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Aspergillus flavus Producing Aflatoxins Isolated from Materials of Commercial Feed in Japan

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Abstract

To investigate both aflatoxin contamination in feed produced in Japan and the distribution and movement of aflatoxin-producing fungi in the feed, a survey experiment was performed. *Aspergillus* fungi were isolated from imported Sudan grass hay, a material used to make total mixed ration (TMR) in Japan. HPLC analysis showed that one isolate produced aflatoxins B_1 and B_2. Isolates were identified as *A. flavus* and *A. terreus* from their morphologies, rDNA-ITS sequences and aflatoxin production. No aflatoxins were detected in the Sudan grass hay source of the isolates or the fermented TMR made from the Sudan grass as one of the raw materials. The fermentation quality of the TMR was good after ensilage for over 60 days, as indicated by increased lactic acid and decreased pH. Only lactic acid bacteria and Bacillus survived in the fermented TMR; no fungi or yeasts were detected. Fermentation is important in preventing aflatoxin contamination of silage.

Discipline: Animal industry
Additional key words: imported hay, rDNA-ITS, total mixed ration

Introduction

Aflatoxins—mycotoxins produced by *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare—are highly toxic and carcinogenic[12,20]. Aflatoxin is metabolized to epoxide by liver cytochrome P450. The epoxide causes DNA injury. As for the aflatoxins, more than ten kinds of relevant compounds are known. Aflatoxin B_1 is the most toxic compound of those. Aflatoxin M_1, one of the toxic aflatoxin relevant compounds, may occur in animal products, e.g. milk and other dairy products after consumption of aflatoxin B_1 contaminated feeds by animals[11]. The misfortune of aflatoxins for domestic animals is extremely rare in Japan. However, the presence of aflatoxins in animal products, the hazard to the health of domestic animals and the resulting economic losses are feared because of the toxicity of strong aflatoxins.

Aflatoxin-producing fungi are found mainly in tropical and subtropical regions[9]. Binder et al.[1] investigated mycotoxins in animal feeds that were produced in dozens of countries in the world. The results showed that regions with a high detection rate of aflatoxin were tropical and subtropical regions. The social problems associated with aflatoxin hardly occur in Japan.

However, recently, *A. flavus* and *A. parasiticus* have been isolated from soil in Kyushu and Okinawa, southwestern Japan[17], and in the Kanto region, central Japan[13]. The Japanese Food and Agricultural Materials Inspection Center announced[3] that aflatoxin B_1 was detected from 23.5% of imported corn. In addition, aflatoxin M_1 that is one of the metabolites of aflatoxin was found in bulk milk though it was at very low concentrations in various places in Japan[16]. This is proof of aflatoxin contamination in the feed. Therefore, it is necessary to investigate both aflatoxin contamination in feed produced in Japan and the distribution and movement of aflatoxin-producing fungi in the feed.

Total mixed ration (TMR) is feed made from roughage, concentrates, minerals, and essential nutrients. Since its high quality and uniformity maximize milk yield and quality, TMR has become a popular feed in Japan. TMR is generally fermented first to improve preservation and palatability. Since it is produced from various materials and is stored for some months, it has a risk of becoming contaminated with aflatoxin-producing fungi. Therefore, contamination should be assessed for the development of measures to reduce aflatoxin concentrations.

Here, aflatoxin-producing fungi and aflatoxin production in TMR were surveyed in Japan. In addition, we

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examined the relation between the fermentation quality of TMR and the concentration of aflatoxin to assess the production of aflatoxin in TMR during storage.

Materials and methods

1. Collection of feed samples

Samples of fermented TMR and its raw ingredients [alfalfa, fescue, Sudan grass, timothy (these hay were made from USA imports), brewer's grain, tofu cake, and formula feed] were obtained from a commercial TMR supplier in southwest Japan in July 2007.

2. Isolation and count of microorganisms

To isolate and count microorganisms in the TMR samples, we added 90 mL of sterile distilled water to 10 g of each sample, mixed and homogenized them, and made serial dilutions of 10^{-1} to 10^{-5}. For incubation, 20 µL of each dilution was applied to MRS medium (Difco Laboratories, Detroit, MI, USA) in 9-cm Petri dishes for lactic acid bacteria (LAB), to NA medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) for aerobic bacteria, and to PDA medium (Nissui) for fungi and yeasts. After treatment in hot water (75°C) for 15 min, 20 µL of each dilution was applied to clostridia count agar (Nissui) for clostridia and to NA medium for bacilli. After 2 days incubation in darkness at 30°C (aerobic for NA and PDA media, anaerobic for MRS and clostridia count agar), colonies were counted. Colonies of fungi were transferred to PDA slants and cultured at 25°C to preserve isolates for the following experiments.

3. Identification of *Aspergillus* fungi

Morphologies of the *Aspergillus* isolates were observed under a light microscope. Small pieces of a colony were vapor-fixed with 1% OsO_4 solution for a day, coated with gold-palladium by ion-sputter (JFC-1100, JEOL Co. Ltd., Akishima, Tokyo, Japan), and observed under a scanning electronic microscope (SEM, Hitachi S-800) according to Koga et al.[7]. Whole genomic DNA was extracted from the isolates as described previously[19]. The internal transcribed spacer (ITS) regions and 5.8S rDNA were amplified by PCR using the ITS1 and ITS4 primer pair[21] by the most common method used to identify *Aspergillus* species[6,14]. Purified PCR products were sequenced by ABI PRISM 3100 automated sequencers (Applied Biosystems, California, USA) according to the manufacturer's recommendations. The obtained sequence data were compared with data on DDBJ by the BLAST program[10]. A phylogenetic tree of the isolates based on the sequences of rDNA-ITS regions was described with some species of *Aspergillus* using the CLUSTAL W program on DDBJ[10].

4. Aflatoxin production experiment

The *Aspergillus* isolates were inoculated in 1 g sterilized corn meal plus 1 mL sterilized distilled water. After incubation for 30 days at 30°C in darkness, 10 mL acetonitrile was added and sonicated. The mixture was filtered through a filter paper (5A, Advantec, Tokyo, Japan). The filtrate was passed through a 0.45-µm membrane filter (DISMIC-13HP, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and then injected into an HPLC (auto sampler: AS-851, degasser: DG-980, pump: PU-880, column oven: CO-860, JASCO Corporation, Tokyo, Japan) equipped with a fluorescence detector (FP-2020 Plus, JASCO Corporation) for qualitative analysis. The HPLC conditions were as follows: mobile phase, acetonitrile/methanol/water = 1/3/6; column, Mightysil RP-18 GP (150 mm × 4.6 mm, 3 µm; Kanto Chemical, Tokyo, Japan); column temperature, 40°C; excitation wavelength, 365 nm; fluorescence wavelength, 450 nm; injection volume, 20 µL; and detection limit (aflatoxin B_1), 200 µg/mL. Authentic aflatoxins B_1, B_2, G_1, and G_2 (Kanto Chemical) were used as standards. We assumed the peak of aflatoxin B_1 (retention time, 15.3 min) to indicate aflatoxin-producing fungi.

5. Fermentation experiments

In the small-scale fermentation experiment, 100 g fresh weight (FW) of each raw TMR product was packed in plastic bags (18 × 26 cm; Hiryu BN-12, Asahi Kasei, Tokyo, Japan). The bags were then degassed and stored in the laboratory (20–25°C) in darkness for 63 days. Triplicate bags were used as replicates. In the commercial-scale experiment, 300 to 500 kg of material was packed into a plastic trans-bag. The bags were degassed and stored in a storehouse (20–30°C) for 64 days. Samples were collected from the upper, middle and lower parts of the packed TMR for replication.

6. Fermentation quality of TMR

Ten grams of fresh material was homogenized with 90 mL distilled water, and then the homogenate was filtered through a filter paper. The pH of the filtrate was measured with a glass electrode pH meter (MP230, Mettler Toledo, Columbus, OH, USA). The ammonium-N was determined by steam distillation of the filtrate. The organic acid (lactic, acetic, propionic, and butyric) contents were determined by HPLC (JASCO). The silage filtrates were shaken with cation exchange resin (Amberlite, IR 120B H AG; Organo, Tokyo, Japan) and centrifuged at 6,500 × g for 5 min, passed through a 0.45-µm membrane filter, and then injected into the HPLC.

The HPLC conditions were as follows: column, Shodex RS pack KC-811 (8.0 mm × 30 cm, Shoko Co., Ltd., Tokyo, Japan); oven temperature, 60°C; monitored wavelength, 450 nm; eluent, 3 mmol L^{-1} $HClO_4$ at 1.2 mL min^{-1}; and post-column reaction reagent, 0.2 mmol L^{-1} bromothymol blue, 8 mmol L^{-1} Na_2HPO_4, and 2 mmol L^{-1} NaOH at 1.2 mL min^{-1}.

7. Analysis of aflatoxins in TMR and ingredients

All the samples were dried in a forced-air oven at 65°C for 48 h and ground with a Wiley mill (ZM200, Retch GmbH, Haan, Germany) to pass a 1-mm screen. Thirty mL of acetonitrile solution (82%) was added to 6 g of dried and powdered sample. Then it was stirred well and filtered through filter paper (5A, Advantec). Six mL of the filtrate was mounted to a multi Multistep #226 AflaZon+® (Romer Labs Inc. Stylemaster Drive Union, USA). The outflow fluid was used as a HPLC analysis sample. The HPLC conditions were the same as in the aflatoxin production experiment. The detection limit was 30 µg/g dry weight (DW) (equaled 20 µg/g FW). The recovery ratio of additional experiment authentic aflatoxin (TMR: dry matter ratio 60%, 100 µg/g DW) was 109.4% (coefficient of variation = 3.2%).

Results

1. Microorganism composition of TMR and ingredients

The number of LAB from the mixed TMR just before fermentation was 9.3 log colony forming unit (CFU) g^{-1} FW. It decreased to 2.7 and 3.9 after small-scale and commercial fermentation, respectively (Table 1). Similarly, the number of aerobic bacteria before fermentation was 5.2 log CFU g^{-1} FW and decreased after fermentation to 3.9 log CFU g^{-1} FW in both. Fungi, yeasts and bacilli were detected from the mixed TMR or its ingredients before fermentation, but they were not detected after fermentation. The fungal counts from Sudan grass, timothy and formula feed were 3.8, 3.0 and 4.2 log CFU g^{-1} FW, respectively, but no fungi were detected from the mixed TMR before fermentation.

2. Identification of fungi

Three *Aspergillus* isolates were obtained from Sudan grass hay (Table 2). All 3 showed characteristic macronematous and colorless conidiophores producing monophialidic conidiogenous cells on the swollen, spherical apex (Fig. 1). Conidia were catenate, light green or light brown, spherical and 0-septate. Colonies of isolate No. 1 were effuse and light brown, but those of Nos. 2 and 3 were effuse and light green (Fig. 1). Number 1 showed 99% similarity to *A. terreus* (DDBJ accession No. AY939788) (Table 2). Numbers 2 and 3 showed 99% similarity to both *A. flavus* isolates (AY939782, AY939785). In the phylogenetic tree, the isolates Nos. 2 and 3 were located in the clade of *A. flavus* in *Aspergillus* sect. Flavi and the isolate No. 1 was in the clade of *A. terreus* (Fig. 2).

3. Aflatoxin production experiment

Isolate No. 2 produced aflatoxins after 30 days incu-

Table 1. Microorganism composition of TMR and its ingredients (log CFU/g FW[†1])

Materials						Clostridia	
	LAB[†2]	Fungi	Yeast	Aerobic bacteria	Bacillus	Black	White
TMR (before fermentation)	9.3	ND	4.9	5.2	4.2	ND[†3]	ND
Alfalfa hay	4.8	ND	4.2	6.2	4.0	ND	ND
Fescue hay	4.4	ND	ND	7.2	5.3	ND	ND
Sudan grass hay	4.9	3.8	3.3	6.3	3.6	ND	ND
Timothy hay	3.7	3.0	ND	7.0	3.5	ND	ND
Brewer's grain	6.3	ND	6.3	4.9	3.2	ND	ND
Tofu cake	8.3	ND	5.7	5.6	ND	ND	ND
Formula feed	4.3	4.2	3.0	4.7	4.5	ND	ND
Fermented TMR (small-scale)	2.7	ND	ND	3.9	ND	ND	ND
Fermented TMR (commercial-scale)	3.9	ND	ND	3.9	ND	ND	ND

[†1]FW: Fresh weight.
[†2]LAB: Lactic acid bacteria.
[†3]ND: Not detected.

Table 2. Identity of rDNA-ITS region and aflatoxin production of the *Aspergillus* species isolated from TMR material

Isolate	Source	Genus	Species	Identity	Production of aflatoxins†
1	Sudan grass	*Aspergillus*	*terreus*	99% (518/520, AY939788)	-
2	Sudan grass	*Aspergillus*	*flavus*	99% (504/506, AY939782)	+
3	Sudan grass	*Aspergillus*	*flavus*	99% (500/505, AY939785)	-

†Detection limit: 200 µg / kg dry weight.

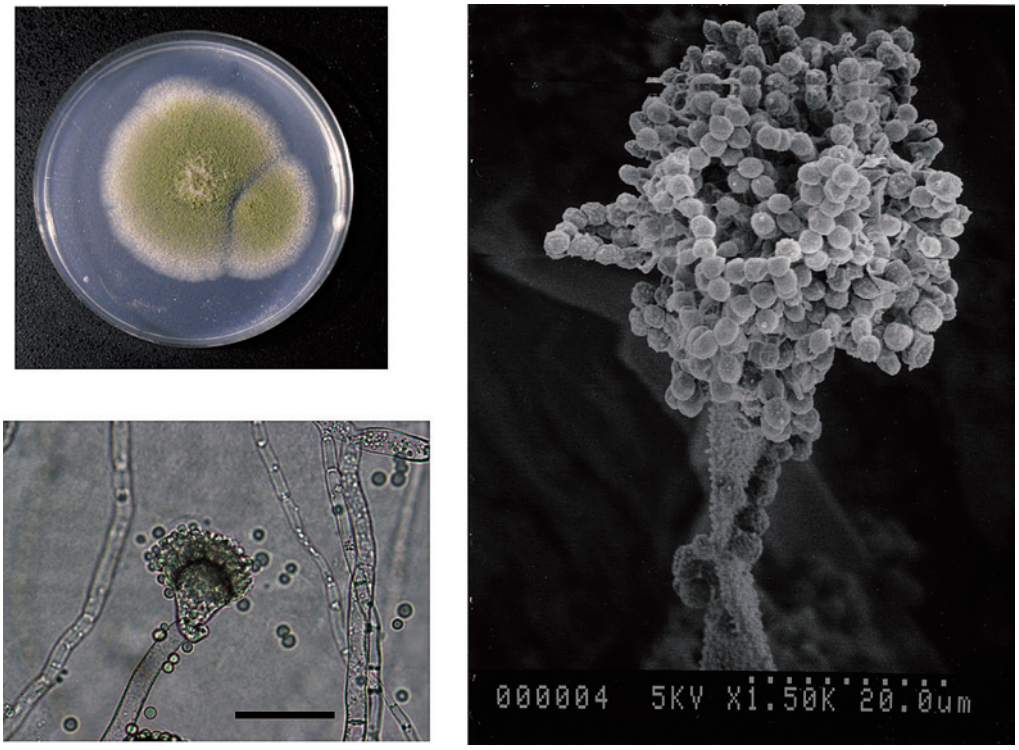

Fig. 1. Isolate No. 2 of *Aspergillus flavus* obtained from TMR material (imported Sudan grass hay)
Colony on PDA medium 5 days after incubation at 25°C (upper left); conidia under a microscope (bottom left, bar: 20 µm); conidia under SEM (right).

bation on corn meal (Table 2), mainly aflatoxin B_1 and a small amount of B_2 (Fig. 3). The concentration of aflatoxin B_1 was 2.1 mg/kg. Aflatoxin G_1 and Aflatoxin G_2 were not detected. No aflatoxins were detected from the other two isolates.

4. Fermentation quality of TMR and its ingredients

In the small-scale experiment, the content of lactic acid increased from 1.10% before fermentation to 3.91% after fermentation, and pH decreased from 4.93 to 4.28 (Table 3). Fungi and yeasts were not detected. In the commercial-scale experiment also, the content of lactic acid increased to 3.47%, and pH decreased to 4.27. The high content of lactic acid and low pH kept the quality of the fermented TMR high after 63 or 64 days of storage.

5. Qualitative analysis of aflatoxins in TMR and its ingredients

Aflatoxins were not detected in the Sudan grass hay that was the source of isolate No. 2. They were also not detected in any fermented TMR made from Sudan grass or in any other samples (data not shown).

Discussion

Three isolates of *Aspergillus* were obtained from the Sudan grass hay, which was imported from the USA. Many other kinds of fungi were also isolated. Isolate No.

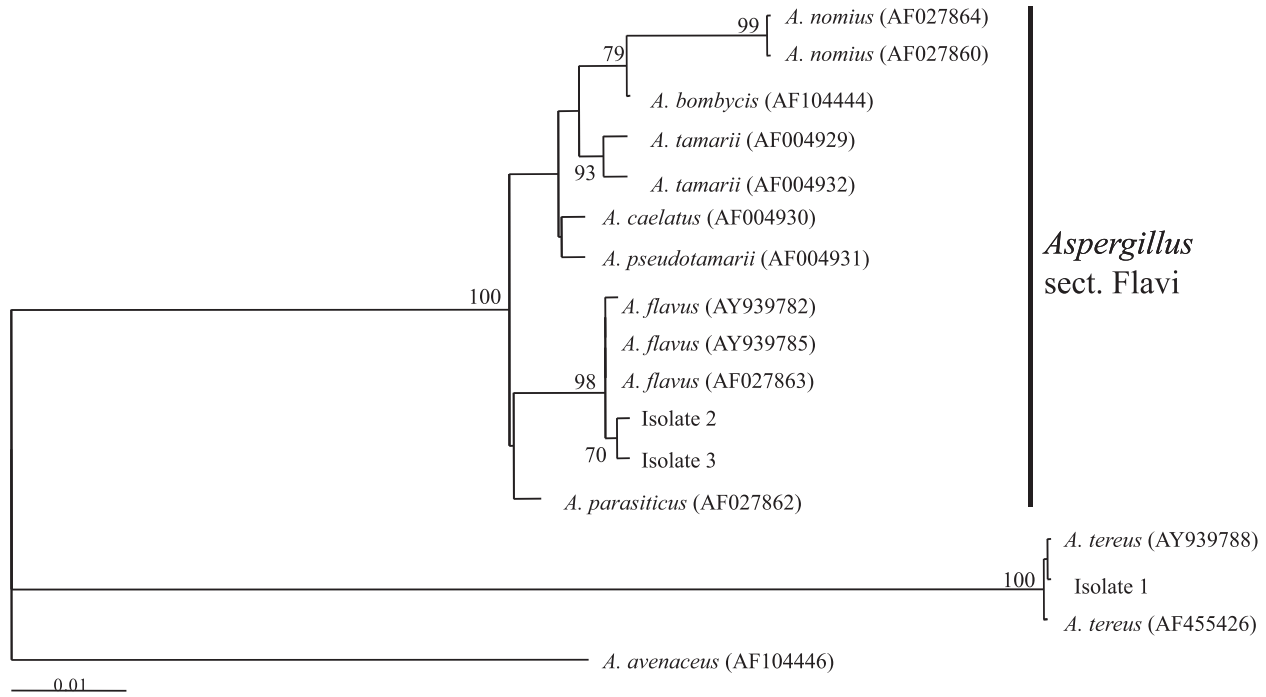

Fig. 2. Phylogenetic tree of *Aspergillus* isolates based on the sequences of rDNA-ITS regions

The tree was described by the neighbor-joining method with bootstrap values on the branches. Bar indicates phylogenetic distance with 1% exchange of the bases. The data of *Aspergillus* species such as *A. nomius*, *A. bombycis*, *A. tamarii*, *A.caelatus*, *A. pseudotamarii*, and *A. parasiticus* of *Aspergillus* sect. Flavi were included in the analysis.

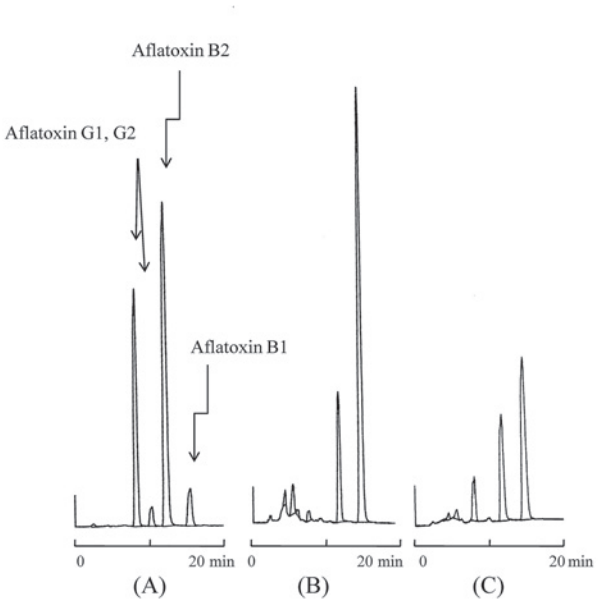

Fig. 3. Chromatogram of aflatoxins

(A) Authentic aflatoxins (B_1, G_1: 200 µg/L; B_2, G_2: 50 µg/L). (B) Culture solution of isolate No. 2. (C) 1:1 mixture of A and B.

1 had characteristics close to *A. terreus*, a common saprophytic species from soil. Numbers 2 and 3 had characteristics close to *A. flavus*, a famous aflatoxin-producing species inhabiting soil and plant surfaces. In the phylogenetic analysis, the isolates Nos. 2 and 3 formed the same clade with *A. flavus* in *Aspergillus* sect. Flavi and the isolate No. 1 was with the clade of *A. terreus.* The isolate No. 2 produced aflatoxin B_1 *in vitro.* In conclusion, we identified isolate No. 1 as *A. terreus* Thom and Nos. 2 and 3 as *A. flavus* Link based on their morphologies, phylogenetic characteristics and toxin productivity.

After the ingredients of TMR had been mixed, that is TMR before fermentation, LAB increased more than the initial LAB counts of each TMR ingredient (Table 1). This is thought to occur because the lactic fermentation began immediately after those materials were mixed. It is assumed the pH was 4.95 (Table 3). The good quality of the TMR was due to the decrease in pH and the increase in lactic acid content after fermentation (Table 3). The LAB count before fermentation was > 5.0 log cfu g^{-1} FW (Table 1), high enough for good silage fermentation[5]. The good quality was shown in the absence of fungi and yeasts from the fermented TMR, as reported previously[16]. Thus, the fermentation quality of the silage was well con-

Table 3. Quality of TMR ingredients and fermented TMR in small-scale and commercial-scale fermentation

Materials	Storage days	pH	SD[†2]	Organic acid content (% FW[†1]) Lactic	SD	Acetic	SD	Propionic	SD	Butyric	NH3–N (g/kg FM)	SD	DM (%)
TMR (before fermentation)	0	4.93		1.10		0.30		0.21		< 0.01	0.23		58.2
Alfalfa hay	0	6.01		0.00		0.00		0.00		< 0.01	1.36		91.3
Fescue hay	0	6.19		0.00		0.00		0.00		< 0.01	0.31		96.4
Sudan grass hay	0	5.80		0.00		0.00		0.00		< 0.01	0.11		99.3
Timothy hay	0	6.41		0.00		0.00		0.00		< 0.01	0.25		95.5
Brewer's grain	0	3.36		1.55		0.22		0.00		< 0.01	0.06		37.0
Tofu cake	0	4.77		0.34		0.13		0.02		< 0.01	0.11		21.2
Formula feed	0	6.18		0.43		0.00		0.59		< 0.01	0.29		94.2
Fermented TMR (small-scale)	63	4.28	0.03	3.91	0.23	1.14	0.06	< 0.01		< 0.01	0.69	0.02	63.2
Fermented TMR (commercial-scale)	64	4.27	0.01	3.47	0.32	1.27	0.10	0.18	0.032	< 0.01	0.64	0.01	59.0

[†1]FW: Fresh weight.
[†2]SD: Standard deviation.

trolled.

It was found that *A. flavus* was present in TMR components, the first report in Japan, and confirmed the production of aflatoxins B_1 and B_2 *in vitro.* As reported previously, *A. flavus* is present in soil in Japan[13,17]. It was thought that the aflatoxin of isolate No. 2 was produced at a high level (present experiment: 2.1 mg/kg). This is because, though the culture method was different, the aflatoxin producing level of a reported strain was 0.012~14.3 mg/kg[13]. However, aflatoxins were not detected in the Sudan grass hay (detection limit: 30 µg/kg DW) from which the *A. flavus* was isolated. Similarly, aflatoxin was not detected from the fermented TMR that was made with this Sudan grass as an ingredient. This detection limit is not necessarily low enough compared with the regulation for aflatoxin concentration of feed in Japan (for dairy cows: 10 µg/kg FW). However, it is thought that the concentration is low enough for judging ingredients not producing the aflatoxin compared with the high aflatoxin-production ability of the fungi. This is shown in the following result. Even if aflatoxin-producing fungi exist in TMR ingredients, TMR doesn't become contaminated with aflatoxin. The reason is thought to be due to nutritional or environmental conditions such as pH and anaerobic fermentation.

Aflatoxin-producing fungi do not occur widely in Japan and occur only in soil. Measures to prevent aflatoxin contamination of feed include not importing contaminated feed, not permitting aflatoxin-producing fungi to infect feed and not preserving feed under conditions that favor fungi. As of 2007, Japan imported 75% of its feed from other countries[8]. This large amount of feed was imported from foreign countries into Japan and fungi infecting the feed could be imported at the same time. The distribution of aflatoxin-producing fungi has been gradually expanding north from Okinawa and Kyushu[9,13]. The origin of the fungi we found here should be investigated, expressly if contaminated in imported feed producing areas or domestic areas. The possibility that imported fungi will naturalize in Japan under a warmer climate should be examined. Therefore, it is necessary to investigate the presence of aflatoxin-producing fungi in the imported feed widely, to investigate the distribution of aflatoxin-producing fungi in feed produced in Japan and to clarify the fermentation quality of fermented TMR where the contamination of the aflatoxin is not caused.

We showed that aflatoxins were not produced in normally fermented TMR, even when the aflatoxin-producing fungi were present in the ingredients. This highlights the role of not preserving feed under conditions that favor the fungi. However, the possibility of good storage and fermentation conditions makes it most important to keep the fungi out of Japan.

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