



Research Paper

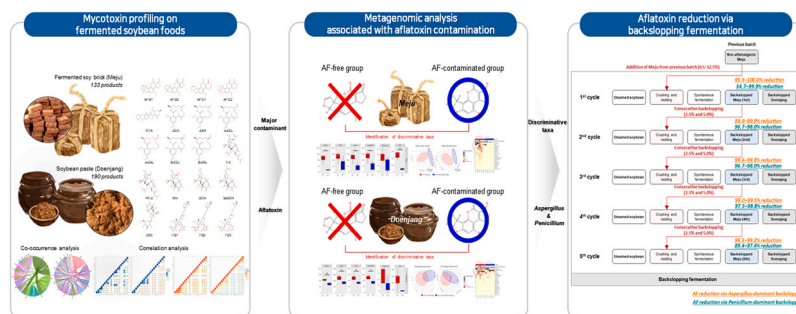
Mycotoxin and microbiome profiling for aflatoxin control in the Korean traditional fermented soybean paste Doenjang

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HIGHLIGHTS

- Aflatoxin (AF) is a major contaminant in South Korean Meju and Doenjang.
- *Aspergillus* and *Penicillium* were unique to AF-free samples and used as inocula.
- Backslopping fermentation using $\geq 2.5\%$ inoculum reduced AF levels by $> 95\%$.
- AF inhibition rates remained $> 90\%$ over five consecutive fermentation cycles.
- Backslopping fermentation is a sustainable solution for artisanal producers.

GRAPHICAL ABSTRACT



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ABSTRACT

Mycotoxin contamination is an important concern in producing traditional fermented soybean paste, though no effective control strategy has been developed. This study investigated the mycotoxin profiles of the intermediate (fermented soybean brick, known as “Meju” in South Korea) and final soybean paste products (“Doenjang”) to identify major contaminants and describe microbial diversity with the mycotoxins. Profiling of 323 Meju and Doenjang samples revealed severe aflatoxin (AF) contamination. Metagenomic analysis revealed that the species richness and phylogenetic diversity were significantly higher in AF-free than in AF-contaminated Meju and Doenjang. Certain *Aspergillus* and *Penicillium* species were more abundant in AF-free than in AF-contaminated Meju and Doenjang. To control AF levels, we developed a novel mycotoxin-reduction approach that preserves the indigenous microbiome by backslopping fermentation of Meju in both *Aspergillus*-dominant and *Penicillium*-dominant modes. Both treatments reduced AF levels by $> 95\%$ at a backslopping rate of $> 2.5\%$. Our results suggested that backslopping fermentation can effectively reduce AF contamination in traditional soybean fermentation, maintaining food safety standards and artisanal practices.

1. Introduction

Fermented soybean products have long been a staple in Asian diets

and are gaining popularity in Western countries [1]. Soybean paste is one of the oldest fermented soybean foods consumed worldwide. It is traditionally produced by fermenting soybeans using starters, such as

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Koji or Meju, combined with brine in a non-sterile environment [2]. Many countries produce unique fermented soybean pastes, including Miso in Japan, Dajiang in China, Taotjo in Indonesia, and Kinema in Nepal [3]. In South Korea, the traditional fermented soybean paste, Doenjang, is a widely consumed seasoning. Traditional manufacturing using spontaneous fermentation has evolved through trial and error over thousands of years and considers many factors depending on the raw ingredients and initial microflora community. Fermentation technology has been developed to reduce variation in product quality and maximize profits. Backslopping fermentation refers to inoculating a new batch of food with a predetermined amount of a successfully fermented culture from a previous batch [4]. This time-honored method, rooted in traditional food production across various cultures, is widely used to accelerate spontaneous fermentation by introducing well-adapted microbial communities [5]. Backslopping promotes rapid fermentation and preserves foods' inherent flavors and aromas, making it particularly valuable for households and small-scale enterprises [6,7]. Studies on backslopping fermentation have predominantly focused on foods where lactic acid bacteria are the primary agents of fermentation, such as sourdough, yogurt, and fermented sausages. These studies aimed to enhance physicochemical qualities, including pH changes, organic acid production, alcohol content, volatile compound formation, and sensory attributes. The technique has been instrumental in ensuring product consistency and maintaining artisanal manufacturing techniques by preserving indigenous microbial communities [5–7]. In contrast, the application of backslopping fermentation in the soybean-based fermented food system, where filamentous fungi dominate the fermentation process, remains underexplored.

Mycotoxins are naturally occurring secondary metabolites produced by filamentous fungi of various genera, such as *Aspergillus*, *Fusarium*, and *Penicillium*. Mycotoxins pose serious health risks and can exert nephrotoxic, teratogenic, estrogenic, hepatotoxic, immunosuppressive, and carcinogenic effects [8,9]. Aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), trichothecenes (including T-2 toxin, HT-2 toxin, deoxynivalenol (DON), and nivalenol (NIV)), and fumonisin B (FB) groups (including FB1, FB2, and FB3) are the most common and important mycotoxins in food products [10]. Therefore, most countries set the maximum regulatory limits of mycotoxins in food and feed, mainly for AFs, type A trichothecenes (T-2 and HT-2 toxin), type B trichothecene (DON), ZEN, and FBs. The maximum permitted levels for mycotoxins vary among food commodities and countries. The European Commission (EC) and the Korean Ministry of Food and Drug Safety (MFDS) have established maximum permitted levels for various mycotoxins in food products [11,12]. These limits include 0.1–15 µg/kg for total AFs (sum of AFB1, AFB2, AFG1, and AFG2), 0.5–20 µg/kg for OTA, 150–2000 µg/kg for DON, 20–350 µg/kg for ZEN, and 200–4000 µg/kg for total FBs (sum of FB1 and FB2). Recently, the EC introduced Commission Regulation (EU) 2024/1038, which set maximum levels of 10–1250 µg/kg for type A trichothecenes (T-2 and HT-2 toxins) in food products [13]. The United States Food and Drug Administration (FDA) also has set regulatory limits of 20 µg/kg for total AFs, advisory levels of 1000 µg/kg for DON, and guidance levels of 2000–4000 µg/kg for total FBs (sum of FB1, FB2, and FB3) [14]. Although the FDA has established a regulatory limit for AFs in foods, AF contamination in the United States corn industry has been reported to result in an annual yield loss ranging from US\$ 52.1–1.68 billion [15].

The production cycle of soybean pastes is lengthy and seasonal [16]. Because the indigenous microbes in the fermentation environment are not artificially controlled, undesirable toxic metabolites from filamentous fungi can be unintentionally produced [17–19]. Meju and Doenjang are sensitive to contamination by various mycotoxigenic fungi, such as *Aspergillus flavus*, *A. ochraceus*, *Fusarium graminearum*, and *F. asiaticum* [20,21], and may suffer co-contamination. Although contamination greatly affects product quality and industry profits, few studies have considered the complete mycotoxin profile of Doenjang, among other fermented soybean products, and the main substrate, Meju, which

directly affects the quality of Doenjang. Further research is needed to identify important mycotoxins and control their production using approaches that respect the products' longstanding traditions and cultural significance.

In this study, we described the mycotoxin profiles of Meju and Doenjang to identify the major contaminants. We also examined the microbial diversity in Meju and Doenjang concerning major mycotoxins, focusing on microbial taxa related to reduced AF levels. Finally, we proposed a method for reducing mycotoxins and preserving the indigenous microbiome through backslopping fermentation.

2. Materials and methods

2.1. Sample collection

A total of 133 Meju and 190 Doenjang samples manufactured through spontaneous fermentation were collected from online retailers, local markets, and households between 2018 and 2024 for mycotoxin profiling. The collected samples were homogenized and stored at −20 °C. Of these, 20 Meju and Doenjang samples naturally contaminated with AF were selected for high-throughput screening. We grouped 10 Meju and 10 Doenjang samples based on AF contamination; each sample was assigned a unique code indicating AF contamination and the type of product: “AF−” AF-free; “AF+,” AF-contaminated; “M,” Meju; and “D,” Doenjang. Samples with no significant differences in the physicochemical factors associated with microbial growth (moisture content, pH, salinity, and amino acid nitrogen content) were selected ($p > 0.05$; Fig. S1). AF levels in AF-contaminated Meju and Doenjang samples were 25.42–685.365 µg/kg and 35.33–89.97 µg/kg, respectively. All samples were equilibrated for 6 h at 20–25 °C before analysis.

2.2. Chemicals, reagents, sample preparation, and ultra-high performance liquid chromatography-tandem mass spectrometry

High-performance liquid chromatography (HPLC)-grade water, acetonitrile, and methanol (MeOH) were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). LC/mass spectrometry (MS)-grade ammonium formate, acetic acid, and formic acid were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Polyethylene glycol 8000, phosphate-buffered saline, polyoxyethylene sorbitol ester (Tween 20), and ZEN metabolites (ZAN, α -ZEL, α -ZAL, β -ZEL, and β -ZAL) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other certified mycotoxin standard solutions were purchased from Romer Labs (Union, MO, USA). FB1, FB2, and FB3 were stored at 4 °C, while other standard solutions were kept at −20 °C.

The quantification of multiple mycotoxins in Doenjang was performed as previously described [10]. For Meju, slight modifications were applied to this method to enhance extraction efficiency, considering the different matrix characteristics, specifically the low moisture content and high levels of soy proteins and lipids. In total, 2 g of Meju sample with 0.2 g polyethylene glycol 8000 was extracted with 10 mL of MeOH:water:acetic acid (79:20:1, v/v/v) by shaking for 30 min at 270 rpm, centrifuged (10,000g, 3 min, 4 °C), and the supernatants were collected in conical tubes. Another 10 mL of the extraction solvent was added to the residue, and the extraction step was repeated (30 min, 270 rpm). The sample extract was centrifuged, and the supernatant was collected in the same tube, frozen at −80 °C for 10 min, and centrifuged again. A 2-mL aliquot of the supernatant was evaporated, redissolved in 4 mL phosphate-buffered saline (0.1 % Tween 20, v/v), and loaded onto a multifunctional Myco 6-in-1 immunoaffinity column (VICAM, Milford, MA, USA). The cartridge was washed with 10 mL water and dried thoroughly under vacuum for 3 min. The immunoaffinity column was treated with 5 mL MeOH (0.2 % formic acid, v/v) to elute the toxins. The eluate was evaporated under a nitrogen stream at 45 °C. The residue was reconstituted with 0.2 mL extraction solvent, treated with 0.2 mL water, and vortexed for 30 s. The reconstituted solvent was filtered through a

polyvinylidene fluoride syringe filter (Whatman, Maidstone, UK) and subjected to LC-MS/MS analysis. The method's performance for the simultaneous determination of multiple mycotoxins in Meju was tabulated in Table S2.

UHPLC-electrospray ionization (ESI) and triple quadrupole MS/MS were performed using an Agilent Infinity 1290 series and Agilent 6470 B triple quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Santa Clara, CA, USA). Data analysis and quantification were performed using Agilent MassHunter Qualitative and Quantitative Analysis software (version 10.0). The chromatographic separation of 20 mycotoxins was performed on a Supelco Ascentis Express C18 (2.1 × 100 mm, 2.7 μm) at 40 °C (Sigma-Aldrich) from a 5 μL injection volume. Gradient conditions were established to achieve complete separation of the mycotoxins, allowing the chromatographic separation of isomers with identical mass-to-charge ratios. Elution was achieved in 20 min using the following gradient of solvent A (5 mM ammonium formate in water containing 1.0 % acetic acid, v/v) and solvent B (5 mM ammonium formate in MeOH containing 1.0 % acetic acid, v/v) with a flow rate of 0.3 mL/min: 0–3 min, 75 % A, 25 % B; 3–14 min, 90 % B; 14–16 min, 90 % B; 16–16.1 min, 25 % B; 16.1–20 min, 25 % B. The MS parameters were as follows: 9 L/min gas flow (ultrapure nitrogen, >99.99 %), 250 °C gas temperature, 30 psi nebulizer, 350 °C sheath gas temperature, 12 L/min sheath gas flow, and −2000 V (ESI-negative mode) and + 3500 V (ESI-positive mode) capillary voltages. The reaction conditions for each analyte are presented in Table S1.

2.3. DNA library construction and sequencing

Genomic DNA was extracted from the Meju and Doenjang samples using a DNeasy PowerSoil Pro Kit (QIAGEN, Germantown, MD, USA), following the manufacturer's instructions. For bacterial amplification, the V3–V4 region of the 16S rRNA gene was amplified using primers 341 F and 805 R (Table S3). The internal transcribed spacer 2 (ITS2) region was amplified for fungal amplification using the ITS3 and ITS4 primers (Table S3). PCR was conducted using the following conditions: predenaturation at 95 °C for 3 min followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The final extension was conducted at 72 °C for 5 min. The amplified products were purified using CleanPCR (CleanNA, Waddinxveen, Netherlands). Purified amplicons were sequenced using an Illumina MiSeq system (San Diego, CA, USA) from CJ Bioscience (Seoul, South Korea).

2.4. Bioinformatics analysis

Raw reads were processed by filtering low-quality (<Q25) reads using Trimmomatic (version 0.32). The paired-end sequence data were merged using VSEARCH (version 2.13.4). The primers were trimmed using the Myers–Miller alignment algorithm at a 0.8 similarity cutoff value. Nonspecific amplicons and grammar profiles were detected using nhmmer in HMMER software (version 3.2.1). Unique reads were extracted, and redundant reads were clustered with unique reads using VSEARCH (version 2.13.4). The EzBioCloud 16S rRNA database was used for taxonomic assignment with VSEARCH, generating a precise pairwise alignment [22]. For Eukarya, the UNITE database was used for taxonomic assignments. Query sequences that matched reference sequences with > 97 % similarity were considered accurate to the species level. Chimeric reads with < 97 % similarity were filtered through reference-based chimeric detection using the UCHIME algorithm and EzBioCloud database. After chimeric filtering, reads not identified at the species level in the EzBioCloud database were compiled, and *de novo* clustering was performed to generate operational taxonomic units (OTUs). Open-reference methods incorporated both reference-dependent and *de novo* detection. Excluding all chimeras, sequences with > 97 % similarity were identified to the species level, and

each was designated as an OTU. For sequences with a similarity of < 97 %, cluster analysis was performed using the CD-hit and UCLUST programs, and each cluster was designated an OTU. OTUs associated with a single sequence were excluded from the diversity analysis.

We grouped the Meju and Doenjang samples concerning AF contamination (AF− and AF+) to compare microbial diversity. Using in-house software developed by CJ Bioscience, we assessed the differences in diversity between the groups based on α-diversity indices, including Ace, Chao1, Jackknife, Shannon, NPSannon, and Simpson, and phylogenetic diversity analyses. The β-diversity distances were calculated using the Bray–Curtis and Generalized UniFrac algorithms and visualized using principal coordinate analysis (PCoA). A dissimilarity analysis [permutational analysis of variance (PERMANOVA)] was performed using 999 permutations. All analyses in Section 2.4 were performed using the EzBioCloud Microbiome Taxonomic Profile—the CJ Bioscience bioinformatics cloud platform (<https://www.ezbiocloud.net/>).

2.5. Investigation of aflatoxigenicity of fungi isolated from Meju

We determined the AF levels in Meju as previously described [23]. Briefly, 10 g of each sample was homogenized in 50 mL 80 % (v/v) MeOH, 25 mL hexane, and 0.4 g NaCl at 6200 rpm for 3 min using an ULTRA-TURRAX instrument (IKA, Staufen, Germany). The homogenized samples were filtered through a Whatman No. 4 filter, and 5 mL of each filtrate was diluted with 35 mL of 0.1 % (v/v) Tween 20 solution. The diluted sample was filtered again using Whatman GF/B, 20 mL was transferred to an AflaTest immunoaffinity column (VICAM), and the column matrix was washed with 15 mL water. AFs were eluted with 4 mL 0.1 % acetic acid in MeOH (v/v), and the eluate was dried under nitrogen gas at 50 °C. The residue was reconstituted in 1 mL 50 % MeOH and filtered through a 0.2 μm polyvinylidene fluoride syringe filter (Whatman). Quantification of AFs was conducted using HPLC on the Agilent 1260 infinity series instrument coupled with a photochemical reactor for enhanced detection and a fluorescence detector (Agilent). Chromatographic separation was conducted on a Waters Symmetry C18 column (150 × 4.6 mm, 3.5 μm; Waters, Milford, MA, USA) with a 50 μL injection volume at 40 °C. The mobile phase comprised solvents A (0.5 % acetic acid in water) and B (0.5 % acetic acid in a MeOH/acetonitrile mixture at an 85:15 v/v ratio). After an isocratic step with 40 % solvent B for the first 8 min, the gradient was linearly changed to 70 % solvent B within 2 min, held constant for 5 min, and then reversed to 40 % solvent B within 2 min. The column was re-equilibrated to initial conditions with 40 % solvent B for 3 min. The flow rate was maintained at 1.0 mL/min. AFs were derivatized with a photochemical reactor (PHRED) and detected at a fluorescence detector (FLD) with excitation and emission wavelengths of 360 and 450 nm, respectively. The method's performance was as follows: the linearity of all analytes, as evaluated by the coefficient of determination, exceeded 0.999. The mean accuracy and precision of the method ranged from 84.5 % to 93.6 % and 2.7–6.7 %, respectively. Additionally, the limits of detection (LOD) and quantification (LOQ) were determined to be 0.03–0.13 μg/kg and 0.10–0.40 μg/kg, respectively.

The aflatoxigenicity of fungi isolated from AF-free Meju was evaluated on potato dextrose agar (PDA) based on the expression of major AF biosynthetic genes. Fungi were isolated from each Meju sample as previously described [18]. Briefly, 10 g of each sample was homogenized in 0.85 % (w/v) saline solution to prepare 10-fold serial dilutions. We spread 0.2 mL of each dilution onto three dichloran rose bengal chloramphenicol (Becton, Dickinson, Le Pont de Claix, France) agar plates for incubation at 25 °C (Sejong Scientific, Bucheon, Korea) over 5–7 d. All colonies were transferred to PDA (Becton Dickinson) for subculturing until a pure culture was obtained. The AF produced by each isolate on PDA was quantified using the HPLC-PHRED-FLD described above. Further confirmation of AF production and its toxic precursor, sterigmatocystin, was also performed using LC-MS/MS analysis. The

instrument was identical to that described in Section 2.2. The mobile phase consisted of water with 0.1 % formic acid (Phase A) and methanol with 0.1 % formic acid (Phase B). The gradient elution program was as follows: 60 % Phase A and 40 % Phase B at 0 min, transitioning to 20 % Phase A and 80 % Phase B at 5 min, maintained until 7 min, followed by a return to 60 % Phase A and 40 % Phase B at 7.5 min, and held constant until 11 min. Chromatographic separation was performed using an Agilent ZORBAX Eclipse Plus C18 column (4.6 × 100 mm, 3.5 μm) with a 0.5 mL/min flow rate at 40 °C. Ionization was conducted in ESI positive mode, with all other MS parameters identical to those described in Section 2.2. The mass transitions for aflatoxins (AFs) are listed in Table S1, and sterigmatocystin was detected using transitions of 325.0 *m/z* → 281.0 *m/z* (quantifier, collision energy 28 V) and 310.0 *m/z* (qualifier, collision energy 40 V).

The presence of three AF biosynthetic genes (*aflO*, *aflP*, and *aflR*) was confirmed using multiplex PCR (Table S2) [24–26]. Aflatoxigenic *A. flavus* (KCCM 60330) was used as a positive control, while *A. flavus* (ATCC 96045), a mutant derived from strain AF36 with the ability to inhibit aflatoxin production, was used as a negative control. Fungal DNA was extracted using a Qiagen DNeasy Ultraclean Microbial Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of DNA were determined from the A260/A280 ratio using a SpectraMax Mini Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA). PCR was performed as previously described [18]. PCR amplification was performed using an AccuPower PCR PreMix Kit (Bioneer, Daejeon, Korea) with 10–20 ng template DNA. Amplification of target DNA was performed under the following conditions in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA): predenaturation at 94 °C for 4 min and then 30 cycles of denaturation at 95 °C for 1 min, annealing at 64 °C for 2 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified products were loaded onto 2 % agarose gel (1 × Tris acetate-EDTA buffer, w/v) and analyzed by electrophoresis at 100 V for 50 min using a PowerPac HC High-Power Current Supply (Bio-Rad). After electrophoresis, the amplification products were confirmed using a Bio-Rad Gel Doc EZ Imager.

2.6. Manufacture of Meju and Doenjang via backslopping fermentation

The soybeans (*Glycine max* L.) used in this study were cultivated in Anseong-Si, Gyeonggi-Do, South Korea, during 2022–2023. They were washed five times with tap water and soaked in tap water for 12 h in stainless-steel containers. The soaked soybeans were manually dehulled and steamed at 121 °C for 60 min at 0.11 MPa using a high-pressure sterilizer (JeioTech, Seoul, Korea) and then placed in a stainless-steel sieve and left to cool to 40 °C. The steamed soybeans were manually crushed and molded into bricks. The backslopping inoculum (freshly fermented Meju) and aflatoxigenic strain *A. flavus* KCCM 60330 were added and mixed with the crushed steamed soybeans. The backslopping inoculum was added at 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, and 12.5 % of the weight of the steamed soybeans. A spore suspension containing 1×10^4 conidia/mL was inoculated at an amount equivalent to 0.5 % of the Meju-sample weight. The degree to which AF production was inhibited was evaluated according to the proportion of backslopping inoculum in 100 g lab-scale Meju. After setting the optimized proportion of backslopping inoculum, Meju was manufactured via a scaled-up approach for commercial yield (i.e., 1.5 kg) comparable to that used by small and medium enterprises. At least three samples were prepared from the control and experimental groups under all conditions. The molded soybean bricks were air-dried at 40 °C for 24 h and spontaneously fermented outdoors for 40 d to produce Meju. The lab-scale Meju samples were air-dried at 40 °C for 12 h and fermented in an incubator for 7 d at 28 °C. The fermented Meju was soaked in 20 % (w/v) brine to a 35:15:60 wt ratio of Meju:salt:water and left to ferment for an additional 60 d in polypropylene containers. The liquid phase was separated and discarded, and the solid fraction was mashed manually and aged for 6

months to produce Doenjang. The manufacturing processes for Meju and Doenjang using spontaneous and backslopping fermentation are illustrated in Fig. S2.

2.7. Evaluation of physicochemical and sensory properties of Meju and Doenjang

We analyzed the Meju and Doenjang samples manufactured via spontaneous and backslopping fermentation regarding moisture content, salinity, pH, amino acid nitrogen content, protease activity, sugar content, and color, as previously described [23]. Seven panelists conducted a descriptive sensory analysis of Meju and Doenjang. This study was approved by the institutional review board of Yong In University (approval no. 2–1040966-AB-N-01–2109-HSR-247–0; Yongin-si, Gyeonggi-do, South Korea). The panelists were trained to correctly discriminate appearance, aroma, and flavor characteristics using the spectrum descriptive analysis method [27]. The descriptive terms used in this study were previously developed based on the consensus of trained panelists [23]. For quantitative sensory analysis, refrigerated Meju and Doenjang samples were equilibrated to room temperature before being served to the panel. At 2 h before the evaluation, 3 g of each sample was transferred to a glass bottle (diameter × height: 5 × 3 cm) and immediately sealed with a lid. A random three-digit code was assigned to each sample. Each sample was placed in a photographic light box (Shenzhen Puluz Technology, Shenzhen, China) in daylight mode to evaluate the sample's appearance. A slice of white bread (2 × 2 × 1 cm) and water with lemons were provided as palate cleansers between each tasting, and the panelists took mandatory 5-min breaks after each tasting to minimize sensory fatigue. A 9-point scale was used to evaluate the intensity of each attribute, ranging from “extremely weak” (1) to “extremely strong” (9), with five repetitions.

2.8. Statistical analysis and data visualization

Statistical analyses were conducted using R (version 4.3.2) with significance set at $p < 0.05$. Chord plots were visualized with RAW-Graphs (<https://www.rawgraphs.io/>). Visualizations of taxonomic composition, microbial diversity, and AF reduction were created using the ggplot2 (version 3.5.1). Correlations were plotted using the ggcorplot (version 0.1.4.1), and clustered heatmaps were generated with the ComplexHeatmap (version 2.18.0). 3D PCoA plots were created with Plotly (version 4.10.1), and group differences were evaluated via the scikit-bio Python library (version 0.5.8). Fungal community succession was plotted using GraphPad Prism 8.

3. Results

3.1. Major hazardous mycotoxins in Meju and Doenjang

AFB1 was the most prevalent mycotoxin in Meju. It was detected in 30.1 % of samples at 0.05–865.35 μg/kg (Fig. 1). In Doenjang, AFB1 was detected in 22.6 % of samples at 0.11–79.10 μg/kg, indicating high levels of contamination in both product types. The regulatory limits set by the Korean Ministry of Food and Drug Safety (10 μg/kg for AFB1 and 15 μg/kg for total AFs) exceeded 16.5 % and 5.3 % of the Meju and Doenjang samples, respectively. AF levels were not significantly correlated with those of other mycotoxins; however, a significant strong positive correlation was observed in terms of both the occurrence (lower limit: 0.21, upper limit: 0.83) and incidence (0.59, 0.89) of AFB1, AFB2, AFG1, and AFG2. ZEN and OTA were other notable contaminants in both Meju and Doenjang. On average, 32.1 % (range, 0.07–1322.06 μg/kg) and 34.6 % (range, 2.98–587.48 μg/kg) of samples were contaminated with OTA and ZEN, respectively, and the contamination levels of 6.2 % of the samples exceeded the regulatory limit for OTA (20 μg/kg). OTA and ZEN were detected more frequently in Doenjang than Meju and exhibited a significant positive correlation regarding mycotoxin

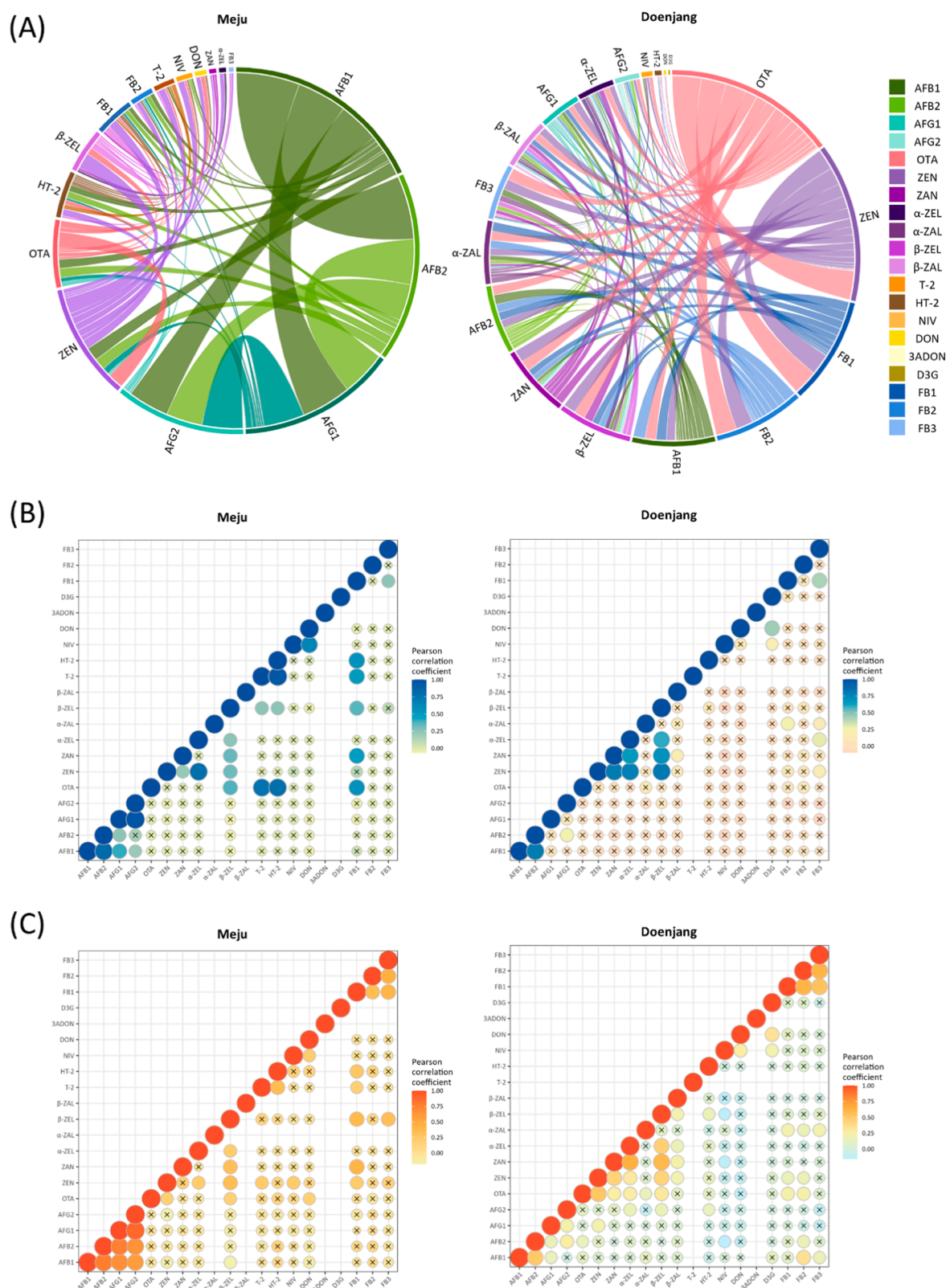


Fig. 1. Multi-mycotoxin profiling in Meju and Doenjang. (A) Chord plot of multi-mycotoxin network. Correlations in (B) occurrence and (C) incidence levels between mycotoxins (× indicates no significant difference, $p > 0.05$). AF stands for aflatoxin, OTA for ochratoxin A, ZEN for zearalenone, ZEL for zearalenol, ZAL for zearalanol, NIV for nivalenol, DON for deoxynivalenol, ADON for acetyl-DON, D3G for DON-3-glucoside, and FB for fumonisins B.

incidence. ZEN levels showed a strong positive correlation with metabolite levels; however, the levels of all ZEN metabolites were very low (1.18–21.44 µg/kg). AFs, ZEN, and OTA co-occurred in 66 % of the samples. Contamination with all trichothecenes was observed in < 10 % of Meju and Doenjang samples, also only at low concentrations (maximum of 175.24 µg/kg). The FB group was detected in < 4.5 % of the Meju samples. In contrast, FB1 was detected in 47.9 % of Doenjang samples but at very low concentrations (maximum of 24.56 µg/kg).

3.2. Microbiome taxonomic profile associated with AF contamination

We performed taxonomic classifications of fungi in Meju and Doenjang to the species level (taxa with a relative abundance of $\geq 1\%$ were plotted, and unclassified OTUs were excluded) to offer insight into the distribution of fungi across groups categorized by AF contamination in Fig. 2. We identified 25 and 16 taxa in the AF-free and AF-contaminated Meju groups, respectively. In the AF-free Meju group, *Densospora* sp. was the predominant fungus (18.9 %), followed by *A. flavus* (14.8 %) and *A. niger* (10.0 %). In Doenjang, 36 and 19 taxa were classified into the AF-free and AF-contaminated groups, respectively. In the AF-free Doenjang group, *Mucor racemosus* (23.9 %) and *A. cibarius* (10.3 %) dominated. In contrast, some yeasts, such as *Zygosaccharomyces rouxii* and *Debaryomyces prosopidis*, were more abundant in AF-contaminated than in AF-free Doenjang. More phyla and species were observed in AF-free Meju and Doenjang samples than in AF-contaminated samples. The bacterial communities are shown in Fig. S3. Regardless of AF contamination, the three most abundant bacteria in Meju were *Bacillus subtilis*, *Enterococcus faecium*, and *B. licheniformis*. *B. subtilis*,

Tetragenococcus halophilus, and *B. licheniformis* were the most abundant species in the Doenjang samples, regardless of AF contamination.

3.3. Microbial diversity associated with AF contamination

We analyzed and compared the fungal microbiome diversity in the Meju and Doenjang samples and observed a significant difference in AF contamination. The α -diversity indices, including OTUs, Chao, and Jackknife, in both the AF-free Meju and Doenjang groups were higher than those in the AF-contaminated groups ($p < 0.05$), indicating higher species richness in the AF-free groups (Fig. 3A). Greater phylogenetic diversity was observed in AF-free than in AF-contaminated Meju ($p < 0.01$). PCoA based on the Bray–Curtis distance metric was used to investigate the grouping of fungi associated with AF contamination. PERMANOVA demonstrated that the β -diversity of the fungal communities in Meju differed depending on the presence of AF at both the genus and species levels ($p < 0.05$; Fig. 3B), but not in Doenjang. Bacteria also showed higher species richness and phylogenetic diversity in both Meju and Doenjang (Fig. S4). However, the PCoA with PERMANOVA found no difference in the bacterial profiles of the AF-free and AF-contaminated groups in either Meju or Doenjang ($p > 0.05$). These findings suggested that AF contamination was strongly associated with the fungal microbiome structure.

3.4. Characterization of mycobiome associated with AF contamination

We characterized the mycobiome based on AF contamination to identify taxa significantly abundant in AF-free samples, aiming to utilize

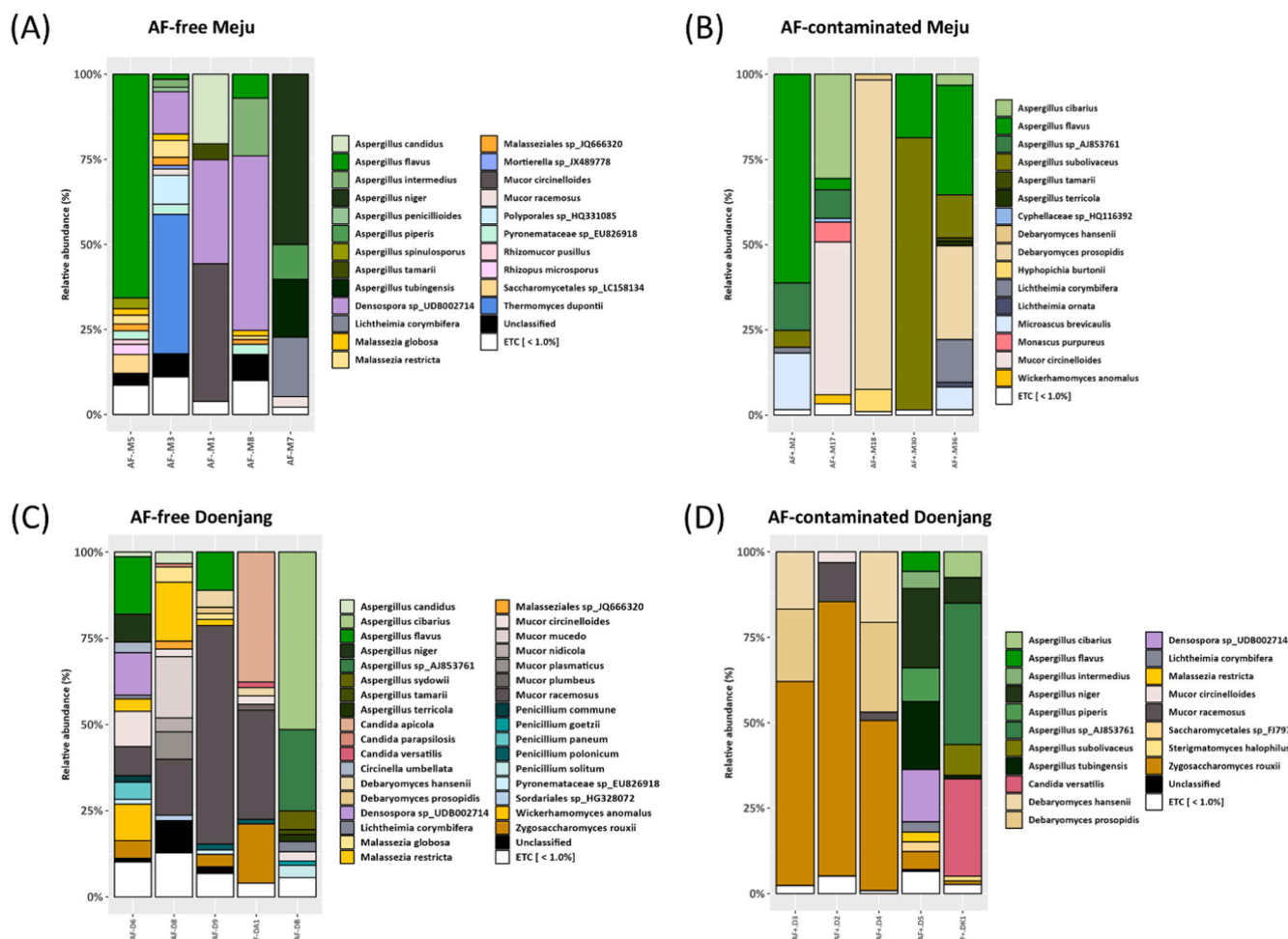


Fig. 2. Classification and relative abundance (%) of fungal taxa in (A, C) AF-free and (B, D) AF-contaminated (A, B) Meju and (C, D) Doenjang at the species level.

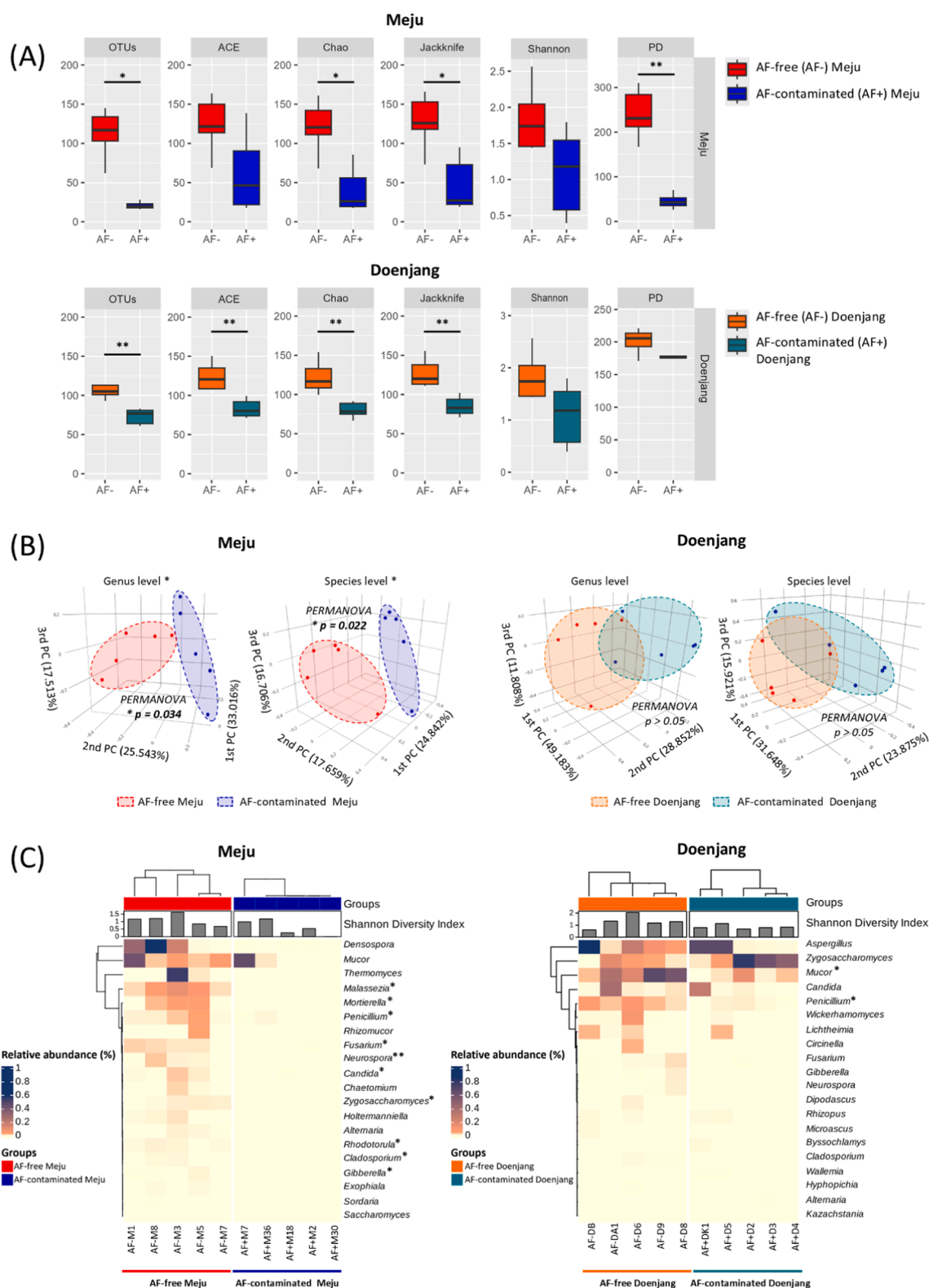


Fig. 3. (A) α -Diversity indices in AF-free (AF-) and AF-contaminated (AF+) Meju and Doenjang samples (Wilcoxon rank-sum test, * $p < 0.05$, ** $p < 0.01$). OTUs, observed taxonomic units; ACE abundance-based coverage estimator; PD, phylogenetic diversity. (B) PCoA of fungal communities with PERMANOVA based on the Bray–Curtis distances, at genus and species levels (* $p < 0.05$), and (C) heatmap with the top 20 taxa in terms of abundance at the genus level in Meju and Doenjang (* $p < 0.05$, ** $p < 0.01$).

these findings as a foundation for developing a reduction strategy. The top 20 taxa (based on *p*-values) were visualized using a heat map (Fig. 3C), which supported the findings of the diversity analysis, indicating greater diversity in AF-free than in AF-contaminated samples. We identified 10 and 2 genera that were more abundant in AF-free Meju and Doenjang (*p* < 0.05), respectively: *Neurospora*, *Malassezia*, *Penicillium*, *Gibberella*, *Mortierella*, *Cladosporium*, *Rhodotorula*, *Candida*, *Fusarium*, and *Zygosaccharomyces* in Meju and *Penicillium* and *Mucor* in Doenjang. *Penicillium* sp. was detected only in AF-free Meju and Doenjang, where it was detected in all samples. At the species level, 23 taxa in Meju and one taxon in Doenjang varied significantly depending on the presence of AF. The top 20 taxa are shown in Fig. S5. Except for four taxa, all others were significantly more abundant in the AF-free group. *A. subolivaceus*, *A. terricola*, *A. parasiticus*, and *A. cibarius* were the most abundant species in the AF-contaminated groups. Among them, *A. subolivaceus*, *A. terricola*, and *A. parasiticus* belong to the *Aspergillus* section *Flavi*. *Aspergillus* was detected across all Meju and Doenjang samples and was thus not an important taxon in the current context. However, the occurrence of various *Aspergillus* species depended on the AF-free conditions in Meju.

3.5. Reduction in AF levels via backslopping fermentation

Backslopping fermentation was applied during the upstream phase of Doenjang production, specifically in the manufacture of Meju. To reflect various producers' diverse artisanal manufacturing environments, we used commercially available Meju samples as backslopping inocula. We employed a three-step process to ensure the non-aflatoxigenicity of the Meju samples used as backslopping inoculum. First, (1) we selected AF-free Meju samples in which none of the four aflatoxins (AFB1, AFB2, AFG1, and AFG2) were detected via HPLC-FLD analysis. (2) None of the isolates from the AF-free Meju samples produced AF or its toxic precursor, sterigmatocystin, as confirmed by HPLC-FLD and LC-MS/MS. Finally, (3) all isolates exhibited at least one deletion in three key AF biosynthetic genes: *afIO* and *afIP* (late pathway genes) and *afIR* (regulatory gene). Only Meju samples satisfying all three criteria (1)–(3) were selected as “backslopping inocula”. The backslopping inocula were grouped according to their dominant genera, resulting in two groups: *Aspergillus*-dominant (*n* = 5) and *Penicillium*-dominant (*n* = 5). These genera were selected based on their significant abundance in AF-free Meju and Doenjang, as described in Section 3.4.

The inhibition of AF production via backslopping fermentation was evaluated by co-inoculating an AF-producing strain during the manufacturing process. First, the reduction in AF levels was evaluated at varying backslopping rates (Fig. 4A). When only an aflatoxigenic strain was inoculated (without a backslopping inoculum), AF production reached 1263.0 µg/kg. However, under the same conditions, adding 0.5 % *Aspergillus*-dominant backslopping inoculum reduced the AF level to 56.5 µg/kg in Meju, demonstrating a 95.5 % reduction. By performing backslopping at a rate of > 1.0 %, the AF production level was reduced to a maximum of 4.6 µg/kg, reflecting a reduction of > 99.6 %. No AF was produced in Meju at a backslopping rate of > 5.0 %. Backslopping fermentation using the *Penicillium*-dominant inoculum reduced AF levels of 572.3 µg/kg (54.7 % reduction) and 241.7 µg/kg (80.9 % reduction) at backslopping rates of 0.5 % and 1.0 %, respectively. *Penicillium carneum*, *P. mexicanum*, and *P. commune* were relatively abundant in the *Penicillium*-dominant inocula. A significant reduction in AF levels was observed with only 0.5 % backslopping fermentation in both groups.

We evaluated the long-term sustainability of AF reduction through repetitive backslopping fermentation (Fig. 4B). The AF reduction effect was monitored across five consecutive cycles of backslopping fermentation, where a portion of the preceding batch was used as the backslopping inoculum for the subsequent cycle. A backslopping rate of ≥ 2.5 % was required to achieve an AF reduction rate of ≥ 95.0 %. Based on these results, the number of consecutive batches that could maintain effective AF control with continuous backslopping at rates of

2.5 % and 5.0 % was further investigated. In *Aspergillus*-dominant backslopping fermentation, a > 95 % reduction in AF levels was sustained through five cycles at a 2.5 % backslopping rate. At a 5.0 % backslopping rate, an even greater reduction of > 99 % was observed and maintained up to the fifth cycle. Similarly, the *Penicillium*-dominant backslopping fermentation also demonstrated robust AF reduction capabilities. A > 90.0 % reduction in AF levels was achieved at a 2.5 % backslopping rate, lasting until the fourth cycle, and a > 95.0 % reduction was successfully maintained through five consecutive cycles at a 5.0 % backslopping rate. Compared to *Aspergillus*-dominant fermentation, the *Penicillium*-based approach showed slightly lower efficacy but consistently maintained substantial AF reduction exceeding 90 % across multiple fermentation cycles.

To investigate fungal community succession during backslopping fermentation, we compared the fungal community of the backslopping inoculum with that of the newly fermented Meju batch prepared using the corresponding backslopping inoculum (Fig. 4C). The results showed that the dominant genera in the backslopping inoculum remained dominant in the new Meju batches, regardless of the dominant genera type. In *Aspergillus*-dominant backslopping fermentation, *Aspergillus* sp. maintained its dominance in the new batch, with a slight increase in relative abundance from 81.6 % to 92.9 %. In comparison, the relative abundance of *Wickerhamomyces* decreased from 16.7 % to 6.4 %. Similarly, in *Penicillium*-dominant backslopping fermentation, *Penicillium* sp. maintained a relative abundance of > 99 % in the new batch. These findings demonstrate that the non-aflatoxigenic fungal community in the backslopping inoculum was successfully transferred to the next batch and persisted as the dominant community until the end of backslopping fermentation.

3.6. Physicochemical and sensory properties of Doenjang and Meju following backslopping fermentation

We analyzed the physicochemical (moisture content, salinity, pH, amino acid nitrogen content, protease activity, sugar content, and color value) of Meju and Doenjang manufactured under spontaneous (control) and backslopping fermentation (Table 1). In Meju, the moisture content was 26.3 % in the control and 20.9–24.1 % in backslopping fermentation samples. The salinity and pH of the control were 14.4 % and 5.5, respectively, while they were 15.3–19.8 % and 5.4–6.0 in the backslopping groups. No significant differences were observed in protease activity across the groups. The amino acid nitrogen content was highest in the *Aspergillus*-dominant backslopping group (*p* < 0.05), while no significant difference was observed between the control and *Penicillium*-dominant backslopping groups. For Doenjang, the moisture content was 56.2 % in the control and 53.1–54.8 % in the backslopping fermentation group. The salinity and pH of the control were 15.7 % and 6.0, respectively, while they were 13.9–18.3 % and 5.0–5.5 in the backslopping groups. The lightness values indicated that the control group was brighter than the backslopping fermentation groups (*p* < 0.05). The amino acid nitrogen content in Doenjang, as with Meju, was highest in the *Aspergillus*-dominant backslopping group (*p* < 0.05). While pH increased in Meju prepared through backslopping fermentation, it decreased in Doenjang, indicating no consistent correlation with backslopping.

The sensory characteristics of Meju and Doenjang were also evaluated. The brightness scores decreased in the backslopping fermentation groups (3.4–5.5) compared to the control (6.5–6.7) in Meju and Doenjang, indicating a darker color in the fermented products. Sourness and saltiness were higher in the backslopping fermentation groups (4.2–5.7 and 4.0–5.7, respectively) compared to the control (3.6–4.1 and 3.5–3.8, respectively). Additionally, Doenjang's *Aspergillus*-dominant backslopping fermentation groups achieved significantly higher umami scores than the control and *Penicillium*-dominant groups (*p* < 0.05). A multiple regression analysis of the overall satisfaction was performed as follows: $Y = 5.559 - 0.193X_1 + 0.352X_5 - 0.217X_6$ (X_1 = brightness of the

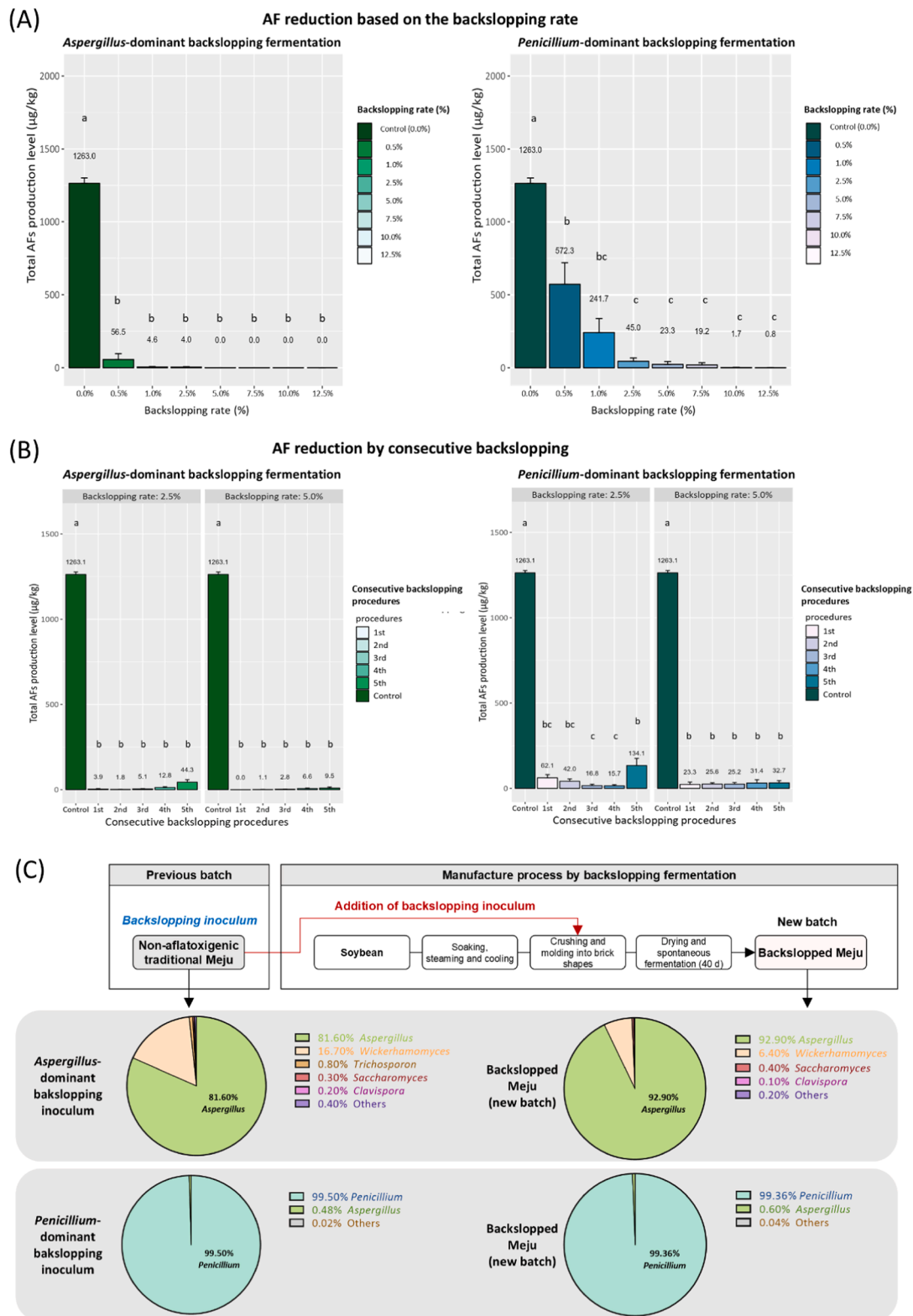


Fig. 4. Inhibition of AF production via backslopping fermentation. (A) *Aspergillus*-dominant and *Penicillium*-dominant fermentation at varying backslopping rates (0.0 % indicates spontaneous fermentation) and (B) under consecutive backslopping procedures. Error bars represent standard errors. Different letters indicate significant differences between groups ($p < 0.05$; one-way ANOVA followed by Scheffe's post hoc multiple range test). (C) Fungal community succession during backslopping fermentation at the genus level.

Table 1
Physicochemical and sensory properties of Meju and Doenjang manufactured via spontaneous (control) and backslopping fermentation.

Food	Property		Control	A-2.5 % ¹	A-5.0 %	P-2.5 % ²	P-5.0 %
Meju	Moisture content (%)		26.3 ± 2.8 ^{a,3}	21.5 ± 6.3 ^b	20.9 ± 7.2 ^b	24.1 ± 6.1 ^{ab}	21.2 ± 6.5 ^b
	Salinity (%)		14.4 ± 3.4 ^b	19.0 ± 2.8 ^a	19.8 ± 3.4 ^a	15.5 ± 1.8 ^b	15.3 ± 3.3 ^b
	pH		5.5 ± 0.6 ^b	6.0 ± 0.6 ^a	5.9 ± 0.6 ^a	5.4 ± 0.4 ^b	5.5 ± 0.5 ^b
	Amino acid nitrogen (mg%)		882.6 ± 419.5 ^{bc}	1082.9 ± 295.4 ^{ab}	1106.2 ± 256.1 ^a	728.08 ± 50.8 ^c	792.5 ± 86.9 ^c
	Protease activity (Unit/g) ^{ns,4}		882.6 ± 128.7	871.3 ± 51.5	769.3 ± 119.7	862.4 ± 108.6	748.8 ± 328.6
	Color value	L ^{ns}	52.4 ± 5.2	47.3 ± 11.7	48.2 ± 12.1	50.6 ± 5.9	48.6 ± 5.9
		a	7.2 ± 0.3 ^c	8.8 ± 1.8 ^a	8.3 ± 2.0 ^{ab}	7.6 ± 0.9 ^{bc}	7.2 ± 0.6 ^c
		b	15.7 ± 0.7 ^b	19.4 ± 4.0 ^a	19.3 ± 3.8 ^a	20.0 ± 2.7 ^a	18.6 ± 2.2 ^a
	Appearance	Brightness	6.7 ± 1.0 ^a	4.5 ± 0.8 ^c	3.4 ± 1.0 ^c	5.5 ± 0.7 ^b	4.6 ± 1.0 ^c
	Aroma	Sour	3.6 ± 1.3 ^c	4.5 ± 1.3 ^b	5.7 ± 1.3 ^a	4.2 ± 1.1 ^{bc}	4.2 ± 1.4 ^{bc}
		Sweet ^{ns}	3.8 ± 1.3	4.8 ± 1.3	4.7 ± 1.6	4.6 ± 1.2	4.7 ± 1.3
		Salty	3.5 ± 1.0 ^d	5.0 ± 1.2 ^{ab}	5.5 ± 1.2 ^a	4.1 ± 1.2 ^{cd}	4.5 ± 1.3 ^{bc}
		Savory	3.6 ± 1.0 ^b	5.1 ± 0.9 ^a	5.0 ± 1.4 ^a	4.4 ± 1.2 ^a	4.8 ± 1.3 ^a
		Off-aroma ^{ns}	4.4 ± 1.9	4.3 ± 1.5	4.6 ± 1.4	3.8 ± 1.5	4.0 ± 1.5
		Overall satisfaction	4.2 ± 1.1 ^b	5.7 ± 0.9 ^a	5.7 ± 1.0 ^a	5.3 ± 0.8 ^a	5.6 ± 1.2 ^a
Doenjang	Moisture content (%)		56.2 ± 1.0 ^a	54.4 ± 0.3 ^{ab}	54.8 ± 1.3 ^{ab}	54.0 ± 0.8 ^b	53.1 ± 1.0 ^b
	Salinity (%)		15.7 ± 1.9 ^{abc}	17.9 ± 0.6 ^{ab}	18.3 ± 1.0 ^a	14.7 ± 2.4 ^{bc}	13.9 ± 2.2 ^b
	pH		6.0 ± 0.0 ^a	5.4 ± 0.0 ^c	5.5 ± 0.0 ^b	5.0 ± 0.0 ^e	5.1 ± 0.0 ^d
	Amino acid nitrogen (mg%)		569.5 ± 27.6 ^c	851.0 ± 16.9 ^b	949.2 ± 13.8 ^a	469.5 ± 32.5 ^d	411.5 ± 11.7 ^e
	Sugar contents (°Brix)		3.6 ± 0.1 ^b	3.6 ± 0.0 ^{ab}	3.7 ± 0.1 ^a	3.4 ± 0.0 ^c	3.5 ± 0.1 ^b
	Color value	L	37.6 ± 0.6 ^a	30.3 ± 0.3 ^c	29.4 ± 0.7 ^c	31.6 ± 0.5 ^b	30.7 ± 0.9 ^{bc}
		a	9.4 ± 0.9 ^a	8.8 ± 0.5 ^{ab}	7.8 ± 0.8 ^b	7.8 ± 0.1 ^b	7.7 ± 0.4 ^b
		b ^{ns}	19.6 ± 1.4	18.1 ± 1.3	17.3 ± 2.5	17.3 ± 1.9	18.1 ± 1.6
	Appearance	Brightness	6.5 ± 1.1 ^a	5.0 ± 0.7 ^b	3.4 ± 1.1 ^c	5.5 ± 0.7 ^b	4.0 ± 1.0 ^c
	Aroma	Sour	4.1 ± 1.4 ^b	5.4 ± 1.4 ^a	5.4 ± 1.3 ^a	4.6 ± 1.3 ^{ab}	4.7 ± 1.2 ^{ab}
		Sweet	3.9 ± 1.0 ^b	4.9 ± 1.3 ^a	5.0 ± 1.8 ^a	4.7 ± 1.1 ^a	5.2 ± 0.9 ^a
		Salty	3.8 ± 1.1 ^b	5.5 ± 1.4 ^a	5.7 ± 1.3 ^a	4.0 ± 1.0 ^b	4.3 ± 1.1 ^b
		Savory	3.9 ± 1.3 ^b	4.7 ± 1.4 ^{ab}	5.4 ± 1.2 ^a	4.0 ± 1.1 ^b	4.1 ± 1.1 ^b
		Off-aroma ^{ns}	4.4 ± 1.7	4.6 ± 1.3	4.9 ± 1.3	3.9 ± 1.2	4.1 ± 1.6
	Flavor	Sour ^{ns}	4.1 ± 1.3	4.8 ± 1.3	4.8 ± 1.4	4.3 ± 1.3	4.8 ± 1.5
		Sweet	3.9 ± 1.2 ^b	5.1 ± 1.5 ^a	5.3 ± 1.7 ^a	4.4 ± 1.5 ^{ab}	4.7 ± 1.4 ^{ab}
		Salty ^{ns}	5.7 ± 1.3	5.7 ± 1.2	5.8 ± 1.4	6.1 ± 0.9	5.8 ± 1.0
		Bitter ^{ns}	5.1 ± 1.8	4.3 ± 1.4	3.6 ± 1.2	4.5 ± 1.6	3.6 ± 1.5
		Savory ^{ns}	4.1 ± 1.5	4.9 ± 1.3	4.9 ± 1.6	3.9 ± 0.8	4.4 ± 1.3
		Umami	3.6 ± 1.3 ^b	5.1 ± 1.5 ^a	4.9 ± 1.6 ^a	4.1 ± 1.3 ^{ab}	4.2 ± 1.4 ^{ab}
		Off-flavor ^{ns}	4.4 ± 1.8	3.9 ± 1.5	4.6 ± 1.5	4.0 ± 1.4	4.2 ± 1.4
		Overall satisfaction	4.2 ± 1.2 ^c	5.5 ± 0.9 ^a	5.4 ± 1.2 ^a	4.7 ± 1.0 ^{bc}	5.1 ± 1.0 ^{ab}

¹ Meju and Doenjang manufactured with 2.5 % and 5.0 % *Aspergillus*-dominant backslopping inoculum (A-2.5 % and A-5.0 %, respectively).
² Meju and Doenjang manufactured with 2.5 % and 5.0 % *Penicillium*-dominant backslopping inoculum (P-2.5 % and P-5.0 %, respectively).
³ Mean ± standard deviation; different letters in a row indicate significant differences (Duncan's multiple range test, $p < 0.05$).
⁴ ns: not significant ($p > 0.05$).

appearance, X_5 = savory, X_6 = off-aroma; $p < 0.01$) for Meju; $Y = 3.197 + 0.211X_4 + 0.173X_8 + 0.300X_{11} - 0.313X_{13}$ (X_4 = salty aroma, X_8 = sweet flavor, X_{11} = savory flavor, X_{13} = off-flavor; $p < 0.05$) for Doenjang. Savory, a key factor positively correlated with overall satisfaction in Meju and Doenjang, was significantly enhanced in backslopping fermentation groups. Overall satisfaction scores were significantly higher in the backslopping fermentation groups for Meju and Doenjang ($p < 0.05$), highlighting the sensory improvements associated with the backslopping process.

4. Discussion

Studies on the microbiome of fermented soybean food are needed to inform the development of strategies for reducing mycotoxin contamination and public health risks. In this study, we identified the major mycotoxins in Meju and Doenjang and characterized the associated microbiome to identify a promising mycotoxin control strategy. To investigate contamination patterns, we conducted multi-mycotoxin profiling on 323 Meju and Doenjang samples. AFB1 was identified as the most frequently and highly contaminated mycotoxin in both Meju and Doenjang in our study. Among AFB1-positive samples, 54 % and 19 % exceeded the regulatory limit of 10 µg/kg. OTA and ZEN were identified as significant mycotoxins of concern, with frequent contamination observed notably in Doenjang. These differences in contamination patterns are more likely influenced by production environments than intrinsic product characteristics. Alternatively, the diverse

ingredient composition and the longer fermentation and aging process of Doenjang compared to Meju might also provide more opportunities for exposure to environmental fungi, potentially resulting in additional mycotoxin production. Type A and B trichothecenes were minor contaminants in fermented soybean foods, with incidence rates below 10 % and concentrations under 30 µg/kg, aligning with a previous report [28]. While some mycotoxins co-occurred in Doenjang, very few showed strong relationships. Notably, the most frequently co-occurring mycotoxins in Meju and Doenjang were AF + OTA + ZEN, followed by AF + OTA + FB and OTA + ZEN + FB, which may lead to synergistic toxicity. AFB1 was notably prominent in co-occurring combinations, appearing frequently alongside other mycotoxins. Several studies have reported synergistic effects induced by AFB1 + OTA [29], AFB1 + OTA + FB1 [30], and AFB1 + ZEN + FB1 [31,32]. The combinations of AFB1 + OTA and AFB1 + ZEN have also been identified in previous studies on Meju and Doenjang [10,17,33]. Moreover, these combinations are often observed in other fermented legume foods worldwide, such as AFB1 + OTA in iru (fermented locust beans in Africa) and AFB1 + FBs+ZEN in tempeh (fermented soybeans in Indonesia) [9,34]. Synergistic interactions have been linked to increased cytotoxicity, hepatotoxicity, and mutagenicity, posing a significant risk from consuming fermented soybean foods.

In addition, FB1 also frequently co-occurred with other mycotoxins in Doenjang. Previous studies have reported the isolation of *Fusarium* spp., such as *F. proliferatum* and *F. fujikuroi*, from Doenjang and its raw materials, with an increased distribution of *Fusarium* species observed

during the fermentation of Meju and Doenjang [35,36]. However, in our study, FB1 levels were detected at a maximum of 24.56 µg/kg, which is lower than the levels previously reported in fermented soybean paste (1.85–56.19 µg/kg) and fermented locust bean (32–167 µg/kg), suggesting that FB1 contamination in these products may be of relatively less concern compared to other mycotoxins [28,37]. Overall, mycotoxin contamination, especially AF, in the present study was much more severe than that previously reported for Meju and Doenjang [17–19]. Despite efforts to develop reduction strategies, these products remain highly vulnerable to mycotoxin contamination. AFB1, classified as a Group 1 carcinogen by the International Agency for Research on Cancer, is the most toxic mycotoxin. It highlights the urgent need to prioritize effective reduction strategies to mitigate its presence in these fermented products.

To characterize the microbiomes of Meju and Doenjang and identify the microbes associated with AF contamination, we performed a metagenomic analysis using microbiome taxonomic data. Alpha-diversity analysis demonstrated that fungal diversity was significantly higher in AF-free Meju and Doenjang than in AF-contaminated samples, consistent with previous findings suggesting that AF reduces fungal diversity in medicinal herbs [38,39]. The PCoA with PERMANOVA showed that AF contamination strongly affected fungal community structure, particularly in Meju at both the genus and species levels ($p < 0.05$). However, no clear differences were observed in the fungal communities of AF-free and AF-contaminated Doenjang. Doenjang is manufactured by mixing Meju and brine (prepared using solar salt) and is fermented for approximately half a year in a spontaneous environment. At this stage, red pepper and charcoal are typically added [40], among other complementary ingredients, such as jujube fruit, sesame, and barley. Consequently, Doenjang contains a wide range of microorganisms. This manufacturing process makes it challenging to identify differences in microbiomes based on AF contamination in the final product. Furthermore, we previously found that 77 % of the AF in Doenjang originated from the raw Meju substrate [23], highlighting the need for time-specific AF control, particularly during the Meju manufacturing stage.

First, the fungal community in Meju and Doenjang was investigated using high-throughput sequencing. In Doenjang, *Mucor racemosus* was the most frequently occurring fungus in all samples. Doenjang is typically manufactured in late winter, as the low temperature provides favorable conditions for the growth of *Mucor* species. Furthermore, *Zygomycetes* fungi, including *Mucor*, generally require high water activity for rapid growth, making them well-adopted from the early stages of fermentation in Doenjang [41]. Additionally, our results observed the presence of *Densospora* sp. in AF-free Meju groups. This genus, belonging to the family *Mucoromycota*, has rarely been reported in fermented soybean foods, and its role in fermentation remains largely unexplored [42]. Advancements in fungal metagenomic databases may enable future investigations to revisit unclassified OTUs, providing opportunities to elucidate their potential roles in fermentation dynamics and mycotoxin regulation.

Meanwhile, with no significant differences, *A. flavus* showed a high relative abundance in both AF-free and AF-contaminated Meju groups. *A. flavus* is a representative aflatoxin-producing fungus, whereas *A. oryzae* is classified as a Generally Recognized as Safe (GRAS) strain. Both are commonly associated with fermented soybean products and share a highly similar genome, as they diverged from a common ancestor [43,44]. This high similarity renders metagenomic analysis based on ITS region sequencing unable to differentiate between the two strains, potentially introducing biases in interpretation. However, metagenomic analysis provides the advantage of quantifying the relative abundances of taxa and facilitating diversity analyses to identify discriminative strains between groups. Therefore, we utilized high-throughput sequencing data to compare microbial compositions based on AF contamination.

Next, we focused on the unique taxa to AF-free Meju and Doenjang. *Penicillium* was the only taxon occurring in AF-free Meju and Doenjang,

but it was not found in AF-contaminated samples. Various *Penicillium* spp. have been found in Meju and Doenjang, among which *P. polonicum* is the most prevalent [45,46]. In our study, various *Penicillium* species, including *Penicillium carneum*, *P. mexicanum*, and *P. commune*, were isolated from Meju and Doenjang. *Penicillium* produces a cheesy smell in fermented soybean foods and contributes to the flavor of soy sauce [47]. Of course, several *Penicillium* spp. exert antifungal (inhibition rate of 35–70 %) and anti-aflatoxigenic (inhibition rate of 27–54 %) effects *in vitro* [48–50]. At the species level, 40 % of the differentially prevalent taxa belonged to *Aspergillus*. Atoxigenic *Aspergillus* is currently the most widely used biocontrol agent for reducing AF contamination and bioremediation in cereal crops [51]. Biocontrol strategies using *A. flavus* AF36 and Afla-Guard GR (*A. flavus* NRRL 21882; registered with the U.S. Environmental Protection Agency) to compete with naturally occurring mycotoxigenic strains have been demonstrated to effectively reduce AF contamination in the field [52]. In this study, we found that the fermentation of Meju using *Aspergillus*- or *Penicillium*-dominated inoculum effectively reduced AF contamination during Doenjang production.

AF levels were reduced by 95.5–100.0 % and 54.7–99.9 % through *Aspergillus*-dominant and *Penicillium*-dominant backslopping fermentation, respectively. Notably, 2.5 % backslopping inoculum suppressed AF production by > 95.0 % ($p < 0.05$). This reduction was maintained at > 90.0 % and > 95.5 % through the fifth consecutive cycle at backslopping rates of 2.5 % and 5.0 %, respectively. Considering that the typical production cycle for Meju and Doenjang spans one year, these findings suggest that backslopping fermentation is an effective long-term strategy for AF reduction. Previous studies on fermented soybean-based foods have mostly focused on reducing AF levels *in vitro* or *in situ*. *In situ* models have applied various treatments, including chemical treatment with organic acids (62.0–95.1 % reduction) [53], physical treatment by autoclave (33.6 % reduction) [53], gamma irradiation at 10 kGy (62.2–76.4 % reduction) [54], and ozone treatment at 20 mg/30 min (24 % reduction) [55]. Regarding biological reduction, several studies have highlighted the potential of starter cultures comprising selected microbial strains isolated from Doenjang, such as *A. oryzae* or *B. subtilis*, to suppress AF production *in vitro* studies [56]. However, there remains a significant lack of *in situ* studies, particularly at the real scale of Doenjang production, that evaluate their application within the Doenjang matrix during its production [53–55]. Here, we demonstrated that Meju and Doenjang manufacturing via backslopping could considerably reduce AF levels even at a larger fermentation scale while preserving the traditional fermentation methods.

Our results revealed that the higher amino acid content, darker color, and lower moisture content observed in the *Aspergillus*-dominant backslopping fermentation group indicate the most rapid fermentation of soybeans. The amino acid nitrogen content is a reflective indicator of soy protein hydrolysis, progressively increasing as fermentation advances [57]. Additionally, as fermentation progresses in soybean products, Maillard reactions involving amino compounds and reducing sugars lead to a darker color [58]. In Meju, prolonged ripening has been associated with increased pH, attributed to nitrogen compounds such as amines and amino acids produced from soy protein degradation [59]. In contrast, in the high-salt conditions of Doenjang, organic acids such as lactic acid and acetic acid are produced as fermentation progresses, contributing to a decrease in pH [60]. These findings further corroborate the rapid fermentation process of soybeans observed in this group. Notably, the overall satisfaction scores of Meju and Doenjang produced through backslopping fermentation were significantly higher, indicating that backslopping fermentation contributes to AF reduction and improvements in sensory quality. This sensory improvement is thought to be influenced by the dominant fungi present in the Meju used as the backslopping inoculum. Studies have reported that inoculating Doenjang with *Aspergillus* spp., particularly *A. oryzae*, significantly reduces off-flavors while enhancing savory flavors, leading to a notable increase in consumer preference [23]. Additionally, *Penicillium* spp., including

P. polonicum, *P. commune*, and *P. paneum*, have demonstrated strong positive correlations with aroma compounds, emphasizing their critical role in contributing to the characteristic odorant flavors of traditional soybean paste [61]. Notably, these species showed a strong positive correlation with pyrazine compounds, closely linked to nutty, roasted, and cocoa-like flavors.

Backslopping establishes the best-adapted strain as fermentation progresses, serving as a basis for the traditional or natural starter [62]. These strains determine the indigenous microbiome in the fermented foods. We selected Meju-containing atoxigenic fungi as the backslopping inoculum and added it during the early stages of new-batch fermentation, allowing the fungi to adapt to the fermentation environment. The biocontrol of aflatoxigenic strains using non-aflatoxigenic strains operates through competitive exclusion mechanisms. Atoxigenic strains deplete essential nutrients, such as carbon and nitrogen sources, critical for aflatoxin biosynthesis [63,64]. They also occupy physical space on food surfaces, thereby inhibiting the proliferation of toxigenic strains [65].

Additionally, native strains already adapted to the local environment often exhibit resource and space competition mechanisms, further enhancing the ecological advantages of non-aflatoxigenic strains [66]. In this context, using backslopping inoculum confirmed as non-aflatoxigenic is an effective strategy to mitigate AF contamination. By leveraging non-toxicogenic and ecologically competitive strains, this approach reduces the risk of AF production and supports the preservation of microbial communities adapted to fermented soybeans.

Therefore, the backslopping fermentation of Meju preserves the traditional manufacturing method and produces Doenjang with characteristic aroma and taste profiles while providing effective AF control. Despite effectively reducing AF levels, backslopping fermentation did not fully prevent AF contamination or remove AF-producing fungi, indicating a limitation of this study. Future research should aim to optimize fermentation conditions for complete AF inhibition and investigate the direct AF reduction capabilities of specific fungal strains identified as key discriminative taxa. Additionally, tracking microbial shifts to elucidate the mechanism of AF reduction and assessing this method's scalability and practical applications in diverse fermentation environments are recommended.

5. Conclusion

This study identified AF as a major contaminant in fermented soybean products, particularly Meju and Doenjang, and proposes a novel approach for addressing contamination. Multi-mycotoxin profiling and microbiome taxonomic analysis showed that *Aspergillus* and *Penicillium* species were strongly associated with AF-free conditions. Using backslopping fermentation, an artisanal food production method, we developed a mycotoxin-reduction strategy to preserve fermented soybean foods' indigenous microbiome and quality. To preserve the unique fungal community of Meju through backslopping fermentation while mitigating AF contamination, it is crucial to ensure the non-aflatoxigenicity of the Meju used as the backslopping inoculum. Backslopping using atoxigenic *Aspergillus*-dominant or *Penicillium*-dominant inoculum reduced the levels of AF by > 95 % at inoculation rates of ≥ 2.5 %. This reduction was maintained over five consecutive fermentation cycles, presenting a sustainable solution for AF contamination control. Compared with previous methods, including chemical, physical, and irradiation treatments, backslopping fermentation produced comparable or superior results while maintaining the sensory and physicochemical properties of the product. Backslopping fermentation offers a practical solution for small-scale artisanal producers, ensuring food safety and preserving traditional manufacturing practices. To the best of our knowledge, this is the first effective procedure applying the concept of backslopping to reduce AF contamination in traditional fermented soybean paste. The high contamination levels in control samples emphasize the need for improved Meju manufacturing strategies and

demonstrate the potential of backslopping fermentation in soybean product manufacturing. Furthermore, the findings of this study hold significant implications for both the domestic distribution and international export potential of Korean traditional fermented foods, offering a viable strategy to mitigate economic losses associated with AF contamination.

Environmental Implication

This study provides an environmentally friendly approach to reducing mycotoxin contamination in traditional fermented soybean foods, which can enhance food safety while preserving microbial diversity and sensory quality. The research supports sustainable artisanal food production practices by identifying aflatoxin as a major contaminant in Meju and Doenjang and proposing a backslopping fermentation method. This method mitigates public health risks associated with mycotoxins and minimizes the need for chemical interventions, thereby reducing environmental impact. The findings contribute to developing safer, more sustainable food systems and support preserving traditional fermentation practices.

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CRediT authorship contribution statement

Hyang Sook Chun: Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Woo Jun Sul:** Software, Methodology, Investigation. **Sang Yoo Lee:** Methodology, Formal analysis, Data curation. **Su Been Park:** Visualization, Software, Formal analysis. **So Young Woo:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2025.137777](https://doi.org/10.1016/j.jhazmat.2025.137777).

Data availability

Data will be made available on request.

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