meju* samples

Matrix	Parameters	Toxins (μg/kg)			
		AFB1	AFB2	AFG1	AFG2
Meju	No. of not detected samples[1)]	49	53	55	54
	No. of positive samples	7	3	1	2
	Mean (μg/kg)	2.246	0.116	0.769	0.048
	Positive median (μg/kg)	1.250	1.020	42.290	1.340
Nuruk	No. of not detected samples	7	17	18	18
	No. of positive samples	11	1	0	0
	Mean (μg/kg)	0.602	0.004	N.D.[2)]	N.D.
	Positive median (μg/kg)	0.880	0.070	N.D.	N.D.

[1)] <LOD; [2)] Not detected (LOD: 0.06 μg/kg for AFB1, 0.05 μg/kg for AFB2, 0.14 μg/kg for AFG1, 0.05 μg/kg for AFG2).

flavus after centuries of culturing[36)]. It is often difficult to distinguish *A. flavus* from *A. oryzae* using current identification methods because their phenotypes are quite similar and easily affected by the environment. They are also confused because of the high degree of genetic similarity. Thus, it is highly possible that some of the isolates identified as *A. flavus* might in fact be *A. oryzae*. It has been reported that *A. flavus* var. *oryzae* (*A. oryzae*) found in fermented Korean foods is recognized as a safe and effective microorganism for producing various fermented foods and industrial products[34)]. Meanwhile, the growth of *A. fumigatus*, *A. niger*, and *A. clavatus* in *Nuruk* and *Meju* was similar to that described above. In addition, previous studies of *Nuruk* and *Meju* have primarily focused on identifying the fungi at the genus level[38,39)] or have emphasized the diversity of bacteria[3,38,40)] and yeast[41)]. Compared with those reports, this study provides relatively detailed information about the identities of *Aspergillus* fungi in *Nuruk* and *Meju* at the species level.

To investigate the aflatoxigenicity of the isolates, we performed mPCR assays using specific primers targeting aflatoxin biosynthetic genes *aflR*, *aflO*, and *aflP*. All three genes were expressed in 10.6% of the isolates from *Nuruk* samples and in 19.2% of the isolates from *Meju* samples. In the aflatoxin biosynthesis pathway, the gene *aflR* encoding a Gal4 zinc finger transcription factor works as the internal cluster activator, and the genes *aflO* and *aflP* encode essential enzymes intervening at the end of the biosynthetic pathway. The loss of any of these genes will result in the loss of aflatoxigenicity. The integrity of the aflatoxin biosynthetic pathway is necessary for aflatoxin production; however, some strains possessing the entire aflatoxin biosynthetic pathway still cannot produce aflatoxins. For example, the nonaflatoxin-producing *A. flavus* strain AF36 has been applied in agricultural environments to competitively exclude the aflatoxigenic, *Aspergillus* spp. This strain cannot produce aflatoxin even though the aflatoxin biosynthetic pathway is intact[42)]. Similarly, according to our results, among all the isolates that showed amplification of all three targeted genes, only five strains from *Meju* produced aflatoxins. Strains of *A. oryzae* also have the aflatoxin biosynthetic cluster but it appears to be nonfunctional because of deletions, frameshift mutations, and base pair substitutions in some of the genes [43)]. Considering the phenotypic similarities between *A. flavus* and *A. oryzae*, it is possible that some of the nonaflatoxigenic *A. flavus* isolates may in fact be *A. oryzae*.

The natural occurrence of aflatoxins in *Nuruk* and *Meju* was also investigated. Although the detection frequency of aflatoxins in *Meju* samples (12.5%) was lower than that of *Nuruk* samples (61.1%), the contamination level was higher in *Meju* samples than in *Nuruk* samples of total aflatoxins. Particularly, the strain that produced the highest concentration of aflatoxins (9,025.81 μg/kg for AFB1 and 93.12 μg/kg for AFB2) in PDA media was isolated from the sample that was contaminated by the highest concentration of aflatoxins. Thus, the corresponding strains are considered to have produced aflatoxins in the *Meju* samples. To the best of our knowledge, there have been no reports of aflatoxins in Korean *Nuruk* or in fungi isolated from *Nuruk*. However, in the present study, aflatoxins were detected in 11 of the 18 *Nuruk* samples, at an average concentration of 0.602 μg/kg for AFB1 and 0.004 μg/kg for AFB2. The AFs levels of positive *Nuruk* samples were below the regulation limit (10 μg/kg for AFB1 and 15 μg/kg for total AFs) permitted by the Korean MFDS. However, aflatoxins are known to accumulate in the human body and even extremely low levels of aflatoxins in foods can cause serious health problems with long-term consumption, so level of aflatoxins in foodstuffs should be As Low As Reasonably Achievable (ALARA) with continuous monitoring. Interestingly, none of the isolates from *Nuruk* samples were found to be

aflatoxigenic using mPCR analysis of aflatoxin cluster genes or HPLC detection of aflatoxin production in the culture medium. This result might be caused by the loss of aflatoxigenic fungal cells during the manufacturing process.

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국문요약

메주와 누룩은 한국 전통 발효 식품에 사용되는 스타터로, *Aspergillus*속 곰팡이나 aflatoxin에 노출되기 쉽다. 본 연구에서는 우리나라에서 시판되는 57개의 메주 시료와 18개의 누룩 시료로부터 *Aspergillus* 속 곰팡이를 분리하고 동정하였다. 분리주의 aflatoxin 생성 가능성을 평가하기 위하여 multiplex PCR을 통해 aflatoxin 생합성 유전자 (*aflO, aflP, aflR*)를 확인하고, 이들 분리주에 의해 생성되는 aflatoxin 함량을 HPLC로 조사하였다. 뿐만 아니라 시판 메주와 누룩 시료 중 aflatoxin 함량을 분석하였다. 그 결과, 메주 시료로부터 130개, 누룩 시료로부터 47개 균주가 분리되어 총 177개의 분리주를 확인 및 동정하였다. 각각 메주와 누룩으로부터 분리된 19.2% (25/130), 10.6% (5/47)의 분리주가 3 종류의 aflatoxin 생합성 유전자를 모두 보유하였으며, 그 중 메주로부터 분리된 5개의 분리주가 실제로 aflatoxin을 생성하였다. 시판 메주와 누룩 시료 중 aflatoxin 함량을 분석한 결과, 88% (51/58)의 메주 시료의 aflatoxin 오염 수준은 모두 검출한계 미만으로 나타났고, 누룩 또한 시료의 39% (7/18)가 검출한계 미만으로 확인되었다. 메주와 누룩에서 분리된 분리주 중 aflatoxin 생합성 유전자를 모두 보유하거나 배지 상에서 aflatoxin 생성을 보여준 aflatoxigenic 균주는 존재하였으나 유통되고 있는 시료에서 aflatoxin 오염 빈도는 낮은 수준임을 확인할 수 있었다.

Conflict of interests

The authors declare no potential conflict of interest.

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