Feed and feed storage factors in relation to aflatoxin M1 contamination in bulk milk of smallholder dairy farms

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Abstract
The aim of the study was to determine feed and feed storage factors associated with aflatoxin M1 (AFM1) contamination in bulk milk of dairy farms. The study was conducted from May to July 2016, at all smallholder farms in Mae Wang dairy cooperative, Chiang Mai, Thailand. Data on feed and feed storage factors were collected from the farmers using interviews and observations. For feed, we included type of roughage and physical appearance of concentrated feed, and for feed storage factor, we included storage method of roughages. AFM1 concentration was measured using the Charm® ROSA® MRLAFMQ (aflatoxin M1) Test. Fisher’s exact chi-square test was used to determine the association of feed and feed management factors with AFM1 contamination. From a total of 67 farms, 50 farms were included in the analysis. AFM1 contamination was observed in 46% of the samples. Farms using factory-corn silage had a significantly higher percentage of AFM1 contamination (62.5%) than farms that did not use factory-corn silage (30.8%). AFM1 contamination in farms that used concentrates with cracked pellets was significantly higher (64.3%) than in those that did not (22.7%). For feed storage, roughage stored in piles within the barn was associated with significantly higher AFM1 contamination than that stored outside (61.5% and 29.2%, respectively). In addition, AFM1 contamination for roughage piles with mold on the surface was higher (60%) than for roughage piles without mold (25%). Our results indicate that type of feed and feed storage factors are associated with AFM1 contamination in bulk milk.

Introduction
Aflatoxin M1 (AFM1) is a mycotoxin found in milk, which is metabolized from aflatoxin B1 (AFB1) consumed by cattle through their feeds, and is classified by the International Agency for Research on Cancer as a group 2B human carcinogen¹. Owing to the high consumption of milk and milk products by humans, AFM1 contamination of milk is one of the greatest public health concerns, especially in children²,³. Problems due to AFM1 contamination in milk have been reported in many countries, including Thailand²,³.

The main factor determining AFM1 concentration in milk is the level of AFB1 concentration in cattle feeds⁴,⁵. Findings reported by Suriyasathaporn and Nakprasert² suggest that dairy farm mismanagement causes AFM1 contamination in commercial pasteurized milk. Improper environment in feed storage rooms, including temperature and humidity, facilitates fungal infection and promotes AFB1 production in dairy cow feeds⁶,⁷,⁸,⁹. AFM1 contamination in milk samples is also related to improper environment of feed storage rooms¹⁰. In addition, use of commercial concentrates with abnormal characteristics, such as having cracked pellets or having a positive result to fluorescent light, increased the risk of AFM1 contamination¹¹.

Many countries, including Thailand, use mixed rations of roughage and concentrates¹⁰,¹¹. Most studies have investigated AFM1 or AFB1 contamination in concentrates and related raw materials due to their long duration of storage¹⁰,¹¹,¹². A recent study showed that storing roughage for a short time also increased the risk of AFB1 contamination¹¹. However, it is necessary to know the characteristic factors of
roughage related to AFM\textsubscript{1} contamination in milk. Therefore, the objective of this study was to determine the factors of abnormal feed characteristics, type of roughage and feed storage method associated with AFM\textsubscript{1} contamination in bulk milk.

**Materials and Methods**

**Study design and data collection**

An observational cross-sectional study was performed from May to July 2016, at 67 smallholder dairy farms from Mae Wang dairy cooperative, Chiang Mai Province, Thailand. Most farms had, along with milking cows, five to sixty animals, and fed their cows with a mixed ration of roughage and commercial concentrates. Data on feed and feed storage method were collected on the same day of milk collection. The majority of commercial concentrates were purchased from the cooperative. Types of roughage originated from post-harvest agricultural plants, including grass, hay, and maize, or were by-products from corn processing factories, including dry ground outer layer of corn seed (corn dust), dry corn cover, and boiled corn cobs with husks (factory-corn silage). Feed and feed storage factors included characteristics of commercial concentrates (with or without cracked pellets), keeping roughage in piles within cow barns (yes or no), and having mold on the surface area of roughage piles (yes or no).

**Milk collection and AFM\textsubscript{1} measurement**

At each farm, 30 ml of bulk tank milk was collected in a plastic tube, stored on ice, and immediately transported to the Faculty of Veterinary Medicine, Chiang Mai University. Concentrations of AFM\textsubscript{1} were determined by an immunoreceptor assay utilizing Charm\textsuperscript{R} ROSA\textsuperscript{R} (Rapid One Step Assay) lateral flow technology, using the Charm\textsuperscript{R} MRL aflatoxin M1 quantitative test (MRLAFMQ) (Charm Sciences Inc., Lawrence, MA, USA). Test kits were from kit lot 016 (Exp. 10/2016). Samples were kept at 0°C to 7°C and mixed well using vortex mixer before testing. A milk sample (300 µl) was added to the MRLAFMQ test strip and incubated at 40°C on the ROSA\textsuperscript{R} incubator for 15 min. The test strips were removed and inserted into the Charm EZ READING instrument for 1 min, to determine AFM\textsubscript{1} concentrations in the milk samples. Samples with AFM\textsubscript{1} concentrations greater than 40 ppt were considered contaminated with AFM\textsubscript{1}.

**Statistical analysis**

Data on feed and feed management factors were expressed as percentages. The association of feed and feed management factors with AFM\textsubscript{1} contamination was analyzed using Fisher’s exact chi-square test (SAS, 1997). The significant level was set at \( P < 0.05 \) for all analyses.

### Results

Out of the 67 farms, 17 were excluded due to incomplete data collection. Twenty-three (46%) of the 50 milk samples in the final analysis were contaminated with AFM\textsubscript{1} (Fig. 1).

The distribution of feed used is shown in Table 1. Twenty-eight farms (56%) used commercial concentrates with cracked pellets. Regarding type of roughage, dry corn cover (78%) was used in most farms, whereas corn dust was used in only six farms (12%). Due to the high incidence of AFM\textsubscript{1} contamination in the milk samples (46%), all feed had high levels of AFM\textsubscript{1} contamination ranging from 22.7% to 64.3% for farms using and not using concentrates with cracked pellets. Farms using grass had a lower percentage of AFM\textsubscript{1} contamination than farms using another type of roughage. Comparing all feed types, farms that used factory-corn silage (62.5%) or concentrates with cracked pellets (64.3%) had significantly higher percentages of AFM\textsubscript{1} contamination than those that did not (30.8% and 22.7%, respectively) \( (P = 0.046, P = 0.005) \).

AFM\textsubscript{1} contamination in bulk milk based on facility and feed storage factors are reported in Table 2. Our results show that farms that had fewer than 15 milking cows exhibited significantly lower rates of AFM\textsubscript{1} (23.5%) contamination than larger farms (57.6%) \( (P = 0.035) \). As for feed storage method, roughage stored in piles within the cow barn was associated with significantly greater AFM\textsubscript{1} contamination than roughage stored outside (61.5% and 29.2%, respectively) \( (P = 0.027) \). In addition, roughage piles with mold on the surface were associated with higher percentages of AFM\textsubscript{1} contamination than those without (60% and 25%, respectively) \( (P = 0.021) \).

**Fig. 1** Distribution of AFM\textsubscript{1} contamination in smallholder dairy farms in Mae Wang dairy cooperative, Chiang Mai, Thailand.
Table 1  Percentage of AFM$_1$ contaminated milk in relation to feed type and presence.

| Factor                  | Yes  | No   | P-value (α = 0.05) |
|-------------------------|------|------|--------------------|
|                         | Total (n) | AFM$_1$ positive (n) | % | Total (n) | AFM$_1$ positive (n) | % |                   |
| Concentrates with cracked pellets | 28 | 18 | 64.3 | 22 | 5 | 22.7 | 0.005 |
| Corn                    | 14 | 7 | 50.0 | 36 | 16 | 44.4 | 0.760 |
| Grass                   | 21 | 8 | 38.1 | 29 | 15 | 51.7 | 0.398 |
| Hay                     | 32 | 16 | 50.0 | 18 | 7 | 38.9 | 0.559 |
| Factory-corn silage     | 24 | 15 | 62.5 | 26 | 8 | 30.8 | 0.046 |
| Dry corn cover          | 39 | 19 | 48.7 | 11 | 4 | 36.4 | 0.515 |
| Corn dust               | 6 | 3 | 50.0 | 44 | 20 | 45.5 | 1.000 |

Table 2  Percentage of AFM$_1$ contaminated milk in relation to facility and feed storage factors.

| Factor                        | Yes  | No   | P-value (α = 0.05) |
|-------------------------------|------|------|--------------------|
|                              | Total (n) | AFM$_1$ positive (n) | % | Total (n) | AFM$_1$ positive (n) | % |                   |
| Milking cows ≤15             | 17 | 4 | 23.5 | 33 | 19 | 57.6 | 0.035 |
| Keeping roughage pile within barns | 26 | 16 | 61.5 | 24 | 7 | 29.2 | 0.027 |
| Keeping roughage in plastic bags outside barns | 11 | 7 | 63.6 | 39 | 16 | 41.0 | 0.305 |
| Using plastic sheets for roughage | 20 | 11 | 55.0 | 28 | 10 | 35.7 | 0.242 |
| Mold found on surface area of roughage | 30 | 18 | 60.0 | 20 | 5 | 25.0 | 0.021 |

Discussion

This study analyzed AFM$_1$ contamination using Charm® ROSA® MRLAFMQ, a rapid method for detection of AFM$_1$ in milk. This is a routine screening method for periodic checks of AFM$_1$ contamination in milk before sending it to factories, especially in European countries. Based on the test guidelines, milk samples with AFM$_1$ concentration of 40 ppt or greater are considered contaminated. According to the EU regulations, samples with concentrations exceeding the European Union maximum residue levels (EU MRL) (> 0.05 ppb or > 50 ppt) were considered AFM$_1$-contaminated. The difference in the cutoff values for the Charm® ROSA® MRLAFMQ guidelines and the EU MRL might be due to limitations of the test. However, this study uses cutoff value of the test in order to minimize this limitation.

Overall, 46% of bulk milk was AFM$_1$-contaminated, with concentrations ranging between 0 ppt and 100 ppt (Fig. 1). Ruangwises and Ruangwises\textsuperscript{3}) reported that in central Thailand, 47.5% of milk samples contaminated with AFM$_1$ had concentration levels exceeding the EU MRL value. In addition, AFM$_1$ in commercial pasteurized milk throughout Thailand ranged from 4 ppt to 293 ppt\textsuperscript{12}, which is a similar range as reported in the present study. Feed-related factors, such as feed characteristics and feed positive to blue-greenish yellow fluorescence test related with AFM$_1$ contamination in milk, have been previously reported\textsuperscript{5,10}. This study found that farms using concentrated feeds with cracked pellets had a higher percentage of AFM$_1$ contamination in milk than did farms using concentrated feeds without cracked pellets. This finding is supported by a previous study showing that cracked particles of concentrated feeds increased the risk of AFM$_1$ contamination in raw bulk milk\textsuperscript{10,13}. Broken or fine pellets indicate low quality of raw ingredients\textsuperscript{12}, which increases risk of contamination with fungi and AFB$_1$\textsuperscript{14,15}.

Among the six types of roughage, hay, factory-corn silage, dry corn covers, and corn dust had long durations of storage time outside barns. In contrast, corn and grass were generally transported to dairy farms immediately. Out of the four types of roughage with longer storage duration, factory-corn silage was the only wet roughage with very high humidity. It is well documented that in areas of high humidity, high temperature, and improper storage conditions, fungal growth is favored and there is an increased amount of mycotoxins produced in silage\textsuperscript{8,16}. Therefore, the characteristic wetness of factory-corn silage might greatly increase fungal growth and aflatoxin presence\textsuperscript{8,10,11}.

Farms with greater than 15 milking cows had a higher percentage of AFM$_1$ contamination. This might relate to the duration of feed storage times due to the high amount needed for farm maintenance. Storing factory-corn silage for 2 weeks increased AFM$_1$ contamination by approximately twofold compared to the contamination before storage\textsuperscript{11}. This might be due
to increased levels of fungi and aflatoxin contamination in maize. The high risk of AFM1 contamination in farms storing roughage in piles within barns might be explained by the high humidity within barns, which is favorable for mold growth and increased AFB1 production. Increased risk of AFM1 in milk because of increased mold growth is also supported by our result showing that farms using roughage piles that have mold on the surface had higher percentages of AFM1 contamination than farms using roughage piles without mold (Table 2). Other studies have also found that mold on the surface increases the amount of AFB1 in the feed. These criteria might be used as a tool for monitoring the risk of AFM1 contamination in dairy farms.

**Conclusion**

Feed and feed management, which includes type of roughage (factory-corn silage), farm size, cracked pellets of concentrated feed, mold appearing on the surface of feeds, and roughage stored in piles within barns are associated with AFM1 contamination in bulk milk.

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