The Occurrence of Aflatoxin M1 in Fresh Milk and Its Possible Effects to Public Health

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Abstract. Aflatoxins are group of mycotoxins produced by *Aspergillus* sp., which is commonly found in agricultural products. Aflatoxins have carcinogenic, mutagenic, teratogenic and immunosuppressive properties for humans and animals. Aflatoxin B1 (AFB1) is the most toxic type of aflatoxins in nature compared to the others, such as AFB2, AFG1, and AFG2. Aflatoxin M1 (AFM1) is a hydroxylated product of AFB, which commonly occurs in milk. Although AFM1 toxicity is lower than AFB1, the risks have been a global concern, particularly for children. The objective of this study was to determine the occurrence of AFM1 in fresh milk and to evaluate the risks to public health. Fresh milk samples (n = 52) were collected from Magelang and Wonosobo, Central Java. Samples were extracted and cleaned up on SPE cartridges. The AFM1 was detected by high performance liquid chromatography (HPLC) on a reversed-phase C18 column under fluorescence detection**.** The results indicated that the samples contained AFM1 and AFM2 metabolites, as well as AFB1 as the parent compound. AFM1 was detected in 38 samples out of 52 samples (73.1%) and 26 of the positive samples (68.4%) were above the maximum residue limit regulated by FAO (0.5 ng/mL). The concentration of AFM1 in the positive samples ranged between 0.003 ng/mL to 34.202 ng/mL, with an average of 5.061 ng/mL. Apart from AFM1, 2 samples contained AFM2 metabolites (3.8%) and 22 samples contained AFB1 (42.3%) as the parent compound with averaged concentrations of 0.267 ng/mL and 0.032 ng/mL, respectively. From the results obtained, it can be concluded that most of the samples were contaminated by AFM1 and posed health risks to humans, especially children who are more susceptible to aflatoxin toxicity. It is also a concern that the immunosuppressive properties of AFM1 and AFB1 may lead to an increase in sensitivity to infectious diseases.

Keywords: aflatoxin M1 · milk · public health · immunosuppressive

1 Introduction

Aflatoxins are the most toxic group of mycotoxins produced by fungi belongs to Aspergillus species, particularly *A. flavus, A. parasiticus*, and *A. nomius*. The fungi commonly found in crops, cereals, nuts, and spices [1], as well as in animal products. Indonesia is a tropical country where high temperatures and humidity favor the growth of

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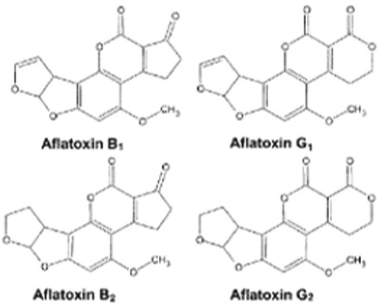


Fig. 1. Major types of aflatoxin in nature

Aspergillus sp. and aflatoxin production. Therefore, aflatoxin contamination is difficult to avoid and becomes a national concern. Contamination with aflatoxins not only reduces the economic value of products, but also causes serious health problems in humans and animals.

There are 4 types of aflatoxin found in nature, namely aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (Fig. 1) [2]. The aflatoxins, especially the B group have carcinoglenic, mutagenic, teratogenic and immunosuppressive properties to humans and animals [3, 4]. The diseases caused by aflatoxin called aflatoxicosis occasionally occur due to consumption of aflatoxin-contaminated foods and feeds.

Among the other aflatoxins, AFB1 is the most prevalent and the most toxic. The toxin was classified as group 1A carcinogenic agent to humans by The International Agency for Research on Cancer (IARC). Food-borne diseases associated with aflatoxin toxicity have been widely reported, particularly in developing countries. Acute aflatoxicosis due to exposure to high levels of aflatoxin, characterized by severe liver damage, jaundice, hemorrhage, oedema and death. Meanwhile, the chronic aflatoxicoses due to low level exposure leads to immunosuppression and cancer developments [1]. The synergistic of AFB1 with hepatitis B virus potentially increase the risk of liver cancer [5–7]. Aflatoxin also correlated with the impaired growth, stunting, and kwashiorkor of children [8, 9].

Aflatoxin M1 (AFM1) is a hydroxylated product of AFB1 formed during the detoxification process. The term "aflatoxin M1" was derived from "milk aflatoxin", however the toxin is also found in other animal products such as liver, kidney, meat, and eggs due to the consumption of aflatoxin-contaminated feed [10–12]. The AFB1 was converted into AFM1, involving the activation of the cytochrome P-450 enzyme [1]. According to Agus et al. (2010) [13], AFM1 is rapidly excreted in milk after feeding contaminated diets containing 50–250 μg/kg AFB1 to dairy cattle for 21 days. The conversion of AFB1 to AFM1 was approximately 0.1 to 0.3% and the AFM1 residue still remained in the milk after 5 days of treatments. Tsakiris et al., (2013) [14] reported that the excretion of AFM1 was between 12 and 24 h after AFB1 intake and the withdrawal interval was about 2–3 days after giving AFB1-free diets to the animals, and the carryover rate of AFM1 in milk varied between 1 and 6%.

Contamination of AFM1 in fresh milk and milk products have been published worldwide [9, 15–19]. In Indonesia, contamination of AFM1 was detected in fresh dairy

milk from west Java and Lampung provinces with concentrations below the maximum residue limit of 0.5 ng/m [20]. Earlier, Nuryono et al. (2009) [21] also reported AFM1 content in fresh milk collected from Yogyakarta. A surveillance conducted by Sumantri et al., (2019) [22] revealed the presence of AFM1 in various dairy milk products from Yogyakarta. AFM1 was detected in 92.5% of 42 samples at an averaged concentration of 216 ng/L.

As milk is an important protein source to fulfill daily nutritional requirements, it is a concern that the presence of AFM1 in milk leads to health hazards, especially for young children. Similar to AFB1, AFM1 is also classified as human carcinogen group 1 [23]. The immunosuppressive effects of AFM1 toxicity, which lead to increased sensitivity to infectious diseases, are a greater concern, particularly in young children. In vitro and in vivo studies conducted indicated suppressive effects of AFM1 on both innate and adaptive immune responses [1, 24].

Concerning the risk, many countries in the world have regulated the maximum residue limits (MRL) of aflatoxins, including AFM1. The European Union (EU) regulation 1881/2006 set the MRL of raw milk at 0.05 ng/g, which is lower than the Codex Alimentarius recommendation of 0.5 ng/g. Indonesia set the MRL at 0.5 ng/mL (BSN, 2009), which is similar to the level adopted by other Asian countries [25]. The objectives of this study were to determine the occurrence of AFM1 in fresh milk and to evaluate the possible risk of the toxin to public health, particularly to children who are vulnerable to the toxicity.

2 Materials and Methods

2.1 Sample Collection

Fresh milk samples (n = 52) were collected from 15 dairy farms in Magelang and 7 dairy farms in Wonosobo, Central Java, in 2019. During samples collection, there were no contact to the animals, therefore no ethical conflict to concern. The samples were collected from milking canes or buckets used by the farmers during the milking process. After milking hours, at least two samples were collected from each farmer, either early in the morning or late in the afternoon. Before collection, the fresh milk were homogenized and placed in 500 mL plastic bottles. The samples were frozen during transportation and stored at -20 °C before the analysis. Apart from fresh milk, the animal feed samples were also collected from the same farms for the analysis of AFB1. The type of feed samples collected included grass, corn residuals, rice straws, rice hulls, concentrates, and wheat pollards.

2.2 Detection of AFM1 and Other Aflatoxins in Milk by HPLC

The aflatoxins in the samples were analyzed by a modified AOAC method (2000). Briefly, 20 mL of fresh milk sample was diluted with 20 mL hot water (80 °C), mixed, and poured into an SPE C18 cartridge, which was primarily conditioned by 5 mL methanol followed by 5 mL water. The cartridge was rinsed with 10 mL of 5% acetonitrile in water then reprime with 150 μL acetonitrile. For further clean up, a silica cartridge was conditioned

with 5 mL diethylether and attached to the C18 cartridge. The two cartridges were rinsed with another 5 mL diethylether. Then, the silica cartridge was detached and washed with 2 mL diethylether and the solvent was discarded. The AFM1 was eluted from the cartridge by adding 7 mL dichloromethane-ethanol mixture (95:5, v/v), collected into a 10 mL vial, and evaporated to dryness under a nitrogen stream.

In order to increase sensitivity, the dried extract was derivatized by adding 50 μL trifluoroacetic acid (TFA) and 200 μL n-hexane, then allowed to react for 15 min. The extract was further evaporated to dryness under nitrogen stream. The extract was re-diluted with 0.5 mL of the mobile phase and ready for AFM1 detection on HPLC.

The AFM1 was detected on an HPLC system (Waters Millipore Model e2695) equipped by reversed-phase C18 under fluoresence detection ($\lambda_{excitation}$ 365 nm and $\lambda_{emission}$ 425 nm). The mixture of water-methanol-acetonitrile (55/20/25, v/v/v) was used as the mobile phase at a flow rate of 1 mL/minute. Apart from AFM1, the AFM2 metabolite and AFB1 as the parent compound were also detected in the same HPLC system.

2.3 Detection of AFB1 in Feed by dc-ELISA

Considering feed as the source of AFB1, which leads to AFM1 contamination in milk, all types of feed given to the dairy cattle were collected and analyzed. Feed samples (n = 56) such as grass, corn residuals, rice straws, rice hulls, concentrates, and wheat pollard were collected from the same farms where the fresh milk samples were collected.

The feed samples were extracted in methanol-water (7:3, v/v) using a mixer for 3 min, filtered through filter paper (Whatman # 41) or centrifuged at 3000 rpm for 10 min. AFB1 in the samples were detected using the in-house enzyme-linked immunosorbent assay kit using a direct competitive format (dc-ELISA). The ELISA kit was conditioned at room temperature prior to the sample analysis. A serial of AFB1 standards at concentrations of 0; 0,1; 0,3; 1.1; 3.3; 10 and 30 μg/mL, as well as the sample extracts, were pipetted (100 μL) and put into each well of the mixing plate, mixed with 100 μL of the enzyme conjugate, then 75 μL of the mixtures were transferred into the antibody-coated plate (in duplicate). The plate was incubated at room temperature for 30 min to allow the enzymatic antibody-antigen reaction, the solution was discarded and the plate was washed 3 times with distilled water, then allowed to dry. The substrate was prepared by mixing substrates A and B (97:3, v/v), then 100 μL was pipetted into each well, and incubated for 15 min at room temperature. To stop the reaction, 50 μL of stop reagent was added to each well and the absorbance was read on the ELISA reader at 450 nm.

Upon the data analysis, the absorbance was converted to inhibition percentage using the formula:

$$\%\,\textbf{Inhibition AFB1 standard} = \{\mathbf{1} - (\mathbf{A_{std}} - \mathbf{A_{blank}})\} \times \mathbf{100}/\mathbf{A_{control}} - \mathbf{A_{blank}}$$

$$\%\,\textbf{Inhibition of sample} = \{\mathbf{1} - (\mathbf{A_{spl}} - \mathbf{A_{blank}})\} \times \mathbf{100}/\mathbf{A_{control}} - \mathbf{A_{blank}}$$

The graph was created by plotting the concentration of AFB1 standards against the % Inhibition, and the concentration of AFB1 in the samples using the linearity regression of the standards ($y = ax + b$) and multiplying by the dilution factor of 4. If the absorbance of the samples was out of range, the samples were further diluted accordingly.

3 Results

3.1 AFM1 and Other Aflatoxins in Fresh Milk

Table 1 presents the occurrence of AFM1 in the samples collected from small farms in Magelang and Wonosobo, Central Java in 2019. The analysis of the fresh milk samples revealed that AFM1 were detected in 38 out of 52 samples (73.1%). The concentration of AFM1 in 26 of the positive samples (68.4%) were above the maximum residue limit (MRL) regulated by the Indonesian Government (BPOM, 2018), as well as by Codex Alimentarius and FAO (0.5 ng/mL, ppb).

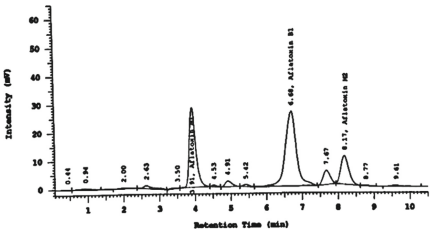
Apart from AFM1, the AFM2 metabolite and AFB1 as the parent compound AFM2 were also analyzed in the milk samples. The analytical results revealed that AFM2 was detected in 2 samples (3.8%), while AFB1 was detected in 22 samples (42.3%) with and averaged concentration of 0.267 ng/mL and 0.032 ng/mL, respectively. The performance of HPLC chromatograms of AFM1, AFM2 and AFB1 in the positive samples are shown in Fig. 2.

3.2 AFB1 in Feed of Dairy Cattle

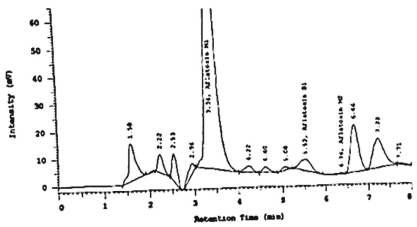
The results of feed samples analysis by ELISA method showed the presence of AFB1 as presented in Table 2. AFB1 was detected in all the samples analyzed, however the toxin was not detected in the grass samples.

Table 1. AFM1 in fresh milk collected from Magelang and Wonosobo, Central Java

Sampling location	Number of sample (N)	Possitive sample (%)	Range AFM1 in positive sample (ng/mL)	Average AFM1 in positive sample (ng/mL)
Magelang	32	28 (87.5%)	0.004–16.486	1.696
Wonosobo	20	10 (50.0%)	0.003–34.202	8.425
Total	**52**	**38 (73.1%)**	**0.003–34.202**	**5.060**



Standard AFM1, AFM2, AFB1



AFM1, AFM2, AFB1 in fresh milk sample

Fig. 2. The performance of AFM1, AFM2 and AFB1 chromatograms in fresh milk samples compared to the standards

Table 2. AFB1 in feed samples of dairy cattle collected from Magelang and Wonosobo

Sampling location	Type of sample	Number of sample (N)	Possitive sample (%)	Range AFB1 in possitive sample (ng/g)	Average AFB1 in possitive sample (ng/g)
Magelang	Grass	6	0 (0%)	–	–
	Peanut	2	2 (100%)	15.8–41.3	28.5
	residuals	11	11 (100%)	2.8–89.0	36.1
	Rice straws	3	3 (100%)	28.3–102.5	65.4
	Rice hulls	6	6 (100%)	14.4–114.8	68.9
	Concentrates	2	2 (100%)	5.9–15.8	10.85
	Wheat pollards				
	Sub Total	**30**	24 (80%)	2.8–114.8	209.7
Wonosobo	Grass	7	0 (0%)	–	–
	Corn residuals	3	3 (100%)	26.9–117.8	72.4
	Rice straws	4	4 (100%)	15.4–52.2	29.2
	Rice hulls	5	5 (100%)	11.9–102.5	53.9
	Concentrates	6	6 (100%)	31.2–125.4	66.6
	Wheat pollards	1	1 (100%)	–	4.2
	Sub Total	**26**	19 (73.1%)	15.4–125.4	226.3

4 Discussion

The analytical results from this study indicated a high incidence of AFM1 contamination in the fresh milk samples. Most of the samples from Magelang and Wonosobo contained AFM1 as the major metabolite in milk with a prevalence of 87.5% and 50%, respectively. The findings were supported by Sumantri et al., 2019 [22] who found AFM1 in 83.3% of fresh milk samples from Yogyakarta. Although AFB1 was detected in 42.3% of the positive samples, the average concentration was low (0,032 ng/mL). The incidence of AFM1 contamination in fresh milk samples from Magelang was higher than that from Wonosobo.

It was revealed that the occurrence of AFM1 in the milk is due to the presence of AFB1 in the feed materials. The concentration of AFM1 excreted in milk depends on the AFB1 content in the feed. In this study, the feed concentrate was the major source of contamination, followed by corn residual and rice hull. The total concentration of AFB1 in cattle feed from Magelang and Wonosobo was 209.7 ppb and 226.3 ppb, respectively which were above the MRL for dairy cattle according to SNI. 3148.1:2009 i.e. 200 ppb (BSN, 2009). The analytical results indicated that the cattle were continuously exposed to AFB1 that led to the formation of AFM1 excreted in the milk. Therefore, the AFM1 concentrations found in this study were relatively higher than other studies [13, 20] AFB1 and AFM1 were found to affect cytotoxicity and related gene changes in bovine mammary epithelial cells [26]. In order to minimize the AFB1 level in feed, the selection of feed ingredients and feed management are important.

The presence of AFM1 with concentrations above the MRL poses a threat to public health. Considering that AFM1 is heat resistant and remains stable during processing,

for instance, the pasteurization or condensation process, the risk of contamination should be anticipated. Sumantri et al., 2019 [22] detected AFM1 in 83.3% fresh milk, 93.7% of pasteurized milk, and 100% of recombined milk at concentration levels ranging between >50 to 500 ng/L. Meanwhile, Wijaya et al., 2017 [27] also reported the occurrence of AFM1 in 85% of powdered milk and 65% in sweetened milk. The findings indicated that processing cannot remove AFM1 from milk products.

In this study, 68.4% of the positive samples containing AFM1 exceeded the MRL set by the Indonesian government, Codex Alimentarius and FAO (0.5 ng/mL). This condition can cause serious public health hazards, particularly to children, who are more sensitive to aflatoxin exposure. Referring to the risk assessment studied by Restiani et al., 2020 [28], the hazard index (HI) value of infants aged 0–6 months and 6–12 months were >1 which indicated the AFM1 causes health risk to young children, while the HI value of children aged 1–3 years was <1 which means less health risk. The HI Index depends on the daily intake. Considering younger infants consume more milk compared to the elders, the risk to AFM1 related health hazard such as liver cancer, hepatitis, and stunting is higher [7, 29–31].

5 Conclusion

Aflatoxin contamination is hard to avoid, but can be managed to minimize the risk. Risk management of aflatoxin contamination can be performed by implementing the HACCP concept, where the contamination is controlled in each step of processing and involving all stakeholders. For instance, to minimize the risk of AFM1 contamination in milk, farmers should pay attention to the quality of feed given to the animals, avoid using moldy feed materials, perform dilution by mixing a larger volume of milk before processing, if possible apply biological control technologies to degrade AFM1 in processing milk products, perform aflatoxin monitoring program in milk and milk products, enhance laboratory capability to analyze the aflatoxin contamination, and perform epidemiological studies to do risk analysis and risk assessment of milk products to identify cause and effect loops.

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