# Final Report

# Mold growth, aflatoxin production, and cytotoxicity detoxification against aflatoxin after supplementing organic acid in by product corn silages

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## Mold growth, aflatoxin production, and cytotoxicity detoxification against aflatoxin after supplementing organic acid in by product corn silages

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# Table of Contents

# ABSTRACT

**Project Code: MRG5980020**

**Project Title: Mold growth, aflatoxin production, and cytotoxicity detoxification against aflatoxin after supplementing organic acid in by product corn silages**

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**Project Period: 2 years 6 months**

The objectives of this study were to determine factors related to mold and AFB1 contamination in by-product corn processing factories as dairy roughage when keeping in small holder farm conditions, evaluate the efficacy of organic acid on reducing the contamination of *Aspergillus flavus* and AFB1 in by product corn silage, and identify the factors associated with these contaminations.

According to the first objective, by-products from corn processing factories including 123 samples of dried corn covers (dried corn), and 108 samples of boiled corn cobs with husks (boiled corn) were collected from small holder dairy farms in Chiang Mai, Thailand. They were collected from 3 areas including surface base position, surface high position, and the center of feed piles. Mold contamination was determined by spread plate technique. Analysis for AFB1 was conducted by a commercial enzyme-linked immunosorbent assay. The differences of AFB1 concentrations among feed pile's positions, mold contaminations and other variables were separately evaluated between the

dried corns and the boiled corns using generalized mixed model. Results show that the AFB1 concentration of boiled corns (14.72+0.71) ng/Kg was significantly higher than dried corns (6.54+2.54 ng/Kg). Only 12.2% (n=13) of the dried corns had AFB1 concentrations more than the limitation values (20 ng/Kg feed). Results from statistical analysis showed that the center of feed piles had lower AFB1 concentrations than base and high surface positions. The presences of *Cladosporium* spp. had a lower risk of AFB1 contamination. These results suggest that type of samples, sources and the contamination of *Claddosporium* spp. are factors related to AFB1 contamination.

For evaluate the efficacy of organic acids on reducing the contamination of *Aspergillus flavus* and AFB1 in by product corn silage, three types of organic acid including acetic acid, lactic acid, and propionic acid were tested for their inhibition efficacy against *Aspergillus flavus*. Among these, acetic acid had highest potential for reducing fungus; therefore, it was selected for further studies. By-product corn silages were separated into 3 groups: corn husk, corn cob and mixed. Each group was autoclaved, inoculated with *A. flavus* suspension and added with 1.25% of acetic acid. Corn samples with or without acetic acid were kept in a different conditions (exposed, non-exposed oxygen and light) in room temperature. Fungal count, water activity, pH and AFB1 concentration were measured after adding acetic acid at 12 h, day 7, 14 and 21. The results demonstrated that acetic acid reduced the contamination of *A. flavus*, and exhibited much reduced percentage of increasing AFB1 contamination when compared to that of without. The AFB1 concentration in sample exposed with oxygen higher than without ($p$=0.050). Corn cob had significant lower concentration of AFB1 than corn husk ($p = 0.0002$) and mixed corn ($p = 0.0001$). It can be concluded that acetic acid had effectiveness in inhibiting fungal growth, and decreasing an accumulation of AFB1. Moreover, storing feed in non-oxygen condition could be useful for reducing AFB1 contamination.

Keywords (3-5 words): Aflatoxin B1, Corn silage, Organic acid, Acetic acid, Detoxification, Fungal growth

# บทคัดย่อ

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วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาปัจจัยที่ส่งผลต่อการปนเปื้อนเชื้อราและAFB1เศษเหลือของข้าวโพดจากโรงงานที่ใช้เป็นอาหารหยาบหลักภายในฟาร์มโคนมรายย่อย และศึกษาประสิทธิภาพของกรดอินทรีย์ในการลดการปนเปื้อนเชื้อรา *Aspergillus flavus* และ รวมทั้งปัจจัยที่เกี่ยวข้องกับการปนเปื้อน

จากวัตถุประสงค์แรก ทำการเก็บตัวอย่างเศษเหลือของข้าวโพดจากโรงงาน 231 ตัวอย่าง แบ่งเป็นชนิดแห้ง 123 ตัวอย่าง และชนิดเปียก 108 ตัวอย่าง จากฟาร์มโคนมรายย่อยในจังหวัดเชียงใหม่ โดยเก็บตัวอย่างจากกองอาหาร 3 ตำแหน่ง ได้แก่ บริเวณฐาน พื้นผิวด้านนอก และตรงกลางกองอาหาร หาปริมาณเชื้อราด้วยวิธี spread plate และวิเคราะห์ปริมาณอะฟลาท๊อกซินบีวันโดยใช้เทคนิค ELISA วิเคราะห์ความแตกต่างของปริมาณอะฟลาท๊อกซินจากตำแหน่งที่ต่างกันและตัวแปรอื่น ๆ ในข้าวโพดชนิดเปียกแยกจากข้าวโพดแห้งด้วยวิธี generalized mixed model ผลการศึกษาพบว่าตัวอย่างเศษเหลือของข้าวโพดชนิดเปียกมีปริมาณ AFB1 (5.63 ± 0.30 พีพีบี) น้อยกว่าชนิดแห้ง (14.72 ± 0.71 พีพีบี) ($P<0.05$) โดยร้อยละ 12.2 (15 ตัวอย่าง) ของตัวอย่างทั้งหมดมีปริมาณของ AFB1 มากกว่าค่าที่กำหนด (20 นาโนกรัม/อาหาร 1 กิโลกรัม) ผลจากการทดสอบทางสถิติพบว่าตัวอย่างอาหารที่เก็บจากกลางกองอาหารพบความเข้มข้นของอะฟลาท๊อกซินบีวันต่ำกว่าบริเวณพื้นผิว ข้าวโพดที่ปนเปื้อนเชื้อรา *Cladosporium* spp. มีความเข้มข้นของอะฟลาท๊อกซินบีวันต่ำกว่าข้าวโพดที่ปนเปื้อนเชื้อราชนิดอื่น ($P<0.05$) การศึกษานี้สรุปได้ว่าชนิดของตัวอย่างและการปนเปื้อน *Cladosporidium* spp. ส่งผลต่อปริมาณการปนเปื้อนอะฟลาท๊อกซินบีวัน

สำหรับประสิทธิภาพของกรดแอซิติกในการลดปริมาณเชื้อราและอะฟลาท๊อกซินในเศษเหลือข้าวโพดจากโรงงานที่เป็นอาหารหยาบสำหรับโคนม รวมถึงศึกษาปัจจัยที่มีผลต่อการปนเปื้อนของเชื้อราและสารพิษดังกล่าว ตัวอย่างข้าวโพดโรงงานจะถูกแบ่งออกเป็น 3 กลุ่ม ได้แก่ เปลือก

ข้าวโพด ฝักข้าวโพด และกลุ่มที่ผสม ข้าวโพดทั้งหมดจะถูกฆ่าเชื้อด้วยหม้อนึ่งไอน้ำแรงดันสูง ใส่สารละลายสปอร์ของเชื้อแอสเปอร์จิลลัส ฟลาวัส (*Aspergillus flavus*) และกรดแอซิติกความเข้มข้น 1.25% ของน้ำหนักแห้ง ตัวอย่างข้าวโพดทั้งที่เติมและไม่เติมกรดแอซิติกจะถูกเก็บในสภาวะที่แตกต่างกัน (สัมผัสอากาศ, ไม่สัมผัสอากาศและแสง) ในอุณหภูมิห้อง ทำการวัดปริมาณเชื้อรา ค่าแอคทีวิตี้ของน้ำ (Aw) ค่าความเป็นกรดด่าง (pH) และปริมาณอะฟลาท๊อกซินในตัวอย่างทั้งหมดหลังการเติมกรดที่ 12 ชั่วโมง 7 14 และ 21 วัน ผลการทดลองพบว่า การใส่กรดแอซิติกทำให้ปริมาณเชื้อราลดลงอย่างมีนัยสำคัญ ($P$=0.0119) ความเข้มข้นของอะฟลาท๊อกซินบีวันมีค่าเพิ่มขึ้นในช่วงระยะเวลาการเก็บรักษาแต่พบว่าตัวอย่างที่ใส่กรดแอซิติกมีเปอร์เซ็นต์การเพิ่มขึ้นของอะฟลาท๊อกซินบีวันน้อยกว่าเมื่อเปรียบเทียบกับกลุ่มที่ไม่ใส่กรด ข้าวโพดในกลุ่มที่สัมผัสอากาศมีปริมาณอะฟลาท๊อกซินบีวันสูงกว่ากลุ่มที่ไม่สัมผัสอากาศ ($P$=0.0424) โดยฝักข้าวโพดมีความเข้มข้นของอะฟลาท๊อกซินบีวันน้อยกว่าเปลือกข้าวโพด ($P$=0.0002) และผสม ($P$=0.0001) อย่างมีนัยสำคัญทางสถิติ ดังนั้นจึงสามารถสรุปได้ว่ากรดแอซิติกมีประสิทธิภาพต่อการยับยั้งการเจริญเติบโตของเชื้อราและช่วยลดการสะสมของอะฟลาท๊อกซินบีวัน นอกจากนี้การเก็บอาหารภายใต้สภาวะไม่สัมผัสอากาศจะช่วยลดการปนเปื้อนของอะฟลาท๊อกซินบีวันได้

คำหลัก : อะฟลาท๊อกซินบีวัน, ข้าวโพดหมัก, กรดอินทรีย์, กรดอะซิติก, การลดพิษ, การเจริญของเชื้อรา

# EXECUTIVE SUMMARY

## Introduction to the research problem and its significance

Aflatoxins ( AF) are toxic substances produced by fungal species especially *Aspergillus flavus* and *Aspergillus parasiticus* (Iqbal et al., 2010). They are classified as Group1 human carcinogens by the International Agency for Research on Cancer of the World Health Organization (IARC) (IARC, 2002). Among many forms of AF, AFB1 and AFM1 are the most hepatotoxicity and carcinogenic and responsible for many serious diseases in human and animals (Creppy, 2002; Masoero et al., 2007). AFM1 is a major metabolite of AFB1, which is formed when dairy cows ingest feed contaminated with AFB1 (Creppy, 2002; Bhat et al., 2010).

For dairy industry in Northern Thailand, the shortage of feed in terms of quality and quantity, especially during dry season, is one of the most serious problems face to the farmers. To overcome this problem in the last few years, by-products from agriculture and industries such as corn stove (after harvesting baby corn or sweet corn) intended to use as dairy feedstuff. There are 2 types of by-products including dried corn covers (dried corn), and boiled corn cobs with husks (boiled corn). According to the problems of AFM1 contamination in either commercial pasteurized milk (Suriyasathaporn and Nakprasert, 2012) and school milk (Ruangwises and Ruangwises, 2009), it might be related to the use of these by-products. Therefore, determination of mold and AF production and also identify risk factors of AF contamination in these by-products is useful to evaluate risk of AFM1 in milk.

Regarding to boiled corn, a ton of these by-products is packaged in a plastic bag from factory and transported to dairy farms as corn silages. Due to its characteristics and optimal temperature of mold growth, the by-products had high production rate of

AFB1 after keeping for a week in small-holder farms condition (Mongkon et al., 2014). Generally, most farmers manage the by-product corn silages by keeping as sold until use. After opening, the packaged corn silages are kept with poor sealing and air can expose. These conditions promote mold growth and consequently produce AFB1 to contaminate in feeds. One of the best control strategies for AFM1 is to control AFB1 and fungal contamination in feeds (Suriyasathaporn and Nakprasert, 2012; Mongkon et al., 2014). For a decrease of the transfer of aflatoxins from feed to milk, researchers have tried various methods including physical, chemical, or biological treatments (Bhat et al., 2010; Mendez-Albores et al., 2008; kabak et al., 2006; Mokoena et al., 2006). Among these methods, organic acid have been used to reduce the transfer of dietary AFB1 to milk AFM1. Recent studies found that organic acid can effectively inhibit the growth of fungi and aflatoxin production (Mokoena et al. 2006; Mendez-Albores et al., 2008; Shukla et al., 2002). Furthermore, organic acids were reported as an efficient detoxification for AFB1 by reducing toxicity against animal cell lines (Shukla et al., 2002; Aiko et al., 2015).

In Thailand, because of commercial organic acid is very cheap and be easy to use in farm practice, hence, supplementing of the organic acid can be extended to real situations for inhibit mold growth and removing aflatoxin in contaminated corn silage. However, no previous study has examined the effect of commercial organic acid in reducing mold and aflatoxin in by-product corn silage. Therefore, objectives of this study were to determine the efficacy of supplemented organic acids in by-product corn silages on inhibition of fungal growth, AFB1 production, and AFB1 detoxification on animal cell lines.

# LITERATURE REVIEW

## *Aflatoxin*

Aflatoxins (AF) are toxic substances produced by fungal species especially *Aspergillus flavus* and *Aspergillus parasiticus* (Iqbal et al., 2010). Major forms of AF include AFB1, AFB2, AFG1, AFG2 and AFM1 (Han et al., 2013; Bhat et al., 2010; Creppy, 2002). Among many forms of aflatoxin, AFB1 and AFM1 are responsible for many serious diseases in human and animal health (Creppy, 2002). Cows consumed feeds contaminated AFB1 result in losses of their weight gains, dietary intake reduction; liver function impairment, reduction of milk yield and quality and impairment of immune response (Masoero et al., 2007; Creppy, 2002).

Factors that facilitate fungal infection and promote AFB1 production are inoculum availability, weather conditions, pestinfestation during crop growth, maturation, harvesting and storage (Lopez-Garcia et al., 1999). When dairy cows ingested AFB1 contaminated feed, it metabolites in liver to form AFM1 and subsequently excretes in milk (Masoero et al., 2007; Creppy, 2002). The level of conversion from AFB1 into AFM1 in milk is influenced by many factors including 1) feed-related factors, for example quantity, characteristics of feeds consumed and concentrations of contaminated AFB1, 2) cow factors, for example milk yield, lactation stage, breed and time during the day, and 3) other factors, such as weather and/or geographical location of dairy farms (Masoero et al., 2007; Iqbal et al., 2010; Kabak et al., 2006). Taking into account all the relevant considerations, the predicted rate of AFB1/AFM1 carry-over from feedstuff into milk is approximately 0.3–6.0% (Heshmati and Milani, 2010).

Toxic effects of both AFM1 and AFB1 are hepatotoxicity and carcinogenic. They are classified as Group1 human carcinogens by the International Agency for

Research on Cancer of the World Health Organization (IARC) (IARC, 2002). In human, AFM1 is the great concerns with respect to public health because of high consumption of milk and milk products, especially in children. Therefore, to avoid the risk of aflatoxin contamination, several countries and agencies have established acceptable limits for aflatoxin in feeds and milk. For example, European Union has set the maximum residue limit (MRL) for AFB1 in cow feeds and raw milk are 5 µg/kg and 50 ng/kg, respectively (European Commission, 2006). In the United States, the Food and Drug Administration (FDA) has set the MRL for total aflatoxin in cow feeds and raw milk that should be less than 20 µg/kg and 500 ng/kg, respectively (FDA, 2000). In china, the maximum level of AFB1 in feed is 10 µg/kg (China General Administration of Quality Supervision, Inspection and Quarantine, 2001) and that of AFM1 in raw milk is 500 ng/kg (China Ministry of Health, 2011).

***The contamination of aflatoxin in feed, milk and milk product in Thailand***

In Thailand, some researchers have carried out surveillance about occurrences of aflatoxin in animal feed, milk and milk products (Ruangwises, 2010; Suriyasathaporn and Nakprasert, 2012; Ruangwises and Ruangwises, 2009). Ruangwises and Bantaokul (2010) reported that about 60% of dairy roughage feed samples and 66% of concentrated feed samples collected from ten farms in Ratchaburi and Phetchaburi province were contaminated with AFB1 in the range of 15,000-41,000 µg/kg. Meemark and Sakdinun (2008) found 78.42% of dairy feed samples collected from dairy farms in Ratchaburi, Kanchanaburi, Petchaburi, Nakhonpathom and Samutsakhorn provinces (366 samples) contaminated with AFB1 with their concentrations ranging between 0.40-23.97 µg/kg, indicating some severity of AFB1 contamination problems in feed.

For milk and milk products, studies in Thailand have been reported some degree of problems about AFM1 contamination. Suriyasathaporn and Nakprasert (2012) found the contamination of AFM1 in pasteurized milk from supermarkets and from school milk project. Milk samples contaminated with AFM1 were 40% ranging between 35–11,000 ng/kg. Ruangwises and Ruangwises (2009) found the contamination of AFM1 in all of 150 pasteurized milk samples from the School Milk Project in Thailand, but their AFM1 concentrations were within the U.S. regulatory limit of 500 ng/kg. The highest concentration of AFM1 found in school milk samples was 114 ng/Kg. However, most studies showed that AFM1 levels in Thailand were sometime higher than that defined by the EU regulatory limit or higher than 50 ng/kg (Ruangwises and Ruangwises, 2009; Mongkon et al., 2014). This is emphasized by the fact that the AFM1 molecule cannot be inactivated by thermal processing used in the dairy industry, i.e. pasteurization and ultra-high-temperature treatment. According to these results, the contamination of AFM1 in milk from the school milk project which distributes to children all of Thailand should be highly concerned. Therefore, the surveillance of the aflatoxin contamination in animal feed, milk and milk products in Thailand should be performed continuously in order to provide safety of food for the consumers especially children.

***Role of Organic acid for inhibit mold growth and detoxify AFB1 production***

Organic acids occurring in foods are additives or end-products of carbohydrate metabolism of Lactic acid bacteria (LAB). Lactic and acetic acids are the main products of the fermentation of carbohydrates by LAB. These acids, generally recognized as safe agents for the preservation of foods, diffuse through the membrane of the target organisms (Axelsson, 1990) in their hydrophobic un-dissociated form and then reduce cytoplasmic pH and stop metabolic activities. Mechanisms other than cytoplasmic pH reduction have been associated with organic acids (Kabara & Eklund, 1991). It is hypothesized that organic acids act on the plasmic membrane by neutralising its electrochemical potential and increasing its permeability, leading to bacteriostasis and eventually to the death of susceptible organisms, such as mold. Other organic such as phenyllactic acid which have been identified in silos inoculated with LAB also show antifungal activity. It was hypothesized that phenyllactic acid may be acted in a synergy pathway with unidentified antifungal substances produced by LAB (Broberg et al., 2007).

Role of organic acid on AFB1 detoxification has been reported by some researchers. Aiko et al (2015) has reported that lactic acids can convert $AFB_1$ into $AFB_{2a}$, the substance that has 200 times less toxic than $AFB_1$. Mendez-Albores et al (2008) has reported the degradation of $AFB_1$ in sorghum using various concentrations of lactic acid under extrusion conditions. They found that the barrel temperature profile, in combination with the moisture content and lactic acid concentration, significantly affected the extent of aflatoxin reduction in the extruded sorghum. Furthermore, Shukla et al (2002) proposed a mechanism in which $AFB_1$ was reduced to form $AFB_2$ and $AFG_1$ to $AFG_2$ by taking two protons and two electrons from lactic acid.

## OBJECTIVES

1) To determine factors relating to AFB1 contamination in by-product corn processing factories when keeping in small holder farm conditions
2) To determine effects of organic acid supplemented in by-product corn silages on
    - preventing fungal development in contaminated corn silage in different storage times and conditions,
    - control aflatoxins B1 production in relation to storage times and conditions
    - cytotoxic detoxification of aflatoxin supplementary of organic acid in animal cell

# METHODOLOGY

## Determination factors relating to AFB1 contamination in by-product from corn processing factories

### *Sample collection and preparation*

A cross-sectional study was performed during May 2016 to May 2017 using all 82 smallholder dairy farm members of a dairy cooperative in Chiang Mai province, Thailand. One hundred and twenty three samples of dried corn covers (dried corn), and 108 samples of boiled corn cobs with husks (boiled corn) **(Fig.1)** were collected from 3 areas including surface base position, surface high position, and the center of feed piles according to the guidelines of the Department of Livestock Development, Thailand (DLD, 2009). These samples were placed in plastic bags, transported to the laboratory in. Each sample was chopped into 0.5-1 cm size particles and packed at 1-kg in plastic bag and stored in a freezer until analysis.

***Dried corn***

***Boiled corn***

**Fig. 1** Type of corn which was collected

***Assessment of total fungal count***

The total fungal viable count of each sample was determined by using Yeast Extract Agar (YGC) (González Pereyra et al., 2008). Ten grams each of well-ground samples was taken in 90 mL of sterilize sodium chloride (0.9%). The contents were agitated for 30 min with a Worley mix. A serial dilution series ($10^{-1}$–$10^{-5}$) were made using 1 in 9 mL. Fungal culture was aseptic prepared in biological safety cabinet class II. One hundred µl of the suspension from the appropriate dilutions was transferred to Petri plates containing YGC and thoroughly dispersed on the agar plates using sterilized bent pasteur pipettes (Sander, 2012). All the Petri plates were incubated at 25°C for 3-5 days (ISO 13681: 1995). The numbers of colonies of different fungi were counted and the total fungal viable count per gram of sample was calculated as follows. The colonies of fungal in each plate were counted only on plates with colonies concentrations in the range of 10 to 150 colonies (Tournas et al., 2001). Each sample was done in duplicates. The results were displayed as colony forming unit (CFU) per gram dry weight of feed (Maturin and Peeler, 2001).

$$N = \frac{\sum c}{[(1 \times n_1) + (0.1 \times n_2) \times (d) \times 20 \times \%DM]}$$

N = Number of colonies per gram of dry weight, roughage

∑c = Sum of the number of fungi counted in each plate

n1 = Minimum number of plates to be counted for fungus ($10^{-3}$)

n2 = Maximum number of plates to be counted for fungus ($10^{-4}$)

d = Amount of minimal dilution which was found fungus (1000 times)

% DM = Percentage of dry matter of roughage

***Fungal identification***

The physical appearances of each colony on the plates were examined (Macroscopic examination) for their texture, color, shape, size and the pigment of the colonies on the culture medium. This information was used to assist in the diagnosis of fungal isolation. In addition, microscopic examination was performed to look at the fungus and spore formation.

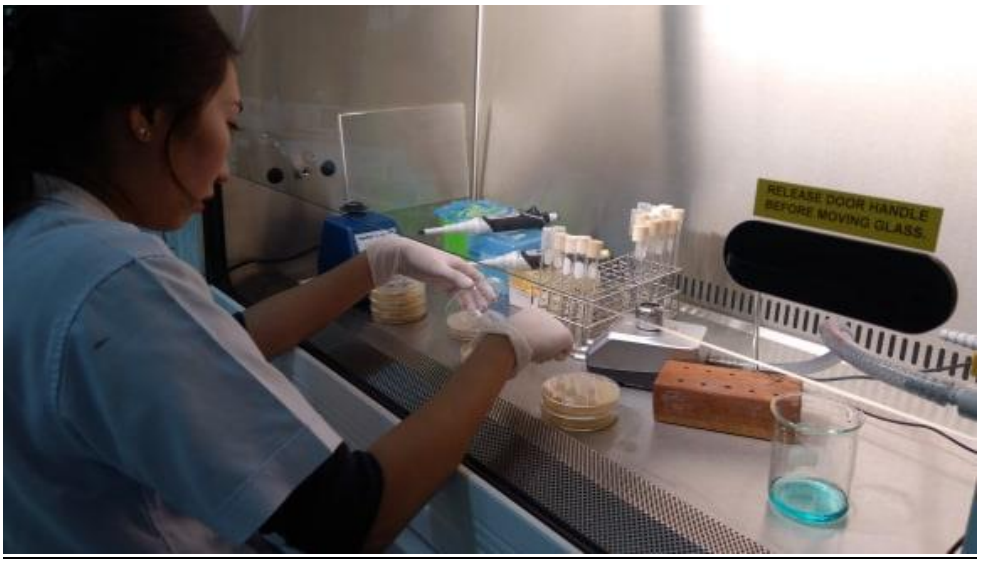


**Fig.2** Total fungal count procedure

***Water activity measurement***

Water activity is the amount of water that is beneficial to the growth and chemical reactions of microorganisms. Water activity can be defined as equilibrium relative humidity (ERH), where the food does not absorb or lose water (Karel et al., 1975).

The water activity was determined using Decagon Devices **(Fig.3)**. Each sample was tested in duplicates. A basic measuring technique utilizes a sealed dish or container with the sensor mounted above the test samples.


**Fig.3** Water activity instrument

***Aflatoxin B1 analysis***

Each feed sample was carefully mixed and finely ground, and then extracted by putting 20 g of the ground sample into an Erlenmeyer flask to which 100 ml of 70% methyl alcohol was added. The flasks were shaken at 300 rounds per min for 30 min, after that the mixture was filtered by Whatman no. 4 paper. The AFB1 level was measured by DOA-Aflatoxin ELISA test kit (Postharvest and Processing Research and Development Division, Thailand) **(Fig.4)**. The analysis was performed according to the kit instruction. Briefly, 50 µl of either AFB1 standards (0, 0.2, 0.5, 1, 2 ng/ml) or the diluted samples were added into the antibody coated wells; then 50 µl of Aflatoxin B1-Horseradish Peroxidase (AFB1-HRP) conjugate was added to each well, slightly shaken, and incubated at room temperature for 30 min. The contents of the well were dumped into the appropriate waste container and the plate was washed 3 to 5 times by 0.01M phosphate buffer saline with 0.5% Tween 20 (PBS-T). Tetramethylbenzidine substrate (100 µl) was added to each well and incubated for 10 min at room temperature. The reaction was stopped by adding 100 µl of 0.3M phosphoric acid. The solutions were read at 450 nm using an automated MicroELISA spectrophotometer reader. The results of absorbance were expressed as percentage of maximal binding as follow:

% maximal binding = B/B0 x 100

B= mean absorbance of feed sample

B0= mean absorbance of AFB1 standard at 0 ng/ml

The standard curve was generated by plotting the concentration of AFB1 with % maximal binding in Log base 10 values.


**Fig.4** Aflatoxin ELISA test kit, extraction procedure

***Statistical Analysis***

Data was described as frequencies for categorical variables. Feed samples were considered contaminated with AFB1 at concentrations higher than 5 µg/kg, the regulatory limit of the European Food Safety Authority (EFSA) (European Food Safety Authority, 2004). For univariable analysis, associations of feed characteristics with AFB1 contamination were separately determined using Fisher's exact chi-square test. The final model of factors associated with AFB1 contamination was created by a multiple logistic model using a backward selection method calculated by SAS (SAS Institute, 1997). All factors were first entered and subsequently removed from the model when their P-values indicated by the likelihood ratio tests were higher than 0.10.

# Determination fungal and aflatoxin B1 contamination in by-products corn silage after supplement with organic acids

***Fugal spore preparation***

*Aspergillus flavus* spores were obtained from the National Center for Genetic Engineering and Biotechnology (BIOTEC). The spores were then amplified by subculture on YGC agar and incubated at 25 ° C for 14 days. The spore suspension was prepared by added conidia obtained from agar plate to sterile water, counting conidia using hemoglobin meter (Hemocytometer slide) until spore solution of 1 x $10^7$ spores per milliliter was obtained. The obtained spore suspension was kept at 4 ° C until experiment.

***Sample preparation***

By-products corn silages were collected from dairy farms in dairy cooperative in Chiang Mai province. These samples were placed in plastic bags and transported to the laboratory in foam boxes with ice. After transferred to the laboratory, all samples were mixed and then separated into Corncob, Corn husk, and the mixture of corncob and corn husk. Each sample was chopped into 0.5-1 cm size particles, separated into 8 aliquots, packed at 400 grams in plastic bag and autoclaved at 121 $^{o}$C for 15 min as shown in **Fig.5**.

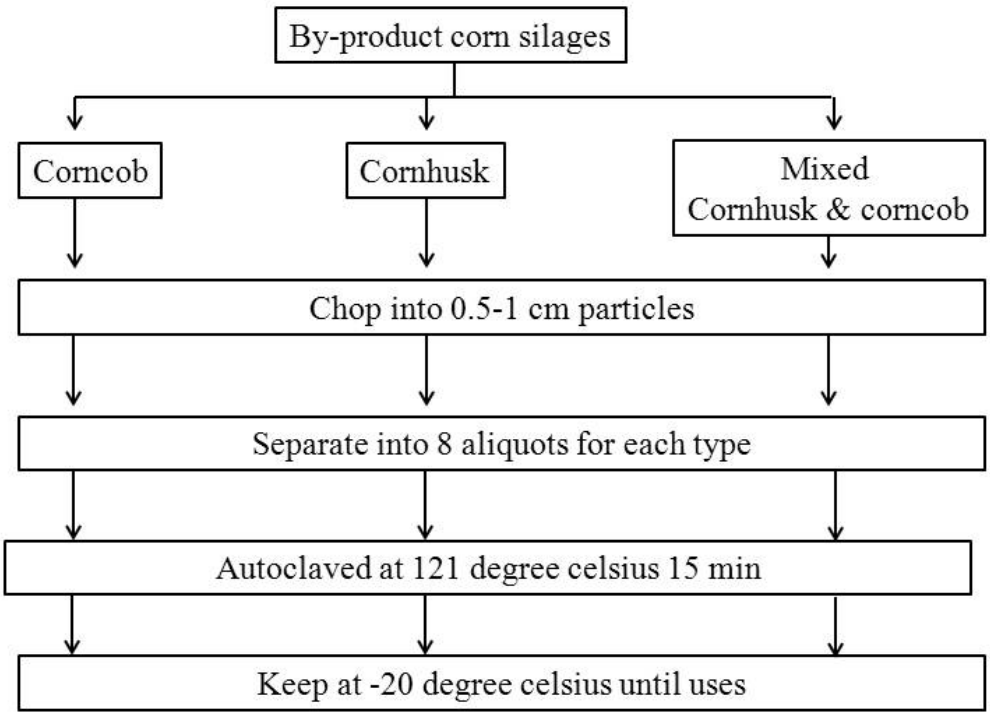


**Fig.5** Preparation for keeping by-product corn silages

***Organic acids preparation***

Organic acids including lactic acid, acetic acid and propionic acid concentration of 12.5, 2.5 and 5% w/w of dry matter feed were added to corn samples containing spore of *A.flavus* to determine their efficacy on reducing fungal count. An acid with highest activities was selected for further study.

**Table 1** The dry matter content of different part of corn

| **Part of corn** | **% dry matter** |
|---|---|
| Corncob | 23.159 % |
| Corn husk | 18.554 % |
| Mixed of corncob and corn husk | 20.721 % |

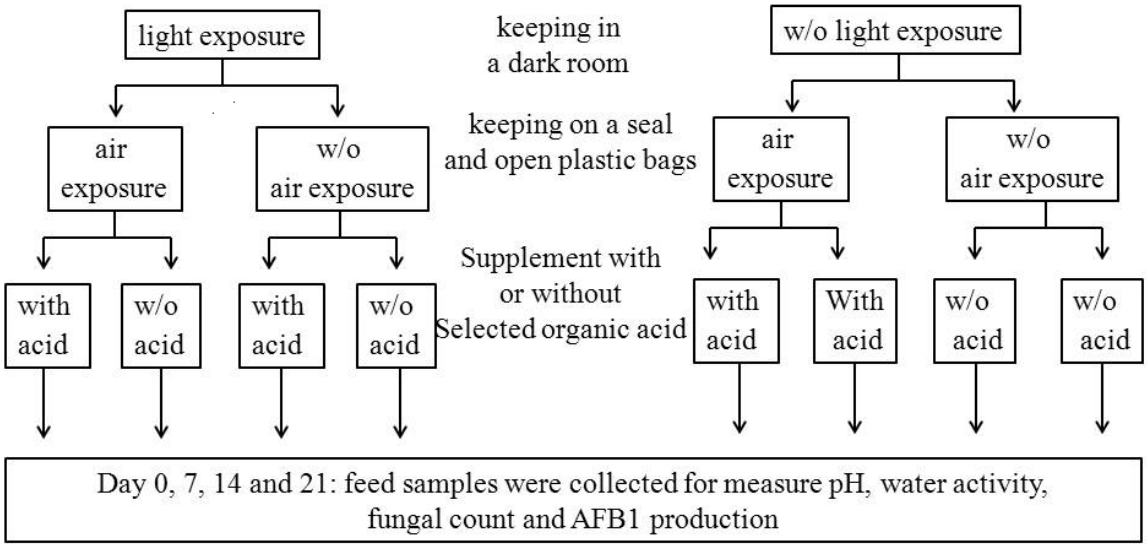


**Fig.6** Experimental design for evaluate the effect of lactic acid supplementation in by-product corn silage on fungal growth, AFB1 production and detoxification of AFB1 cytotoxicity.

***Experimental design***

This study was designed to evaluate 5 factors associated to fungal growth and AFB1 contamination of by-product corn silage. The factors included 1) composition of the corn silages (corn skin, cob, ears, mixed), 2) light exposure (yes, no), 3) air exposure (yes, no), 4) storage time (0, 7, 14 and 21 days), and 5) supplementation with certain concentration of acid which had highest efficacy on reducing fungal count. Each aliquot of prepared samples was thawed, added 2 ml of *A.flavus* suspension containing 1 x $10^7$ spores per milliliter and then was assigned for one of eight groups as shows in **Fig. 6**. From 8 aliquots, 4 of them were kept in a dark room for without light exposure. The plastic bags were sealed with an elastic band or without sealing, as without and with air exposure. For organic acid supplementation, selected acid was dissolved and supplement to the specified feed aliquots at the selected concentration. At day 0, 7, 14, and 21 of study, feed samples were collected for measurement of fungal count, pH and

AFB1 production. The samples of specified aliquots were mixed and then were collected for further analysis. Each experiment was repeated two times.

***pH determination***

The pH of each sample was determined using a slurry pH method. Briefly, mixed 20 g of each chopped corn samples with distilled water at a ratio of 1:2 (Boyd, 1995) in a beaker and allow the solution to saturate for 15 minutes prior measurement (Pudipeddi et al., 2008). The pH value was measured by digital pH meters (Genec, U.K.).

***Determination of Aflatoxin production as describe in previous section***

***Cytotoxicity testing***

***Sample preparation and extraction***

Autoclaved corn samples were added with AFB1 standard to the final concentration of 50 and 100 ppb, immediately added with effective concentration of selected organic acid and incubated at room temperature for 7 days. Aflatoxin containing samples with and without acid were extracted according to the protocols of Candlish et al. (1998) with some modifications. The mixtures of feed samples (12.5 g) with NaCl (1 g) were blended with 62.5 ml of 60%v/v methanol for 1 minute. The extracted samples were filtered and diluted with 62.5 ml distilled $H_2O$, mixed thoroughly and passed through a filter paper (Whatman No. 4). Ten ml of the filtrate obtained was passed through an immuno-affinity column at a flow rate of 2–3 ml/min, washed the column using 10 ml of phosphate buffered saline (PBS) at a flow rate of 5 ml/min. The analyses were then eluted (1 drop/s) using 1 ml of methanol and collected

in an amber vial. The extract was dried in a fume cupboard using $N_2$ gas and stored at 0 °C until use for cytotoxicity analysis.

***Cell culture preparation***

The human cervical cancer cell line or HeLa cell (National Centre for Cell Science, Pune, India) was cultured in plastic flasks as described by Moekona et al (2006). Hela cells was maintained in Dulbecco's modified Eagle's medium (DMEM, HiMedia, India) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, USA) and 1% of antibiotic antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 mg amphotericin B per ml, HiMedia, India). The cultures were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air incubator. They were washed with 5 ml phosphate buffer solution (PBS), trypsinized using 500 µl of trypsin-EDTA (HiMedia, India) and gently rocked to disperse the cells. The number of cells were counted using a haemocytometer and diluted to $1\times10^5$ cells/m

***MTT test***

MTT test to assess the toxicity of AFB1 of extracted samples containing AFB1 with or without treated with acetic acid was carried out as describe by Caruso et al (2009). One hundred microliters of HeLa cell suspension (1×105 cells/ml) and extracted samples reconstituted in methanol were seeded to a 96-well microplate and incubated incubated at 37 °C, 5% $CO_2$ in humidified atmosphere for 48 h. Methanol was used as negative control. After incubation, twenty microliters of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) (Sigma, St. Louis, MO, USA) was added to each well and further incubated for 4 h under the same condition to allow the blue crystals, formazan, to be produced by the mitochondrial enzyme

succinate dehydrogenase. After incubation, the medium was removed and 150 µl DMSO was added to dissolve the blue formazan crystals formed. The color intensity was measured using a microplate reader (Bio-Rad 680, USA) at a test wavelength of 540 nm and reference wavelength of 655 nm.

% Cell viability = [Mean OD values of treated cells / Mean OD values of Control] × 100%

***Statistical analysis***

All the experiments were carried out in triplicate and repeat to confirm the results. The data was reported as mean ± standard deviation and subjected to analysis of variance (ANOVA) to determine the significant difference at $P < 0.05$ level. Data was checked for their normalization and logarithm transformation was used in case of necessary. Effects of factors, including type, air expose, light expose, storage time and LA supplementation, associated with fungal growth, AFB1 contamination, and detoxification efficacy was analyzed using repeated measurement analysis by mixed model analysis. Dependent variables were numbers of total fungal viable count, AFB1 concentration, and efficacy of AFB1 detoxification. Independent variables were types of by-product corn silage, air exposure, light exposure, storage time and LA supplementation. Significant levels were defined at $P<0.05$.

# RESULTS

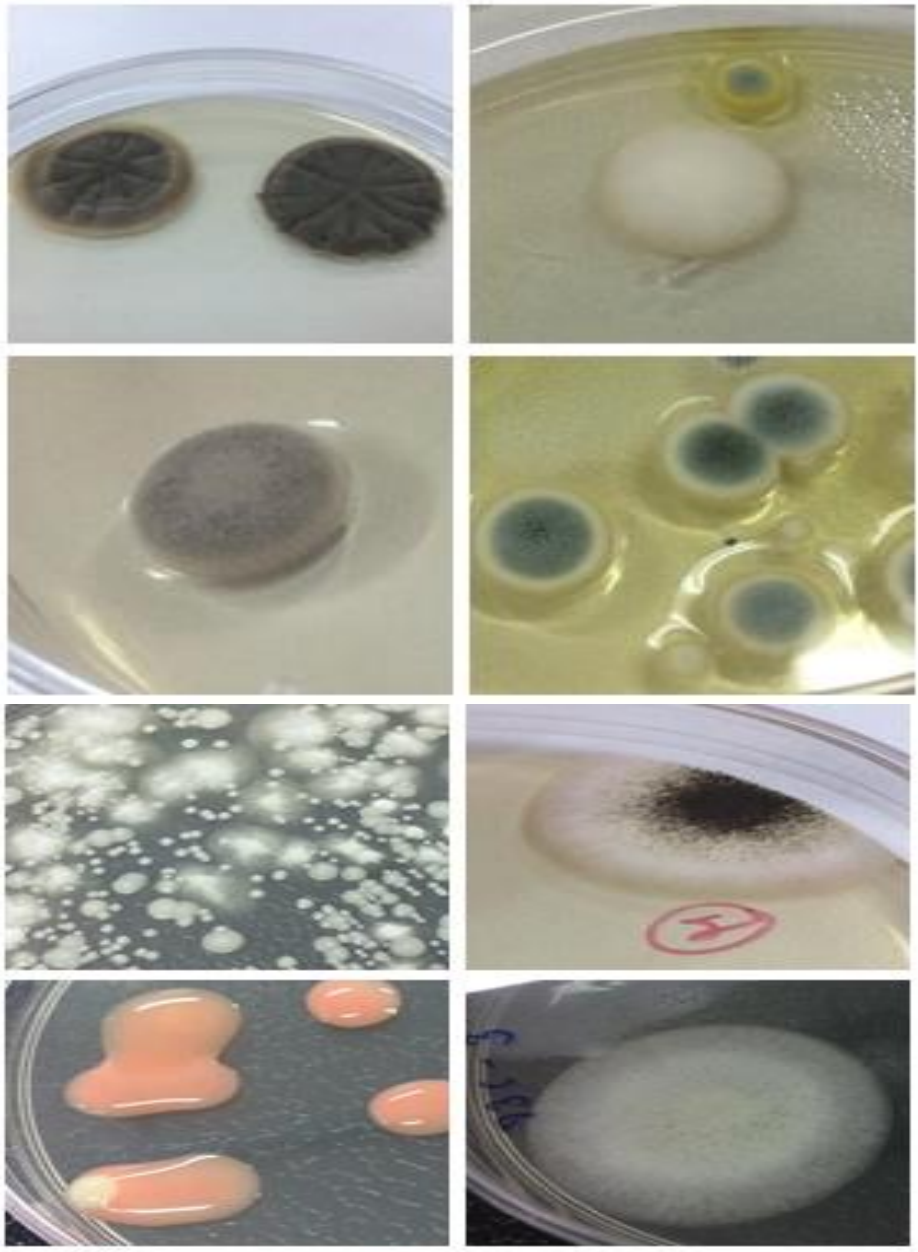

**Fig.7** Macroscopic characteristic of fungal growth on culture medium

Macroscopic and microscopic characteristics of fungal growth on culture medium are shown in **Fig.7** and **Fig.8**.

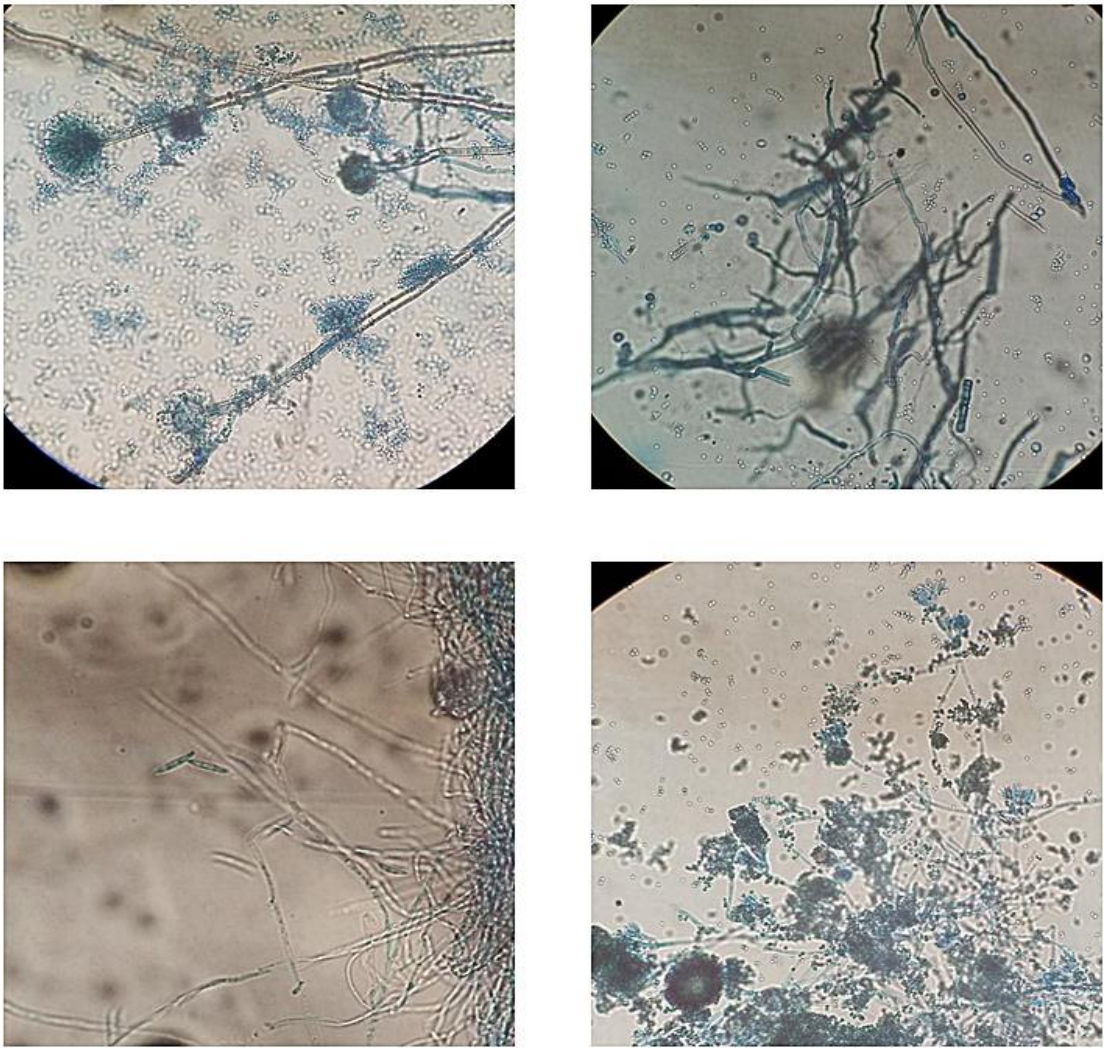

**Fig.8** Microscopic examination of fungal growth on the culture medium

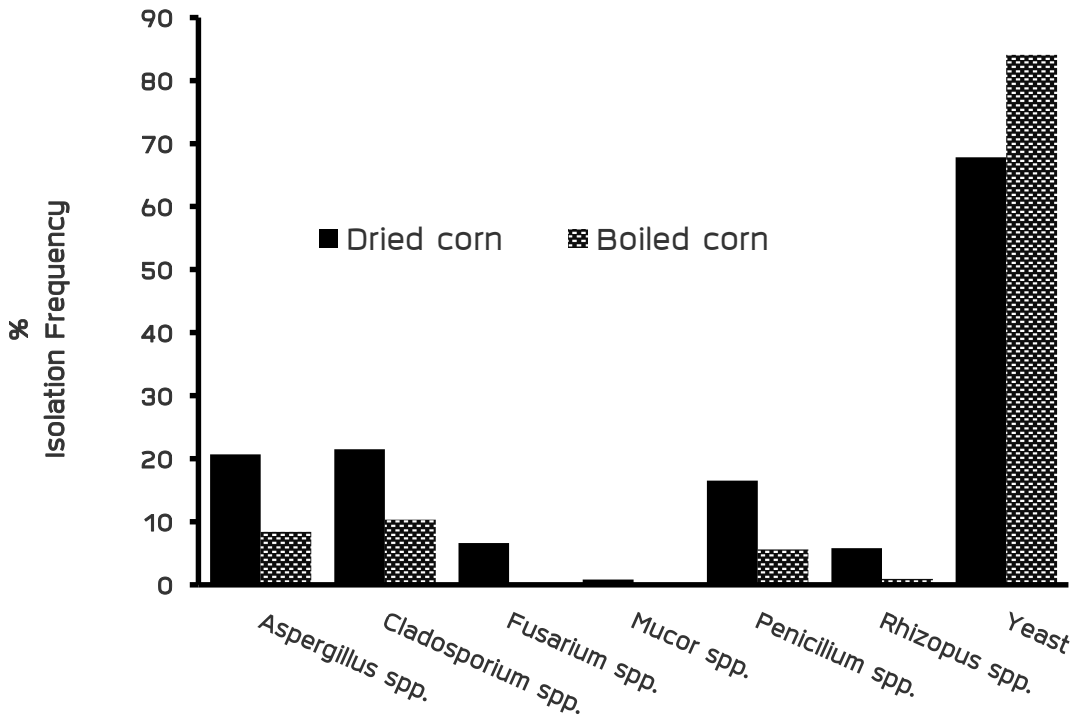


**Fig.9** Percentage of isolation frequency of fungal in corn samples

Percentages isolation frequency of fungal genera in all corn samples are shown in **Fig.9**. The most common fungus was *Aspergillus* spp., *Cladosporium* spp. *Penicillium* spp. yeast and others. These results are in agreement with previous reports from Mudili et al. (2013) Tsedaley and Adugna (2016).

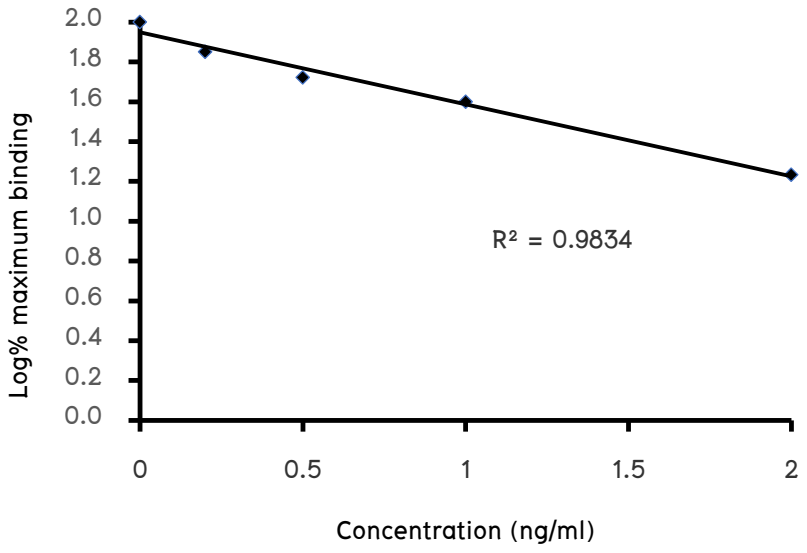


**Fig.10** Calibration curve of AFB1

Calibration curve of AFB1 concentration obtained from the DOA-Aflatoxin ELISA Test Kit is shown in **Fig. 10**. According to a study by Chinaphuri et al (2002), The DOA-Aflatoxin ELISA test kit is capable of detecting the lowest aflatoxin B1 level of 0.4 ppb and the percentage of recovery was between 82 and 100. The efficacy of DOA-Aflatoxin ELISA Test Kit does not differ from Thin-Layer Chromatography and High-performance liquid chromatography.

The water activity, fungal content and Aflatoxin B concentration in the samples of boiled and dried corn at different locations are shown in **Table 2**. It was found that the mean water activity of the dried and boiled corn samples were 0.59 ± 0.19 and 0.99 ± 0.00, respectively. Most of all samples (99.9-100%) were contaminated with fungal microorganism. The average fungal count was $167.11 \times 10^6$ CFU/g. In the samples of boiled corn, the contamination of the fungus was less than dry, with an average of $110.739 \times 10^6 \pm 45.26 \times 10^6$ CFU/g and $215.093 \times 10^6 \pm 75.55 \times 10^6$ CFU/g, respectively. There were no differences ($P > 0.05$) of fungal count in the different collecting positions.

The concentration of AFB1 in the samples of dried corn was higher than boiled corn. The average concentrations were 14.72 ± 0.71 (2.23-50.2) and 6.54 ± 0.30 (0.42-10.67) ppb, respectively (**Table 2**). It was found that 83.6 percent of all samples (153/183 samples) were contaminated with AFB1 more than 5 ppb. Only a few dried corn samples were contaminated with AFB1 above 20 ppb (8.2%, 15/183 samples). Based on the statistical analysis, it was found that the amount of AFB1 presenting in the samples at different collecting positions was not significantly different. It was also found that the amount of fungi did not correlate with the concentration of AFB1 in both types of corn samples ($P > 0.05$).

AFB1 concentrations found in the dried and boiled corn samples in different dairy cooperatives are shown in **Fig.11**. The average AFB1 concentration found in corn

samples from Coop-C was highest, followed by Coop-B and Coop-A. The average AFB1 concentrations in dried corns was 16.05 ± 1.14, 15.39 ± 0.92 and 14.74 ± 0.70 ppb and boiled corns was 4.09 ± 1.83, 8.45 ± 0.32, and 5.68 ± 0.30 ppb, respectively.

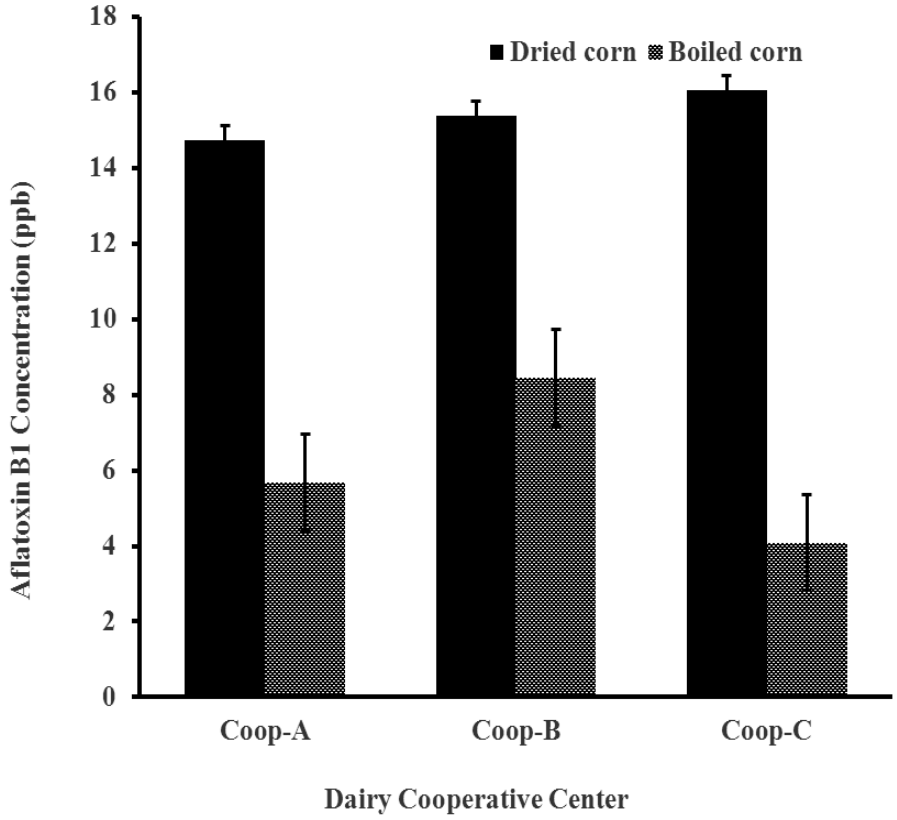


**Fig.11** The chart shows the average concentration of AFB1 in the sample of dried and boiled corns from the different dairy cooperatives

**Table 2** Water activity, fungal count and the concentration of AFB1 in corn samples

| Sample | Water activity | Fungal count x $10^6$(CFU/g) | | | AFB1 Concentration (ppb) | | |
|---|---|---|---|---|---|---|---|
| | Mean±SD | Contamination frequency (%) | Mean±SE | Geometric mean | Contamination frequency (%) | Mean±SE | Sample exceeding regulation limit (%) |
| Dry | 0.595±0.19 | 99.984 | 215.093±75.55 | 5.371 | 100.00 | 14.720±0.71 | 12.20 |
| B | 0.568±0.19 | 99.976 | 160.224±73.73 | 5.077 | 100.00 | 14.808±0.72 | 9.80 |
| S | 0.568±0.16 | 100.000 | 163.357±73.73 | 5.096 | 100.00 | 13.907±0.72 | 12.20 |
| C | 0.584±0.20 | 99.976 | 322.991±73.74 | 5.778 | 100.00 | 15.356±0.72 | 14.60 |
| Wet | 0.996±0.00 | 99.954 | 110.739±45.26 | 4.707 | 99.99 | 6.538±0.30 | 0.00 |
| B | 0.995±0.00 | 99.950 | 113.489±45.70 | 4.732 | 99.97 | 6.166±0.30 | 0.00 |
| S | 0.996±0.00 | 99.960 | 130.711±48.01 | 4.873 | 100.00 | 6.204±0.30 | 0.00 |
| C | 0.996±0.00 | 99.972 | 75.295±46.16 | 4.321 | 100.00 | 6.011±0.30 | 0.00 |

*Sample position : B = Base, S = Surface, C = Centre

*SE : Standard error SD: Standard deviation

*Regulation limit (%) not exceed 30 ppb (Codex Maximum Level , 2003)

Factors related with fungal count in corn samples are shown in **Table 3**. The presence of *Aspergillus* spp. and Yeast in samples significantly increased in fungal numbers. In addition, it was found that the source of samples associated with fungal count. Fungal counts from Coop-A were found more than that from other cooperatives ($P < 0.05$).

**Table 4** shows factors related to AFB1 concentration in corn samples. The results showed that the type of corn, the presence of *Cladosporidium* spp. and the different locations were associated with AFB1 concentrations. The boiled corn samples and the presence of *Cladospordium* spp. were found to have lower AFB1 concentrations than dried corns and samples free from *Cladosporium* spp. infection ($P<0.05$). Moreover, it was found that samples from Coop-B had higher concentrations of AFB1 than that other cooperatives significantly ($P<0.05$). **Table 5** shows the relationship between the storage factors that affected the AFB1 concentration of more than 10 ppb. Results demonstrated that placing corn residues on the ground was found to be more likely to be contaminated with AFB1 than placing corns on cement 13 times (OR=13.73, $P < 0.05$). The boiled corn samples and placing corn samples on the floor with plastic sheet had a lower probability of AFB1 contamination than those of dried corn and corn which are placing on the floor without plastic sheet.

**Table 3** Stepwise linear regression model of factors and quantity of fungus in corn sample

| Variable | Level | Coefficient | Standard Error | T-value | P-value | LS Mean | Geometric Mean |
|---|---|---|---|---|---|---|---|
| *Aspergillus* spp. | With | 1.77 | 0.64 | 2.77 | 0.0062 | 1.33 | 3.78 |
| | Without | 0.00 | | | | -0.30 | 0.74 |
| Yeast | With | 2.78 | 0.58 | 4.80 | 0.0000 | 1.88 | 6.55 |
| | Without | 0.00 | | | | -0.85 | 0.43 |
| Dairy | Coop-C | -2.32 | 0.79 | -2.93 | 0.0038 | -0.16 | 0.85 |
| Cooperative | Coop-B | -2.66 | 0.48 | -5.50 | 0.0000 | -0.59 | 0.55 |
| | Coop-A | 0.00 | | | | 2.29 | 9.87 |

*Transformed fungal numbers to logarithm values before analysis

**Table 4** Stepwise linear regression model of factors related to AFB1 concentrations in corn samples

| Variable | Level | Coefficient | Standard Error | T-value | P-value | LS Mean | Geometric Mean |
|---|---|---|---|---|---|---|---|
| *Cladosporium* spp. | With | -0.22 | 0.09 | -2.29 | 0.0235 | 1.85 | 6.36 |
| | Without | 0.00 | | | | 2.07 | 7.92 |
| Corn type | Wet | -1.04 | 0.07 | -13.66 | 0.0000 | 1.44 | 4.22 |
| | Dry | 0.00 | | | | 2.48 | 11.94 |
| Dairy Cooperative | Coop-A | -0.24 | 0.08 | -3.08 | 0.0024 | 1.90 | 6.69 |
| | Coop-C | -0.25 | 0.12 | -2.03 | 0.0438 | 1.88 | 6.55 |
| | Coop-B | 0.00 | | | | 2.13 | 8.41 |

*Transformed fungal numbers to logarithm values before analysis

**Table 5** Stepwise Logistic Regression model between storage practices for AFB1 contamination

| Variable | Level | Coefficient | Standard Error | Odd ratio | T-value | P-value |
|---|---|---|---|---|---|---|
| Yeast | With | 1.90 | 0.95 | 6.68 | 3.32 | 0.0009 |
| | Without | 0.00 | | | | |
| Floor | Ground | 2.62 | 0.89 | 13.73 | 2.94 | 0.0000 |
| | Cement | 0.00 | | | | |
| Corn type | Wet | -0.94 | 0.91 | 0.02 | -4.48 | 0.0000 |
| | Dry | 0.00 | | | | |
| Ground cover | With | -3.24 | 0.88 | 0.10 | -2.64 | 0.0083 |
| | Without | 0.00 | | | | |

* Yeast is references

## Fungal and aflatoxin B1 contamination in dairy roughage (factory-corn) after supplement with organic acids

**Table** 6 Efficiency of each organic acid on inhibiting fungal growth

| **Organic acid** | **Concentration (%w/w)** | **Mean ± SD Fungal count x $10^5$ (CFU/g)** |
|---|---|---|
| Lactic acid | 1.25 | 4.83 ± 0.89 |
| | 2.5 | 5.45 ± 0.19 |
| | 5 | 6.68 ± 0.58 |
| Acetic acid | 1.25 | 0.00 ± 0.00 |
| | 2.5 | 0.00 ± 0.00 |
| | 5 | 0.00 ± 0.00 |
| Propionic acid | 1.25 | 1.05 ±1.48 |
| | 2.5 | 0.68 ±0.84 |
| | 5 | 1.30 ± 1.83 |

**Table 6** shows the efficacy of lactic, acetic and propionic acid against fungal growth. Results demonstrated that acetic acid at 1.25% up to 5% had efficacy on inhibiting the growth of *Aspergillus flavus*. Therefore, acetic acid at 1.25% was used for further study to determine their efficacy on fungal growth and AFB1 production in different storing conditions.

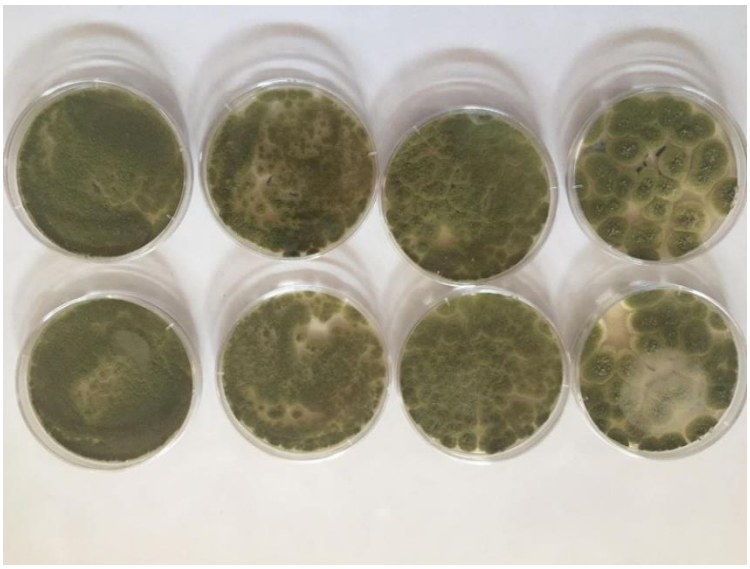

**Fig.10** Physical characteristic of Aspergillus flavus growth on YGC agar

Fungal count, AFB1 concentration, water activity at 12 h, 7, 14 and 21 days are shown in **Table 7**. It was found that the number of *A.flavus* in samples with acetic acid was lower than those without acetic acid throughout the experimental period. In addition, amount of fungal in samples without acetic acid after 12 h decreased significantly in comparison with the control group at 0 h. The fungal number relatively constant between day 7 and day 14 and started to increase on day 21 (**Table 7**).

The water activity ($A_w$) between the samples with and without acetic acid was found to be very similar. The mean pH of samples with acetic acid had lower (3.50 ± 0.008) than those without (3.56 ± 0.007) (**Table 7**).

**Table 7** Fungal count, Aflatoxin B1 concentration, Water activity ($A_w$), pH at 12 hr., day 7, 14 and 21

| Incubation time | Without acetic acid | | | | Acetic acid | | | |
|---|---|---|---|---|---|---|---|---|
| | Fungal count (CFU/g ) | AFB1±SEM (ng/g) | pH | $A_w$ | Fungal count (CFU/g ) | AFB1±SEM (ng/g) | pH | $A_w$ |
| Control[1] | 15763.86 | 849.36 ±29.88 | 3.34 | 1.001 | 14581.62 | 883.61 ±27.25 | 3.30 | 1.001 |
| 12 h | 442.82 | 1349.50± 34.89 | 3.51 | 0.998 | <242.22 | 1199.54 ±25.57 | 3.42 | 0.998 |
| 7 days | <242.22 | 1961.52 ±38.73 | 3.54 | 1.001 | <242.22 | 1764.58± 56.38 | 3.49 | 1.001 |
| 14 days | 253.44 | 854.49± 8.18 | 3.50 | 0.995 | <242.22 | 879.54 ±17.38 | 3.49 | 0.995 |
| 21 days | 2980.38 | 1457.27 ±21.09 | 3.70 | 0.993 | <242.22 | 1547.36 ±18.24 | 3.59 | 0.995 |
| Mean[2] | 979.72 | 1405.70 ±25.72 | 3.56 | 0.997 | <242.22 | 1347.76 ±29.39 | 3.50 | 0.997 |

After spore suspension at 0 hours

**Table 8** Factors associated with AFB1 concentration

| Factors | AFB1 (ng/g) | SEM | *p*-value |
|---|---|---|---|
| Acetic acid | | | |
| With | 1347.77 | 29.39 | 0.4417 |
| Without | 1405.65 | 25.72 | |
| Corn types | | | |
| Corncob | 1102.94 | 12.02 | 0.0002 |
| Corn husk | 1495.56 | 15.42 | |
| Mixed | 1531.64 | 16.64 | |
| Light | | | |
| With | 1382.67 | 9.90 | 0.8732 |
| without | 1370.76 | 11.58 | |
| Oxygen (Air) | | | |
| With | 1453.72 | 11.33 | 0.050 |
| without | 1299.71 | 9.92 | |
| Times | | | |
| 12 h | 1274.48 | 15.29 | <0.001 |
| 7 d | 1862.96 | 24.02 | |
| 14 d | 867.08 | 6.66 | |
| 21 d | 1502.34 | 9.5 | |

Supplementation of acetic in corn samples reduced AFB1 concentration non significantly (P>0.05). Factors influencing the concentration of AFB1 were corn type, oxygen and storing times. Samples exposure with oxygen were found to have higher concentrations of AFB1 that those without (p = 0.050). The results showed that Aflatoxin B1 was significantly lower in corncob than that of cornhusk (p = 0.0002) and mixed group (p = 0.0001) (**Table 8**). Aflatoxin B1 concentrations at different storage periods differed significantly (p <0.01). The average concentration of AFB1 between 12 hours and 7 days is likely to increase. Then, on day 14, the concentration of AFB1 decreased, and on day 21 of the experiment the concentration increased again **(Fig. 11)**

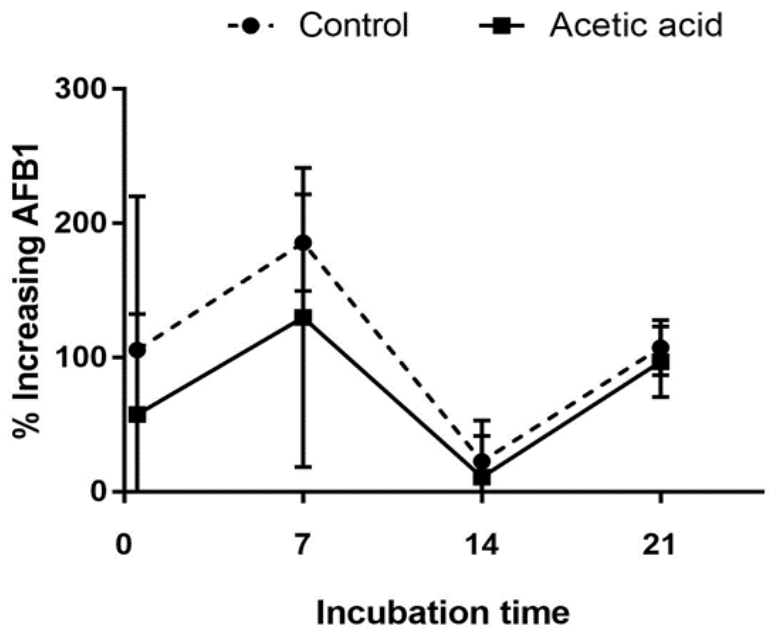


**Fig. 11** Comparison of percent increasing AFB 1 between acetic acid and without acetic acid condition at 12 hr., day 7, 14 and 21

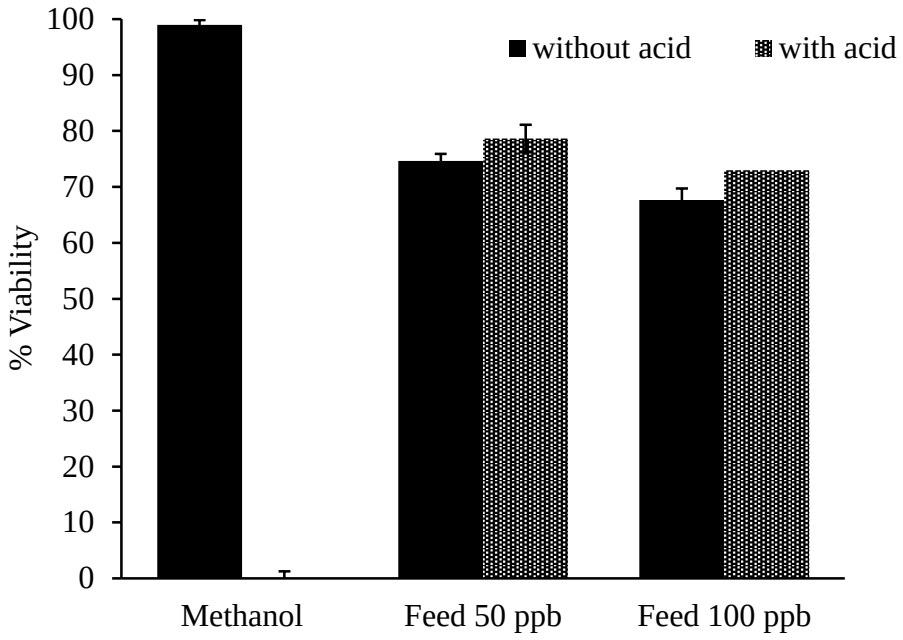


**Fig.12** Toxic effects of aflatoxin extracts with and without supplementary of acetic acid on Hela cell line at 24 hours of exposure

The viability of Hela cell was influenced by the concentration of AFB1 in feed extracts. It was observed that the viability of cell treated with feed containing 50 and 100 ppb of AFB1 without acetic acid was 75% and 68% respectively. Results showed that the viability increased significantly when supplemented with acetic acid in extracted feed containing AFB1 ($P<0.05$) **(Fig.12)**

# DISCUSSION

Over the last two decades, enzyme-linked immunosorbent assay (ELISA) has been favorably accepted. It not only is a tool for rapid and sensitive detection with high sample throughput capacity, but also is relatively inexpensive which suitable for detecting aflatoxin contamination in filed practice for dairy farmers. In this study, the authors used DOA-Aflatoxin ELISA Test Kit which is an in-house (ELISA test) kit produced by Department of Agriculture (DOA) Ministry of Agriculture and Cooperatives. The test kit had been validated for their precision and accuracy for aflatoxin $B_1$ detection in feed samples before used. The correlation coefficient ($R^2$) obtained from calibration curve of AFB1 standard using ELISA kit was 0.9164, indicating a linearity of this test. The accuracy of this test was up to 82-100%. Aflatoxin can be detected at concentrations as low as 0.04 ppb (ng/g). Therefore, the detection of Aflatoxin with this assay is suitable for used.

The dry and boiled corns, by-products from corn processing factories are widely used in dairy farming in the northern part Thailand. Our study found that these corns were contaminated with *Aspergillus* spp., *Cladosporium* spp., *Mucor* spp., *Rhizopus* spp., *Fusarium* spp., *Penicillium* spp. and Yeast. The results were similar to previous studies which reported that *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. are the main fungal found in maize (Rosa et al., 2008; Mudili, V. et al., 2013; Tsedaley and Adugna, 2016). This is consistent with the study of Charoenpornsook et al (2006) who reported many species of these fungi can produce toxins that affect humans and animals. This study found the contamination of fungi in almost all corn samples. The average fungal count of all samples was $167.11 \times 10^6$ CFU/g, 77.7%

(174/224 samples) of total samples. Approximately 77% of samples had amount of fungus exceeds the regulatory limit defined by Animal Feed Quality control Act B.E. 2559 which indicated that the limit of fungal count must not exceed $1x10^5$ CFU/ g. The samples which fungal count excesses the standard value are considered as deterioration. In addition, when referring to the GMP standard of 2008, 92% of samples had fungi higher than standard (1x104 CFU/g) which is indicated the quality of food and inappropriate storage practice of animal feed.

Dried corns had water activity value of 0.59, which was not suitable for fungal growth and toxin formation. However, in this study, fungal contamination was found to be more pronounced in dried corns than in boiled corn. This result different from the previous report form Alonso et al (2011) who found that animal feed with water activity of 0.86-0.97 had higher tended to be contaminated with fungal and fungal producing aflatoxin such as *A.flavus* (Hedayati et al., 2007).

From the statistical analysis, it was found that the presence of *Aspergillus* spp. and yeast in the samples significantly correlated with the amount of fungus. This study in accordance with Keller et al (2013) who reported the amount of fungal in corn samples were higher than the standard and the most common fungus was *Aspergillus* spp. In addition, samples from Coop-A had higher amount of fungus than that of other cooperatives ($P < 0.05$). This may be due to the different storage practices in different cooperatives such as food storage facilities or storage times which affect the growth of the fungus and the contamination of fungal toxins (Mongkon et al., 2017).

Based on the aflatoxin concentration, all samples had aflatoxin contamination of 11.41 ppb. The results consistent with Mongkon et al. (2017) who reported that AFB1 was detected in all corn samples from Chiang Mai area before and after

fermentation, the concentration ranged between 5.5 to 11.4 ppb, respectively. The concentration of aflatoxin in dried corn samples was higher than that of the boiled corns. The concentrations were 14.72 ± 0.71 and 6.54 ± 0.30 ppb, respectively. The average of both samples has passed the Thai and USFDA benchmarks (according to the Codex Maximum Level 2003, Aflatoxin not exceeding 20 ppb) but not passed FAO / WHO standards (AFB1 level not exceeding 5 PPB). However, only 8.2% of all samples especially in dried corn contaminated with AFB1 more than 20 ppb, this is according to Mongkon et al (2017).

Our study found that the amount of fungus and aflatoxin present in the samples at different locations was not significantly different. This is different from the previous study of Gonza'lez Pereyra et al. (2007) which investigated the type and amount of fungus in fermented corn kept in silo. They found that the total fungal count on the top of silo was highest because this position has the greatest exposure to air environment. The different results may be due to samples collected in our study is placed in an open area which has more opportunity to expose with the air, humidity and other factors, hence, the environment is suitable for fungal growth rather than fermentation in silo. It was also found that the amount of fungi was not correlated with the concentration of AFB1 in both types of corn samples. Our study is in agreement with other previous studies (Carvalho et al., 2015; Alonso et al., 2013). Toxins from fungi are more durable than fungus and can be maintained in the animal feed throughout the production and storage process, while the fungus was easily destroyed in non-suitable environment (Carvalho et al., 2015; Alonso et al., 2013). Therefore, the detection of fungal toxins does not necessarily correspond to the presence of fungi in animal feed.

The AFB1 concentration in dried corn higher than boiled corn appeared related to the fungal count which found in higher number in dried corn could be explained by a longer storage period of dried corn when compared to wet corn, this is in agreement with Keller et al (2013) who reported that aflatoxigenic fungi and AFB1 contamination was enhanced during storage. In addition, the dry matter of dried corn is greater than the boiled type. (89.01% for dry and 24.47% for boiled corn). When analyzing the concentration of AFB1, the dried samples were more concentrated in AFB1 than the boiled ones, hence, when cows consume the same amount of both types of corns, cows that eat a dry receive AFB1rather than boiled.

The presence of *Cladosporium* spp. was associated with a decrease in AFB1 concentration in corn samples significantly. This type of fungus is capable of inhibiting the production of AFB1 in the laboratory by interfered aflatoxin formation and transformed AFB1 to the different structure compounds (Cvetnic et al., 2007).

The AFB1 concentration of corn from Coop-B was higher than that of other cooperatives significantly. The management factors of each farm can affect both the amount and concentration of aflatoxin in the samples. Placing corn on the ground are associated with an increase in AFB1 contamination compared to those placed on concrete floors significantly ($P < 0.05$). This can be explained by an increasing exposure to aflatoxigenic fungi from the soil to the pile of corn (Hedayati et al., 2007). In addition, the placement of plastic sheet under the corn pile was significantly related to the reduction of AFB1 contamination ($P < 0.05$) because the plastic sheet can reduce the moisture and fungus from the ground contaminated to the pile of corn.

From our study, it was found that acetic acid can reduce the amount of *Aspergillus flavus.* The result was in accordance with research by Hassan et al (2015) who reported that acetic acid had highest efficacy for reducing the number of *A.flavus*. The mechanism of fungal inhibition is due to acetic acid causes the pH in the cytoplasm of the fungal cell to decrease, resulting in an increase in the acidity of the cell to the extent that it does not grow (Stratford et al., 2009). It was found that most of the microorganisms were easily destroyed in the presence of organic acids because of a decrease in pH in the media (Conner et al., 1990; Farber et al., 1989; Ita and Hutkin, 1991). This phenomenon is due to the hydrophobic effect of organic acids. The proton from acids moves from environment through the membrane into cell. This feature can be achieved by the pH level and the concentration of the solution inside and outside the cell. In acidic conditions, the decrease in pH within the cell is due to the removal of the proton. The cells increase the energy consumption of the protons, these changes will affect the ATP process and metabolic processes of fungal cells results in a reduction in cell growth (Stratford et al., 2009).

In sample without acetic acid, fungal number after 12 h decreased significantly in comparison with the control group at 0 h. The fungal number relatively constant between day 7 and day 14 and started to increase on day 21. The decline of fungal number at 12 h to day 7 is due to the high acidity environment.

The water activity between samples with and without acid was similar. It was found that there was a high level of free water that was beneficial for fungal growth (Aw = 0.94 - 0.99) and aflatoxin production (Pitt and Miscamble, 1995). However, the pH in this study was high from the beginning to the end of the experiment. The

average pH of 3.3 from the beginning is in the pH range of corn silage (pH 3.7-4.2) which represents the fermentation process in samples can inhibit the growth of fungi.

Acetic acid did not significantly decrease Aflatoxin B1 concentrations, however, the experimental group with acetic acid increased the percentage of AFB1 level lower than those without acetic acid. These results in accordance with cytotoxicity testing, in overall, extracted samples from feed containing AFB1 mixed with acetic acid revealed lower toxicity by obtained higher cell viability when compared to that for feed without acetic acid. This can be explained by the properties of organic acid for decomposed toxic AFB1 to AFB2, AFB2a, and AFD1 with has lower toxicity (Ciegler and Peterson, 1968, Pons et al., 1972).

After storage, AFB1 concentration increased on day 7, decreased on day 14 and increased again on day 21. Increasing of AFB1 after storage found in the present study similar with research from Rusul and Marth (1988) who found an increasing in AFB1 over the next 10 days of the storage period. However, decreasing of AFB1 on day 14 might be due to the fluctuation of environment's temperature. The maximum production of AFB1 can be achieved in the range of temperatures between 20-25 $^{o}$C (Bandyopadhyay et al., 2000). The changing of temperature can affect the AFB1 production. An increasing in environmental temperature could reduce the production of AFB1 (Mohale et al., 2013; Mousa et al., 2011). Furthermore, decreasing of aflatoxin could be due to the decay of aflatoxin by *Aspergillus flavus* itself (Hamid and Smith, 1987). Considering on pH, it was found that the reducing of aflatoxin on day 14 relating with the decreasing of environment pH from the beginning. Wheeler et al (1991) reported that AFB1 is reduced to 50 percent at pH 4 and slightly produce at pH 3.

Factors associated with AFB1 production including air exposure and corn compositions. Aflatoxin B1 concentration in group with light exposure was not significantly different from those without. This findings related to previous research which found that light exposure did not affect the production of Aflatoxin B1 from *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett et al., 1978; Mayne et al., 1971).

Aflatoxin B1 concentrations in samples with air exposure were higher than those without air. This result similar with Aisha et al (2013) who reported that AFB1 is produced in an oxygenated condition more that of without oxygen.

According to corn compositions, this research found that corn cobs had lower concentrations of Aflatoxin B1 than that of corn peel and mixed compositions. This difference from Shotwell et al (1980) who found that the amount of Aflatoxin B1 in maize cobs was higher than that of corn peels. However, it was found that the pH of corn cobs had higher acidity than that of corn peels and mixed corn (Table 4). Corn cobs contain Lignocellulose which is a major contributor to an accumulation of acid during fermentation by *Aspergillus niger* (Gusakov et al., 1992) (Itelima et al., 2013) which is relating in decreasing of Aflatoxin B1(Gusakov et al., 1992; Itelima et al., 2013).

The present study demonstrates that adding 1.25%of acetic acid to corn samples effect on inhibition of growth of *A.flavus* while not affecting the reduction of AFB1. However, it was found that the experimental group with acetic acid had the fungal content and the percentage of increase in Aflatoxin B1 was lower than those without. The further study should be done with the higher concentration of acid but not exceeding 5% of dry weigh of corn because this concentration did not affect the

efficiency and health of cattle (Daniel et al., 2013). The temperature also should be controlled because it affecting on fungal growth and AFB1 production.

## CONCLUSION

In conclusion, the average amount of fungus detected in the corn as a mainly roughage used in dairy farms in Chiang Mai was at a higher level than the appropriate standard of feed quality control. This indicates the inappropriate hygiene, food quality and animal feed storage practices of dairy farmers. It was also found that AFB1 contamination in corn, especially dried corn, was higher than that the level of the regulation limit. This high level of contamination might directly affect the cows and humans consuming milk contaminated with aflatoxin. The storage practice of roughage in each farm such as with or without plastic sheet before placing roughage on cement or ground etc., has direct effects on fungal and aflatoxin contamination. Therefore, if the farmer manages to keep the roughage properly, it can reduce the contamination of fungus and production of aflatoxin. Organic acids (acetic acid) helps inhibit the growth of *Aspergillus flavus* in roughage (corn plant), and found to be safe for both humans and animals. Thus, the application of acetic acid may be an option in research and applications in dairy farms in the future.

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# Appendix

# Factors associated with mold and aflatoxin b1 contamination in by-product from corn processing factories as a dairy feed

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**ABSTRACT**

The objective of this study was to determine factors related to mold and AFB1 contamination in the by-products from corn processing factories as a dairy feed. By-products including 123 samples of dry corn covers (dry corn), and 108 samples of boiled corn cobs with husks (boiled corn) were collected from 3 areas including surface base position, surface high position, and the center of feed piles. Mold contamination was determined by spread plate technique. Analysis for AFB1 was conducted by a commercial enzyme-linked immunoabsorbent assay. The differences of AFB1 concentrations among feed pile's positions, mold contaminations and other variables were separately evaluated between the dried corns and the boiled corns using generalized mixed model. Results show that the AFB1 concentration of boiled corns (14.72+0.71) ng/Kg was significantly higher than dried corns (6.54+2.54 ng/Kg). Only 12.2% (n=13) of the dried corns had AFB1 concentrations more than the limitation values (20 ng/Kg feed). Results from statistical analysis showed that the center of feed piles had lower AFB1 concentrations than base and high surface positions. The presences of *Cladosporium* spp. had a lower risk of AFB1 contamination. These results suggest that type of samples, sources and the contamination of *Claddosporium* spp. are factors related to AFB1 contamination.

Keyword : Aflatoxin B1, Contamination, Dairy cow, Feed, Corn

## Introduction

Aflatoxin B1 is the most important toxic substance produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Iqbal, 2010) which contaminated in dairy feeds. Cows consumed feeds contaminated AFB1 result in losses of their weight gains, dietary intake reduction; liver function impairment, reduction of milk production and impairment of immune response (Masoero et al., 2007; Creppy, 2002) resulting in susceptible to vary diseases which contributed to economic loss for dairy farmers. Furthermore, AFM1 which is formed when dairy cows ingest feed contaminated with AFB1 (Creppy, 2002; Bhat et al., 2010) can contaminate in milk and dairy products which is the great public health concerns.

For dairy industry in Northern Thailand, the shortage of feed in terms of quality and quantity, especially during dry season, is one of the most serious problems face to the farmers. To overcome this problem in the last few years, by-products from agriculture and industries such as corn stove (after harvesting baby corn or sweet corn) intended to use as dairy feedstuff. There are 2 types of by-products including dried corn covers (dried corn), and boiled corn cobs with husks (boiled corn). Regarding to boiled corn, a ton of these by-products is packaged in a plastic bag from factory and transported to dairy farms as corn silages. Due to its characteristics and optimal temperature of mold growth, the by-products had high production rate of AFB1 after keeping for a week in small-holder farms condition (Mongkon et al., 2014). Recently, there are some reports demonstrate the problems of AFM1 contamination in either commercial pasteurized milk (Suriyasathaporn and Nakprasert, 2012) and school milk (Ruangwises and Ruangwises, 2009), which is might be related to the use of these by-products.

One of the best control strategies for AFM1 in milk is to monitor fungal and AFB1 contamination in dairy feeds (Suriyasathaporn and Nakprasert, 2012; Mongkon et al., 2014) and identify factors related to the contamination. Therefore, determination of mold and AF production and also identify risk factors of AF contamination in these by-products is useful to evaluate risk of AFM1 in milk.

## Materials and Methods

### Sample collection and preparation

A cross-sectional study was performed during May 2016 to May 2017 using all 82 smallholder dairy farm members of a dairy cooperative in Chiang Mai province, Thailand. One hundred and twenty three samples of dried corn covers (dried corn), and 108 samples of boiled corn cobs with husks (boiled corn) were collected from 3 areas including surface base position, surface high position, and the center of feed piles according to the guidelines of the Department of Livestock Development, Thailand (DLD, 2009). These samples were placed in plastic bags, transported to the laboratory in. Each sample was chopped into 0.5-1 cm size particles and packed at 1-kg in plastic bag and stored in a freezer until analysis.

#### *Assessment of total fungal count*

The total fungal viable count of each sample will be determined by using Yeast Extract Agar (YGC) (González Pereyra et al., 2008). Ten gram each of well-ground samples will be taken in 90 mL of sterilize sodium chloride (0.9%). The contents will be agitated for 30 min with a Worley mix. A serial dilution series ($10^{-1}$–$10^{-5}$) will be made using 1 in 9 mL. Fungal culture was aseptic prepared in Biological safety cabinet class II. One hundred µl of the suspension from the appropriate dilutions was

transferred to Petri plates containing YGC (Yeast extract glucose chloramphenicol agar) and thoroughly dispersed on the agar plates using sterilized bent Pasteur pipettes (Sander, 2012). All the Petri plates were incubated at 25°C for 3-5 days (ISO 13681: 1995). The numbers of colonies of different fungi were counted and the total fungal viable count per gram of sample was calculated as follows. The colonies of fungal in each plate were counted only on plates with colonies concentrations in the range of 10 to 150 colonies (Tournas et al., 2001). Each sample was done in duplicates. The results were displayed as forming unit (CFU) per gram dry weight of feed (Maturin and Peeler, 2001).

$$N = \frac{\sum c}{[(1 \times n_1) + (0.1 \times n_2) \times (d) \times 20 \times \%DM]}$$

N = Number of colonies per gram of dry weight, roughage

$\sum c$ = Sum of the number of fungi counted in each plate

n1 = Minimum number of plates to be counted for fungus ($10^{-3}$)

n2 = Maximum number of plates to be counted for fungus) ($10^{-4}$)

d = Amount of minimal dilution which was found fungus (1000 times)

%DM = Percentage of dry matter of roughage

***Fungal identification***

The physical appearances of each colony on the plates were examined (Macroscopic examination) or their texture, color, shape, size of both the front and the bottom of the colony, and the pigmentation on the culture medium. This information

can be used to assist in the diagnosis of fungal isolation. In addition, microscopic examination is performed to look at the fungus and spore formation.

***Water activity measurement***

Water activity is the amount of water that is beneficial to the growth and chemical reactions of microorganisms. Water activity can be defined as equilibrium relative humidity (ERH), where the food does not absorb or lose water (Karel et al., 1975). The water activity was determined using Decagon Devices. Each sample was tested in duplicates. A basic measuring technique utilizes a sealed dish or container with the sensor mounted above the test sample.

***Aflatoxin B1 analysis***

Each feed sample was carefully mixed and finely ground, and then extracted by putting 20 g of the ground sample into an Erlenmeyer flask to which 100 ml of 70% methyl alcohol was added. The flask was shaken at 300 rounds per min for 30 min, after which the mixture was filtered by Whatman no. 4 paper. The AFB1 level was measured by DOA-Aflatoxin ELISA test kit (Postharvest and Processing Research and Development Division, Thailand).The analysis was performed according to the kit instruction. Briefly, 50 µl of either AFB1 standards (0, 0.2, 0.5, 1, 2 ng/ml) or the diluted samples were added into the antibody coated wells; then 50 µl of Aflatoxin B1-Horseradish Peroxidase (AFB1-HRP) conjugate was added to each well, slightly shaken, and incubated at room temperature for 30 min. The contents of the well were dumped into the appropriate waste container and the plate was washed 3 to 5 times by 0.01M phosphate buffer saline with 0.5% Tween 20 (PBS-T). Tetramethylbenzidine

substrate (100 µl) was added to each well and incubated for 10 min at room temperature. The reaction was stopped by adding 100 µl of 0.3M phosphoric acid. The solutions were read at 450 nm using an automated MicroELISA spectrophotometer reader. The results of absorbance were expressed as percentage of maximal binding as follow:

% maximal binding = B/B0 x 100

B= mean absorbance of feed sample

B0= mean absorbance of AFB1 standard at 0 ng/ml

The standard curve was generated by plotting the concentration of AFB1 with % maximal binding in Log base 10 values.

***Statistical Analysis***

Data were described as frequencies for categorical variables. Feed samples were considered contaminated with AFB1 at concentrations higher than 5 µg/kg, the regulatory limit of the European Food Safety Authority (EFSA) (European Food Safety Authority, 2004). For univariable analysis, associations of feed characteristics with AFB1 contamination were separately determined using Fisher's exact chi-square test. The final model of factors associated with AFB1 contamination was created by a multiple logistic model using a backward selection method calculated by SAS (SAS Institute, 1997). All factors were first entered and subsequently removed from the model when their P-values indicated by the likelihood ratio tests were higher than 0.10.

## Results

**Fig.1**

Percentages isolation frequency of fungal genera in all corn samples are shown in Fig.1. The most common fungus was *Aspergillus* spp., *Cladosporium* spp. *Penicillium* spp. yeast and others. These results are in agreement with previous reports from Mudili et al. (2013) Tsedaley and Adugna (2016).

The DOA-Aflatoxin ELISA Test Kit is capable of detecting the lowest aflatoxin B1 level of 0.4 ppb and the percentage of recovery was between 82 and 100. The efficacy of DOA-Aflatoxin ELISA Test Kit does not differ from Thin-Layer Chromatography and High-performance liquid chromatography. The water activity, fungal content and Aflatoxin B concentration in the samples of wet and dried corn at different locations were shown in Table 1. It was found that the mean water activity of the dried and boiled corn samples were 0.59 ± 0.19 and 0.99 ± 0.00, respectively. Most of all samples (99.9-100%) were contaminated with fungal microorganism. The average fungal count was $167.11x10^6$ CFU/g. In the samples of boiled corn, the contamination of the fungus was less than dry, with an average of $110.739x10^6 \pm 45.26x10^6$ CFU/g and $215.093x10^6 \pm 75.55x10^6$ CFU/g, respectively. There were no differences ($P > 0.05$) of fungal count in the different collecting positions.

The concentration of AFB1 in the samples of dried corn was higher than boiled corn. The average concentrations were 14.72 ± 0.71 (2.23-50.2) and 6.54 ± 0.30 (0.42-10.67) ppb, respectively (Table 1). It was found that 83.6 percent of all samples (153/183 samples) were contaminated with AFB1 more than 5 ppb. Only a few dried corn samples were contaminated with AFB1 above 20 ppb (8.2%, 15/183 samples).

Based on the statistical analysis, it was found that the amount of AFB1 presenting in the samples of maize at different collecting positions was not significantly different. It was also found that the amount of fungi did not correlate with the concentration of AFB1 in both types of corn samples ($P > 0.05$).

AFB1 concentrations found in the dried and boiled corn samples in different dairy cooperatives are shown in Fig.2. The average AFB1 concentration found in corn samples from Coop-C was highest, followed by Coop-B and Coop-A. The average AFB1 concentrations in dried corns was 16.05 ± 1.14, 15.39 ± 0.92 and 14.74 ± 0.70 ppb and boiled corns was 4.09 ± 1.83, 8.45 ± 0.32, and 5.68 ± 0.30 ppb, respectively.

**Fig.2**

**Table 1**

Factors related with fungal count in corn samples are shown in Table 2. The presence of *Aspergillus* spp. and Yeast in samples significantly increased in fungal numbers. In addition, it was found that the source of samples associated with fungal count. Fungal counts from Coop-A were found more than that from other cooperatives ($P < 0.05$). Table 3 shows factors related to AFB1 concentration in corn samples. The results of the study showed that the type of corn, the presence of *Cladosporidium* spp. and the different locations by different dairy cooperatives were associated with AFB1 concentrations. The boiled corn samples and the presence of *Cladospordium* spp. were found to have lower AFB1 concentrations than dried corns and samples free from Cladosporium infection ($P<0.05$). Moreover, it was found that samples from Coop-B had higher concentrations of AFB1 than that other cooperatives significantly ($P<0.05$).

Table 4 shows the relationship between the storage factors that affected the AFB1 concentration of more than 10 ppb. It was found that placing corn residues on the ground was found to be more likely to be contaminated with AFB1 than placing corns on cement 13 times (OR=13.73, P <0.05) The boiled corn samples and placing corn samples on the floor with plastic sheet had a lower probability of AFB1 contamination than those of dried corn and corn samples which are placing on the floor without plastic sheet.

**Table 2**

**Table 3**

**Table 4**

**DISCUSSION**

The dry and boiled corns, by-products from corn processing factories are widely used in dairy farming in the northern part Thailand. Our study found that these corns were contaminated with *Aspergillus* spp., *Cladosporium* spp., *Mucor* spp., *Rhizopus* spp., *Fusarium* spp., *Penicillium* spp. and Yeast. The results were similar to previous studies which reported that *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. are the main fungal found in Maize (Rosa et al., 2008; Mudili, et al., 2013; Tsedaley and Adugna, 2016). This is consistent with the study of Charoenpornsook et al. (2006) who reported many species of these fungi can produce toxins that affect humans and animals. This study found the contamination of fungi in almost all corn samples. The average fungal count of all samples was $167.11x10^6$ CFU / g, 77.7% (174/224 samples) of total samples. Approximately 77% of samples had amount of fungus exceeds the

regulatory limit defined by Animal Feed Quality control Act B.E. 2559 which indicated that the limit of fungal count must not exceed $1x10^5$ CFU/ g. The samples which fungal count excesses the standard value are considered as deterioration. In addition, when referring to the GMP standard of 2008, 92% of samples had fungi higher than standard ($1x10^4$ CFU/g) which is indicated the quality of food and inappropriate storage practice of animal feed.

Dried corns had water activity value of 0.59, which was not suitable for fungal growth and toxin formation. However, in this study, fungal contamination was found to be more pronounced in dried corns than in boiled corn. This result different from the previous report form Alonso et al. (2011) who found that animal feed with water activity of 0.86-0.97 had higher tended to be contaminated with fungal and fungal producing aflatoxin such as *A.flavus* (Hedayati et al., 2007).

From the statistical analysis, it was found that the presence of *Aspergillus* spp. and yeast in the samples significantly correlated with the amount of fungus. This study in accordance with Keller et al. (2013) who reported the amount of fungal in corn samples were higher than the standard and the most common fungus was *Aspergillus* spp. In addition, samples from Coop-A had higher amount of fungus than that of other cooperatives ($P < 0.05$). This may be due to the different storage practices in different cooperatives such as food storage facilities or storage times which affect the growth of the fungus and the contamination of fungal toxins (Mongkon et al., 2017).

Based on the aflatoxin concentration, all samples had aflatoxin contamination of 11.41 ppb. The results consistent with Mongkon et al. (2017) who reported that AFB1 was detected in all corn samples from Chiang Mai area before and after

fermentation, the concentration ranged between 5.5 to 11.4 ppb, respectively. The concentration of aflatoxin in dried corn samples was higher than that of the boiled corns. The concentrations were 14.72 ± 0.71 and 6.54 ± 0.30 ppb, respectively. The average of both samples has passed the Thai and USFDA benchmarks (according to the Codex Maximum Level 2003, Aflatoxin not exceeding 20 ppb) but not passed FAO/WHO standards (AFB1 level not exceeding 5 PPB). However, only 8.2% of all samples especially in dried corn contaminated with AFB1 more than 20 ppb, this is according to Mongkon et al (2017).

Our study found that the amount of fungus and aflatoxin present in the samples at different locations was not significantly different. This is different from the previous study of Gonzalez Pereyra et al. (2007) which investigated the type and amount of fungus in fermented corn kept in silo. They found that the total fungal count on the top of silo was highest because this position has the greatest exposure to air environment. The different results may be due to samples collected in our study is placed in an open area which has more opportunity to expose with the air, humidity and other factors, hence, the environment is suitable for fungal growth rather than fermentation in silo. It was also found that the amount of fungi was not correlated with the concentration of AFB1 in both types of corn samples. Our study is in agreement with other previous studies (Carvalho et al., 2015; Alonso et al., 2013). Toxins from fungi are more durable than fungus and can be maintained in the animal feed throughout the production and storage process, while the fungus was easily destroyed in non-suitable environment (Carvalho et al., 2015; Alonso et al., 2013). Therefore, the detection of fungal toxins does not necessarily correspond to the presence of fungi in animal feed.

The AFB1 concentration in dried corn higher than boiled corn appeared related to the fungal count which found in higher number in dried corn could be explained by a longer storage period of dried corn when compared to wet corn, this is in agreement with Keller et al. (2013) who reported that aflatoxigenic fungi and AFB1 contamination was enhanced during storage. In addition, the dry matter of dried corn is greater than the boiled type. (89.01% for dry and 24.47% for boiled corn). When analyzing the concentration of AFB1, the dried samples were more concentrated in AFB1 than the boiled ones, hence, when cows consume the same amount of both types of corns, cows that eat a dry receive AFB1rather than boiled.

The presence of *Cladosporium* spp. was associated with a decrease in AFB1 concentration in corn samples significantly. This type of fungus is capable of inhibiting the production of AFB1 in the laboratory by interfered aflatoxin formation and transformed AFB1 to the different structure compounds (Cvetnic et al., 2007).

The AFB1 concentration of corn from Coop-B was higher than that of other cooperatives significantly. The management factors of each farm can affect both the amount and concentration of aflatoxin in the samples. Placing corn on the ground are associated with an increase in AFB1 contamination compared to those placed on concrete floors significantly ($P < 0.05$). This can be explained by an increasing exposure to aflatoxigenic fungi from the soil to the pile of corn (Hedayati et al., 2007). In addition, the placement of plastic sheet under the corn pile was significantly related to the reduction of AFB1 contamination ($P < 0.05$) because the plastic sheet can reduce the moisture and fungus from the ground contaminated to the pile of corn.

## Conclusion

In conclusion, the average amount of fungus detected in the corn as a mainly roughage used in dairy farms in Chiang Mai was at a higher level than the appropriate standard of feed quality control. This indicates the inappropriate hygiene, food quality and animal feed storage practices of dairy farmers. It was also found that AFB1 contamination in corn, especially dried corn, was higher than that the level of the regulation limit. This high level of contamination might directly affect the cows and humans consuming milk contaminated with aflatoxin. The storage practice of roughage in each farm such as with or without plastic sheet before placing roughage on cement or ground etc., has direct effects on fungal and aflatoxin contamination. Therefore, if the farmer manages to keep the roughage properly, it can reduce the contamination of fungus and production of aflatoxin.

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**Figure legends**

**Fig.1** Percentage of isolation frequency of fungal in corn samples

**Fig.2** Calibration curve of AFB1

**Fig.3** The chart shows the average concentration of AFB1 in the sample of dried and boiled corns from the different dairy cooperatives

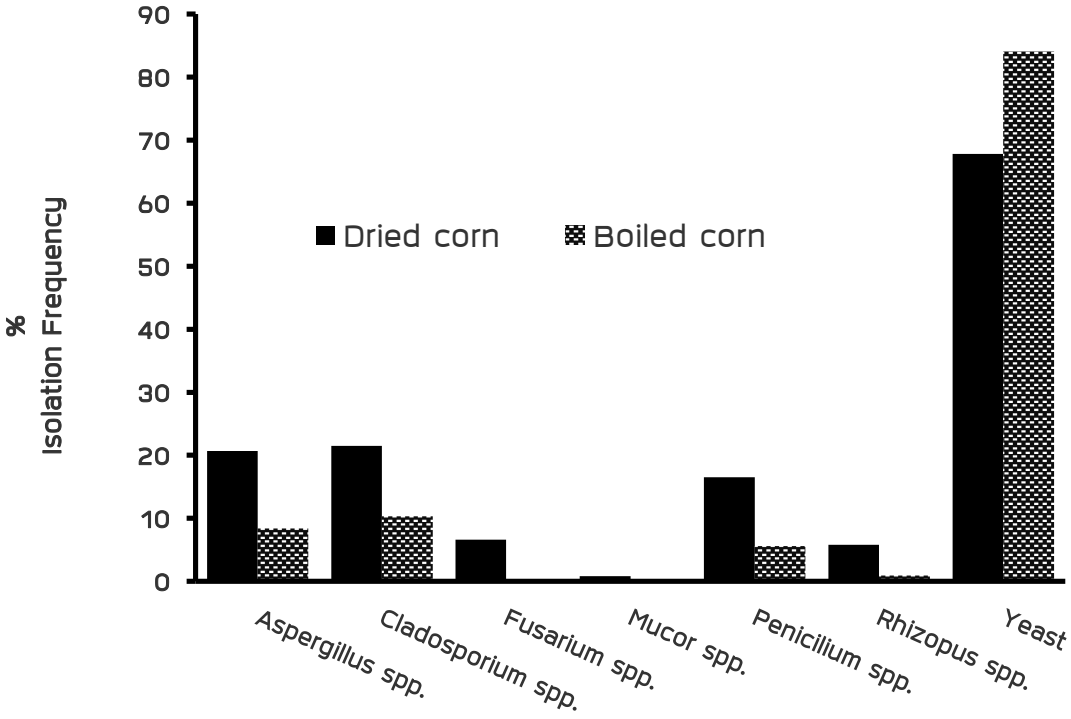
90
80
70
60
50
40
30
20
10
0
%
Isolation Frequency
Dried corn
Boiled corn
Aspergillus spp.
Cladosporium spp.
Fusarium spp.
Mucor spp.
Penicillium spp.
Rhizopus spp.
Yeast

**Fig.1**

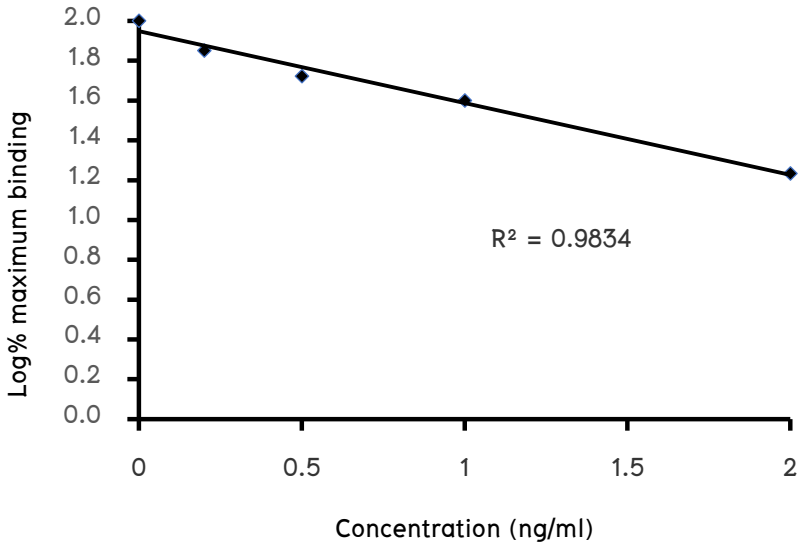
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Log% maximum binding
R² = 0.9834
0
0.5
1
1.5
2
Concentration (ng/ml)

**Fig.2**

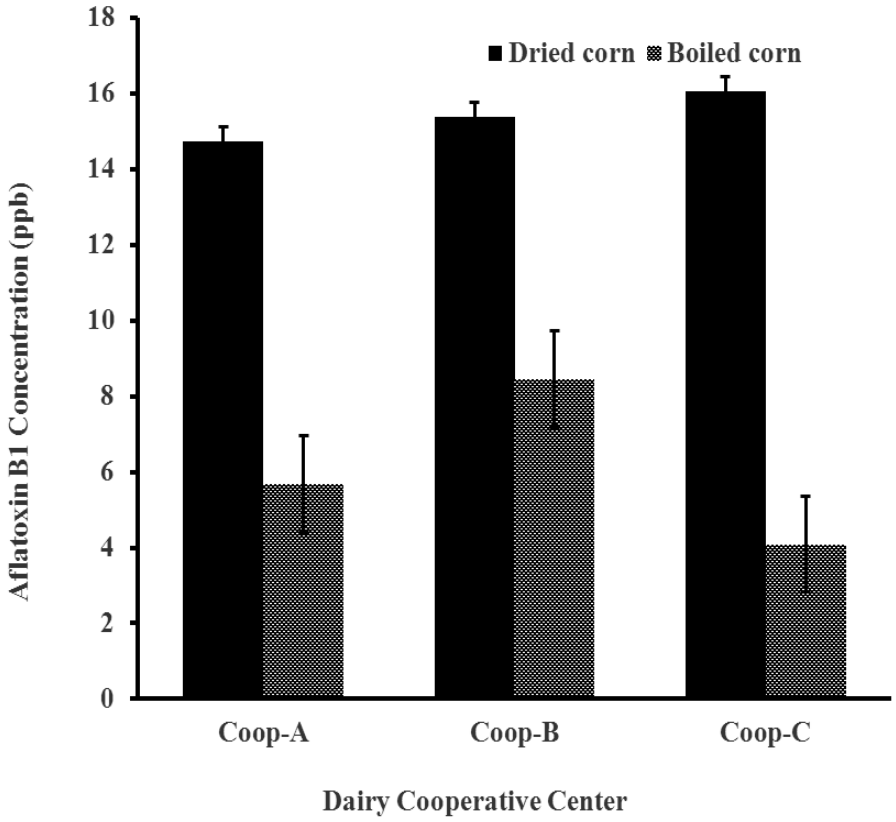
18
16
14
12
10
8
6
4
2
0
Aflatoxin B1 Concentration (ppb)
Dried corn
Boiled corn
Coop-A
Coop-B
Coop-C
Dairy Cooperative Center

**Fig.3**

**Table 1** Water activity, fungal count and the concentration of AFB1 in corn samples

| Sample | Water activity | Fungal count x 10) $^{6}$CFU/g) | | | AFB1 concentration (ppb) | | |
|---|---|---|---|---|---|---|---|
| | Mean±SD | Contamination frequency (%) | Mean±SE | Geometric mean | Contamination frequency (%) | Mean ±SE | Sample exceeding regulation limit (%) |
| Dried corn | 0.595± 0.19 | 99.984 | 215.093± 75.55 | 5.371 | 100.00 | 14.720± 0.71 | 12.20 |
| B | 0.568± 0.19 | 99.976 | 160.224± 73.73 | 5.077 | 100.00 | 14.808± 0.72 | 9.80 |
| S | 0.568± 0.16 | 100.00 | 163.357± 73.73 | 5.096 | 100.00 | 13.907± 0.72 | 12.20 |
| C | 0.584± 0.20 | 99.976 | 322.991± 73.74 | 5.778 | 100.00 | 15.356 ±0.72 | 14.60 |
| Boiled corn | 0.996± 0.00 | 99.954 | 110.739± 45.26 | 4.707 | 99.99 | 6.538± 0.30 | 0.00 |
| B | 0.995± 0.00 | 99.950 | 113.489 ±45.70 | 4.732 | 99.97 | 6.166± 0.30 | 0.00 |
| S | 0.996± 0.00 | 99.960 | 130.711± 48.01 | 4.873 | 100.00 | 6.204± 0.30 | 0.00 |
| C | 0.996± 0.00 | 99.972 | 75.295 ±46.16 | 4.321 | 100.00 | 6.011± 0.30 | 0.00 |

Sample position: B=Base, S=Surface, C=Center

SE: Standard error SD: Standard deviation

Regulation limit (%) not exceed 20 ppb (Codex Maximum level, 2003)

**Table 2** Stepwise linear regression model of factors and quantity of fungus in corn sample

| Variable | Level | Coefficient | Standard error | T-value | P-value | LS Mean | Geometric Mean |
|---|---|---|---|---|---|---|---|
| *Aspergillus* spp. | With | 1.77 | 0.64 | 2.77 | 0.0062 | 1.33 | 3.78 |
| | Without | 0.00 | | | | -0.30 | 0.74 |
| Yeast | With | 2.78 | 0.58 | 4.80 | 0.0000 | 1.88 | 6.55 |
| | Without | 0.00 | | | | -0.85 | 0.43 |
| Dairy cooperative | Coop-A | -2.32 | 0.79 | -2.93 | 0.0038 | -0.16 | 0.85 |
| | Coop-B | -2.66 | 0.48 | -5.50 | 0.0000 | -0.59 | 0.55 |
| | Coop-C | 0.00 | | | | 2.29 | 9.87 |

*Transformed fungal numbers to logarithm values before analysis

**Table 3** Stepwise linear regression model of factors related to AFB1 concentrations in corn samples

| Variable | Level | Coefficient | Standard error | T-value | P-value | LS Mean | Geometric Mean |
|---|---|---|---|---|---|---|---|
| *Aspergillus* spp. | With | -0.22 | 0.09 | -2.29 | 0.0235 | 1.85 | 6.36 |
| | Without | 0.00 | | | | 2.07 | 7.92 |
| Corn type | Boiled corn | -1.04 | 0.07 | -13.66 | 0.0000 | 1.44 | 4.22 |
| | Dried corn | 0.00 | | | | 2.48 | 11.94 |
| Dairy cooperative | Coop-A | -0.24 | 0.08 | -3.08 | 0.0024 | 1.90 | 6.69 |
| | Coop-C | -0.25 | 0.12 | -2.03 | 0.0438 | 1.88 | 6.55 |
| | Coop-B | 0.00 | | | | 2.13 | 8.41 |

*Transformed fungal numbers to logarithm values before analysis

**Table 4** Stepwise logistic regression model between storage practices for AFB1 contamination

| Variable | Level | Coefficient | Standard error | Odd ratio | T-value | P-value |
|---|---|---|---|---|---|---|
| Yeast | With | 1.90 | 0.95 | 6.68 | 3.32 | 0.0009 |
| | Without | 0.00 | | | | |
| Floor | Ground | 2.62 | 0.89 | 13.73 | 2.94 | 0.0000 |
| | Cement | 0.00 | | | | |
| Corn type | Boiled corn | -4.09 | 0.91 | 0.02 | -4.48 | 0.0000 |
| | Dried corn | 0.00 | | | | |
| Ground sheet | With | -2.34 | 0.88 | 0.10 | -2.64 | 0.0083 |
| | Without | 0.00 | | | | |

*Yeast is references

**OUTPUT**

**International conference:**

1. Arpussara Saedan, Jaturaporn Ounnunkad, Tuangporn Butta, Wanpa Techasul, Witaya suriyasathaporn, Vena Jupia, **Wasana Chaisri*** Factors Associated with Mold and Aflatoxin Contamination in by-product from corn-factory using for dairy cow feed. International Conference of Mycotoxicology "Driving Mycotoxin Research Toward Global Food Security", 2018 Febuary 14-15, 2018. Bangkok, Thailand.
2. Tuangporn Butta, Wanpa Techasuk, Jaturaporn Ounnunkad, Arpussara Saedan, Witaya Suriyasathaporn, Vena Jupia, **Wasana Chaisri***. Fungal and Aflatoxin B1 Contamination in dairy roughage (Factory-corn) after supplement with acetic acid. International Conference of Mycotoxicology "Driving Mycotoxin Research Toward Global Food Security", 2018 Febuary 14-15, 2018. Bangkok, Thailand.

**International Conference of Mycotoxicology, 2018 *( ICM 2018 )***
**" Driving Mycotoxin Research Toward Global Food Security "**

**YS04**

# Factor Associated with Mold and Aflatoxin Contamination in by-Product from Corn-Factory Using for Dairy Cow Feed

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The aim of this study was to determine fungal and aflatoxin $B_1$ ($AFB_1$) contamination in by-product from corn-factory using as a dairy cow feed in Chiang Mai. Two hundred and thirthy one samples of corn-factory (123 samples of dry corn and 108 samples of wet corn) were collected from 3 dairy cooperative include Mae On, Mea Wang and Chiang-Mai. The mean of total fungal counts was $167.11x10^6 \pm 44.32x10^6$ CFU/g. Seven fungi genera isolated from corn-factory were *Aspergillus* spp., *Cladosporium* spp., *Mucor* spp., *Rhizopus* spp., *Fusarium* spp., *Penicillium* spp. and Yeast. $AFB_1$ contamination was determined by a commercial enzyme-linked immunosorbent assay (ELISA). Results show that $AFB_1$ concentration ranged from 0.42 to 50.2 ppb. Only 8.2% (n=15) of all samples had $AFB_1$ contamination more than the limitation value (20 ppb). $AFB_1$ level in wet corn ($5.63 \pm 0.30$ ppb) was lower than dry corn $14.72 \pm 0.71$ ppb ($P<0.05$). Total fungal count did not correlate the concentration of $AFB_1$ in both dry and wet corn samples ($P>0.05$). The wet corn and feed samples contaminated with *Cladosporium* spp. had lower of $AFB_1$ concentration than the dry corn and sample contaminated with other fungi ($P<0.05$). These results suggest that type of samples, sources and the contamination of *Cladosporium* spp. are factors related to $AFB_1$ contamination.

***Keywords:*** *Aflatoxin $B_1$; Contamination; Dairy cow; Feed; Corn*

**P07**

# Fungal and Aflatoxin $B_1$ Contamination in Dairy Roughage (Factory-Corn) after Supplement with Acetic Acid

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The objectives of this study were to evaluate the efficacy of acetic acid on reducing the contamination of *Aspergillus flavus* and $AFB_1$ in factory corns as dairy roughage, and identify the factors associated with these contaminations.Factory corn samples were separated into 3 groups: corn husk, corn cob and mixed. Each group of corn samples was autoclaved, inoculated with *A. flavus* suspension and added with 1.25%of acetic acid. Corn samples with or without acetic acid were kept in a different condition (exposed, non-exposed oxygen and light) in room temperature.Fungal count, water activity, pH and $AFB_1$ concentration were measured after adding acetic acid at 12 h, day 7, 14 and 21. The results demonstrated that acetic acid reduced the contamination of *A.flavus*, and exhibited much reduced percentage of increasing $AFB_1$ contamination when compared to that of without.The $AFB_1$ concentration in sample exposed with oxygen higher than samples without oxygen ($p$=0.050). Corn cob had significant lower concentration of $AFB_1$ than corn husk ($p$=0.0002) and mixed corn ($p$=0.0001). It can be concluded that acetic acid had effectiveness in inhibiting fungal growth, and decreasing an accumulation of $AFB_1$. Moreover, storing feed in non-oxygen condition could be useful for reducing $AFB_1$ contamination.

***Keywords:*** *Aspergillus flavus; $AFB_1$ (Aflatoxin $B_1$);acetic acid; roughage*