Aspergillus spp. และสารพิษจากเชื้อราที่เกี่ยวข้องกับอาหารโคนม: อะฟลาทอกซินและกลิโอทอกซิน
(*Aspergillus* spp. and mycotoxins associated with dairy cattle feeds: aflatoxins and gliotoxin)

โดย

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กลุ่มพิษวิทยาและชีวเคมี
สถาบันสุขภาพสัตว์แห่งชาติ

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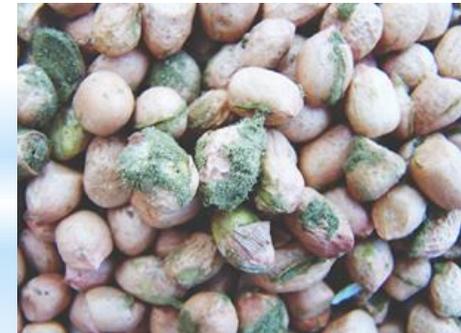
- ❖ **Phurida Sripipattanakul**
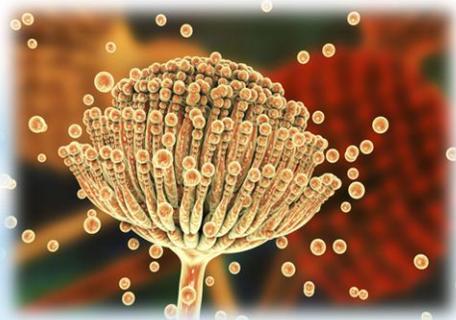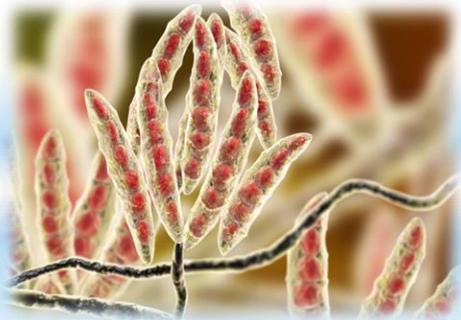
- ❖ **Onin Dejyong**

Introductions

Mycotoxins

❖ **natural compounds produced as secondary metabolites**

❖ **filamentous fungi (*Aspergillus, Penicillium*, and *Fusarium*)**

❖ **contaminate around 25% of global food crops**

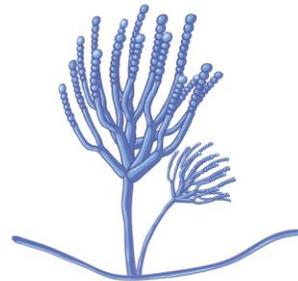❖ **actual contamination rate can range from 60.0% to 80.0%**

Mycotoxins (*cont.*)

❖ **Toxin-producing fungi can be divided into two categories:**

➢ **Field fungi (e.g., *Fusarium* spp.)**

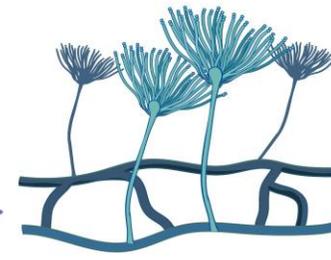➢ **Storage fungi (e.g., *Aspergillus* spp., *Penicillium* spp.)**

The different types of Mold

Bread mould   Penicillium   Aspergillus

Mycotoxins (*cont.*)

❖ **Major mycotoxins**

➢ **Aflatoxins (*Aspergillus flavus, A. parasiticus*)**

➢ **Fumonisins (*Fusarium verticillioides, F. proliferatum*)**

➢ **Ochratoxins (*Aspergillus ochraceus, A. carbonarius, Penicillium verrucosum*)**

➢ **Zearalenone (*Fusarium graminearum*)**

➢ **Trichothecenes (*Fusarium graminearum, F. sporotrichioides, F. poae*)**

➢ **emerging mycotoxins (beauvericin and enniatins) *F. acuminatum, F. avenaceum***

Aflatoxin (AFs)

❖ **Group of secondary metabolites produced mainly by fungal species such as**

       - *Aspergillus flavus*

       - *A. parasiticus*

       - *A. nomius (rarely)*

❖ **Under humid conditions, these fungi are produced in**

       - maize, cottonseed, peanuts, tree nuts (almonds, pistachios, walnuts, etc.)

       - livestock feed

       - medicinal herbs

Aflatoxin (*cont.*)

❖ **Aflatoxin contamination**

    ✓ **can occur at any stage of food production**

        ✓ **from pre-harvest to storage stages of food chain**

❖ **Aflatoxin accumulation is dependent on**

    ➢ **Environmental such as:**

        ✓ **moisture**

        ✓ **temperature**

        ✓ **plant density**

    ➢ **Poor harvest practices**

    ➢ **Improper grain storage**

Aflatoxin (*cont.*)

❖ **The removal of aflatoxins is very difficult due to their**

  ✓ **stability and thermal resistance**

   - **melting point >250˚C**

   - **stable at a pH 3-10**

  ✓ **resistant to food processing**

❖ **Thus remain unchanged throughout the food chain**

Classification of Aflatoxins

❖ **There are more than 20 known aflatoxins, but the four main ones are**

✓ **Aflatoxin B_1 (AFB$_1$)**

✓ **Aflatoxin B_2 (AFB$_2$)**

✓ **Aflatoxin G_1 (AFG$_1$)**

✓ **Aflatoxin G_2 (AFG$_2$)**

AFs B series are produced by
✓ *A. flavus*, *A. parasitcus* and *A. nomius*

AFs G series are produced by
✓ *A. parasitcus* and *A. nomius*

❖ **Named based on their**

✓ **blue (B) or green (G) fluorescence under UV-light**

✓ **Relative mobility, 1 and 2 (higher and lower respectively) by TLC**

Classification of Aflatoxins….

❖ **Other members of the aflatoxin family**

    ❑ **Aflatoxin M_1 (AFM_1) and Aflatoxin M_2 (AFM_2)**
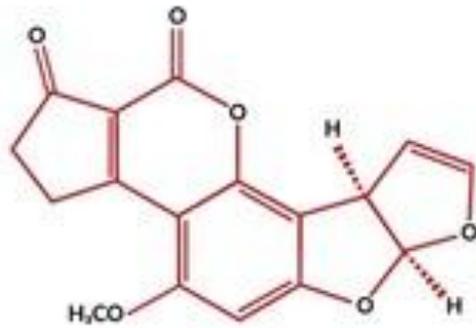
        ✓ **are oxidative forms of Aflatoxin B_1 (AFB_1)**

        ✓ **modified in digestive tract of animals**

        ✓ **isolated from milk, urine and feces**

❖ **The level of toxicity associated aflatoxin varies the types present**
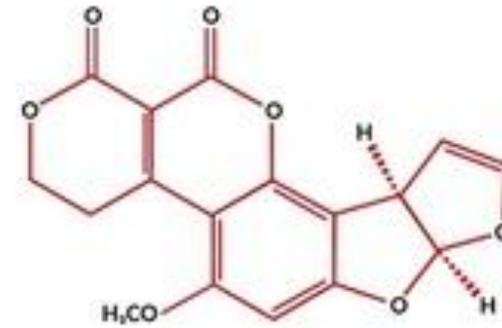
        ✓ **$AFB_1 > AFG_1 > AFB_2 > AFG_2$**
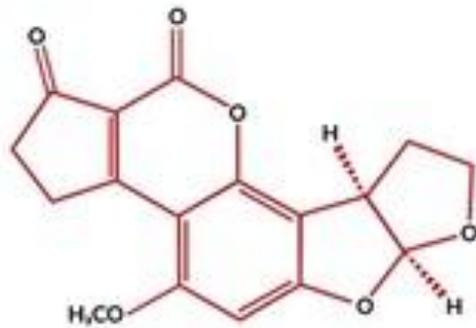
Chemical structure of aflatoxins
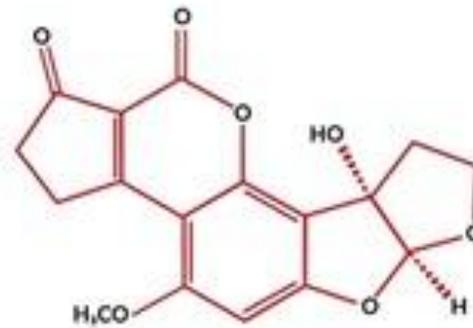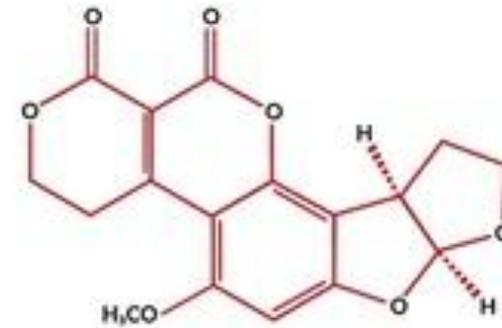
Aflatoxin B₁   Aflatoxin M₁   Aflatoxin G₁

Aflatoxin B₂   Aflatoxin M₂   Aflatoxin G₂

Aflatoxicosis in Animals

The toxic effect can include

- ❖ **Mutagenesis**

- ❖ **Carcinogenesis**

- ❖ **Teratogenesis**

- ❖ **Immunosuppression**

- ❖ **Hepatotoxicity (Liver damage with reduced protein synthesis)**

- ❖ **feed inappetence, poor growth rate, unthriftiness, depression, hemorrhage, icterus, and death**

Health Effects in Dairy Cattle

- ❖ **Reduced feed intake**

- ❖ **Decreased milk production**

- ❖ **Immune suppression and increased disease susceptibility**

- ❖ **Liver damage and reproductive disorders.**

- ❖ **Oral LD_{50} values for AFB_1 in young calves = 0.5-1 mg/kg**

- ❖ **Oral LD_{50} values of AFB_1 estimates range from 0.3-17.9 mg/kg for many animals**

Biotransformed in liver

AFB_1

AFB_1 ingested

AFM_1

AFM_1 excreted in milk
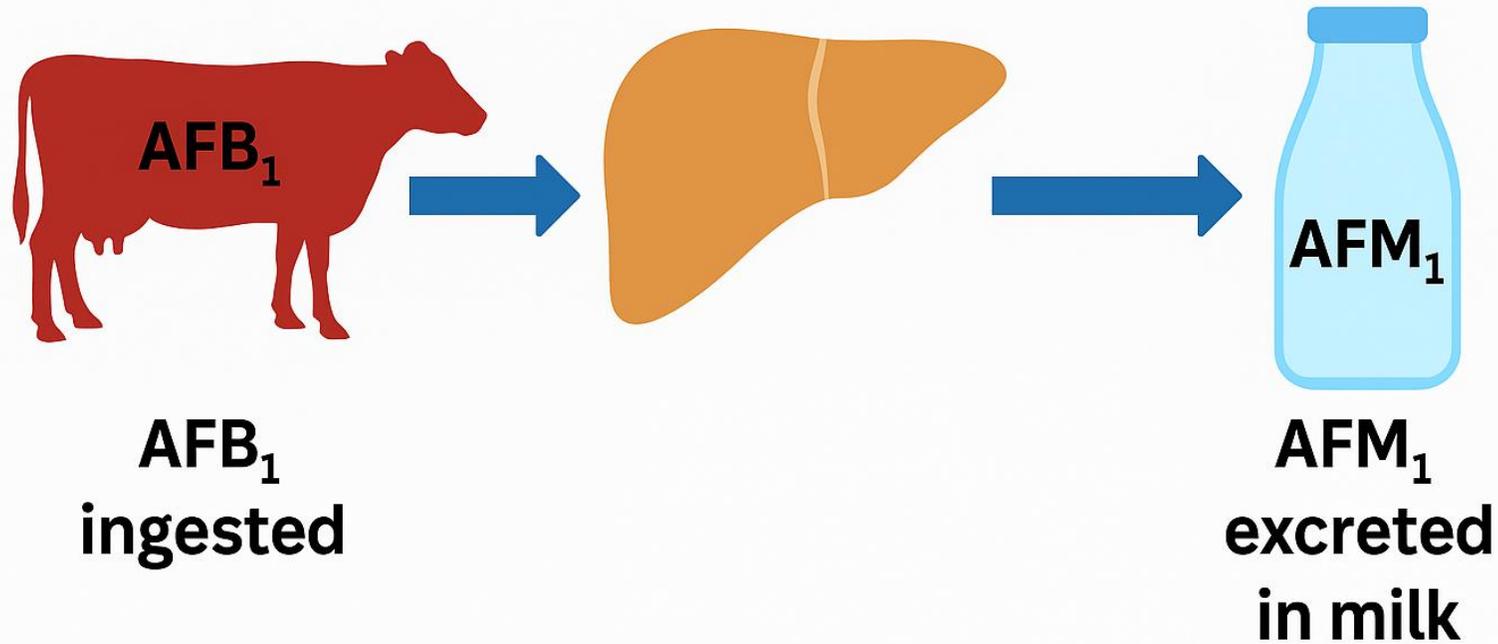
IARC has classified AFB_1 and AFM_1 as human carcinogens (IARC Group 1).

Gliotoxin (GT)

❖ **secondary fungal metabolite**

❖ **Gliotoxin-Producing Fungi (*Aspergillus fumigatus*, *A. flavus*, *Eurotium chevalieri*, *Trichoderma virens*)**

❖ **Contaminate animal feed; complete feed (soybean meal, ground corn, and corn cobs, etc.)**

❖ **Exhibits immunosuppressive, cytotoxic, and genotoxic effects.**

Immunosuppressive Effects

❖ **Inhibits NF-κB activation → reduced cytokine production**

❖ **Impairs lymphocyte activation and defense mechanisms**

Immunosuppressive Effects of Gliotoxin
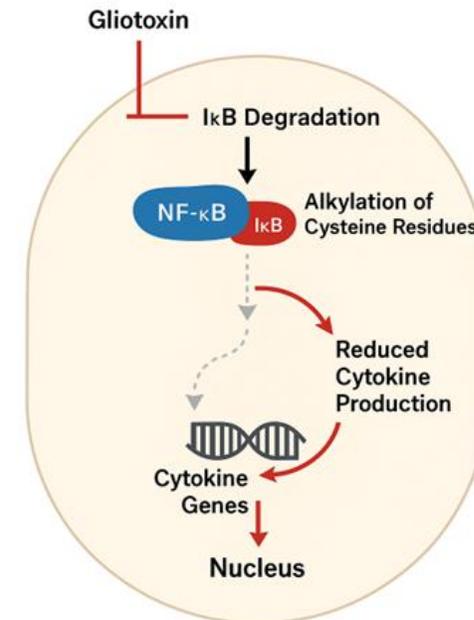
Cytotoxicity and Oxidative Stress

❖ **Produces reactive oxygen species (ROS)**

❖ **Induces apoptosis via mitochondrial damage**

Effects in Dairy Cattle

❖ **Reduced feed intake and milk yield**

❖ **Increased risk of mastitis due to immunosuppression**

❖ **Liver damage; possible mycotoxin residues in milk**

❖ **General Signs: Depression, weakness, rough hair coat, poor growth in young calves, dehydration and diarrhea (if co-exposed with enterotoxins)**

Oral LD$_{50}$ values for GT in mice = 67 mg/kg

Problem Statement and Rationale

❖ **Global Concern: Mycotoxin contamination poses serious risks to animal health and causes economic losses worldwide.**

❖ **Research Gap in Thailand: Most studies focus only on aflatoxins (AFs); few investigate co-contamination with gliotoxin (GT).**

❖ **Importance of Dual Detection: Animal feeds are often contaminated with multiple mycotoxins → increased health risks.**

❖ **Need for Improved Methods: LC/MS/MS requires validation for reliable detection of AFs and GT.**

❖ **Study Significance: Survey AFs and GT in dairy feeds, identify *Aspergillus* spp., and develop validated detection method.**

Objectives

❖ **To investigates the contamination of aflatoxins (AFB1, AFB2, AFG1, AFG2) and gliotoxin (GT) produced by fungi in the genus *Aspergillus* spp. in dairy cattle feed samples collected from farms in the Bangkok metropolitan area.**

❖ **To examines the molecular characteristics of fungi in the genus *Aspergillus* and investigates the genetic relationships among the isolates in correlation with the levels of aflatoxins (AFs) and gliotoxin (GT) detected.**

❖ **To develop and validate a method for the detection and quantification of aflatoxins (AFs) and gliotoxin (GT) in dairy feed samples using LC/MS/MS technique.**

Materials and Methods

Part I: Fungal Isolation & Molecular characteristics of fungi
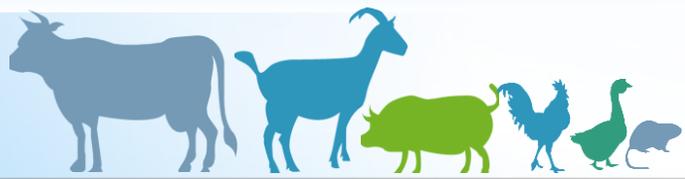
Sample collection

→ **10 complete feeds**

→ **10 corn husks**

→ **20 Others**

Fungal isolation
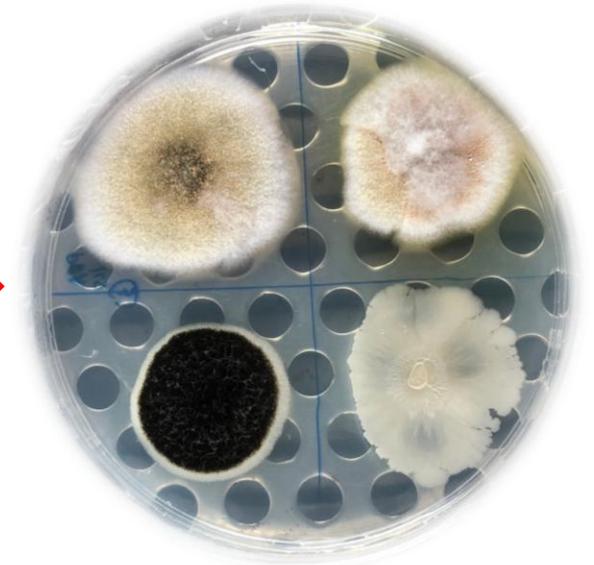
10 complete feeds

10 Corn husks

20 Others

Cultured in PDA and
incubated at 37°C for 5 days

Pure culture in PDA and
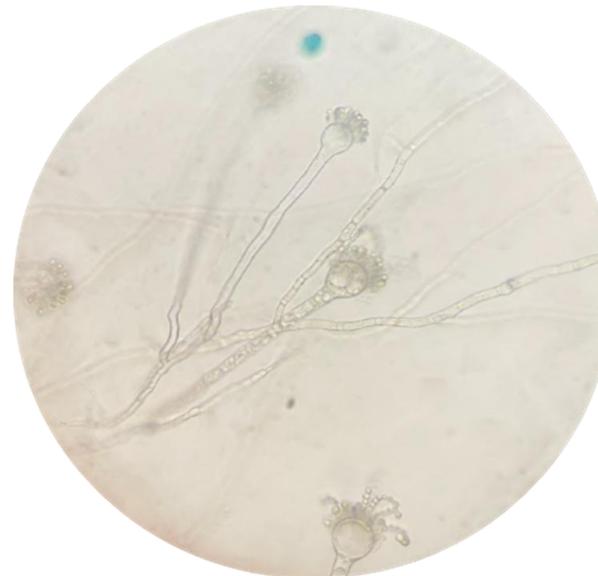incubated at 37°C for 5 days
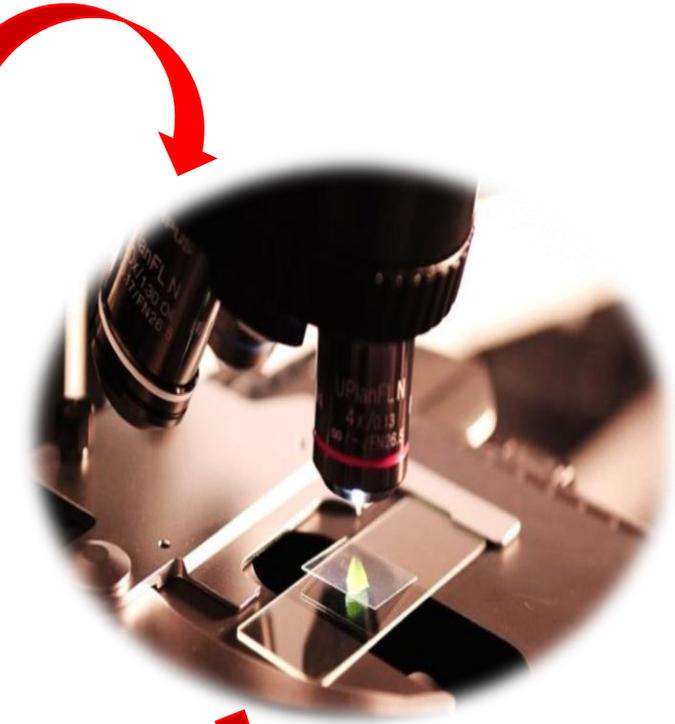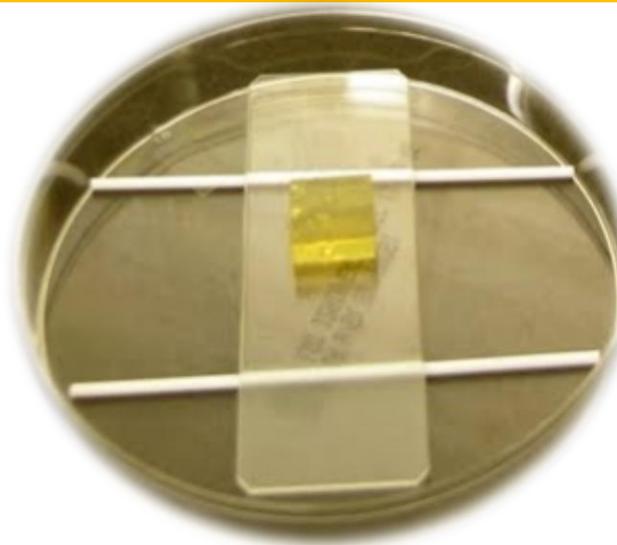
24

Morphological Identification

Transferred growing fungi from samples

↓

Cultivated fungi PDA medium

↓

Incubated for 5 days at 37 °C

↓

Checked the fungal culture using morphological data

↓

Observed morphology under compound microscope

DNA extraction and PCR amplification

+ 1 - 2 µl Lysate
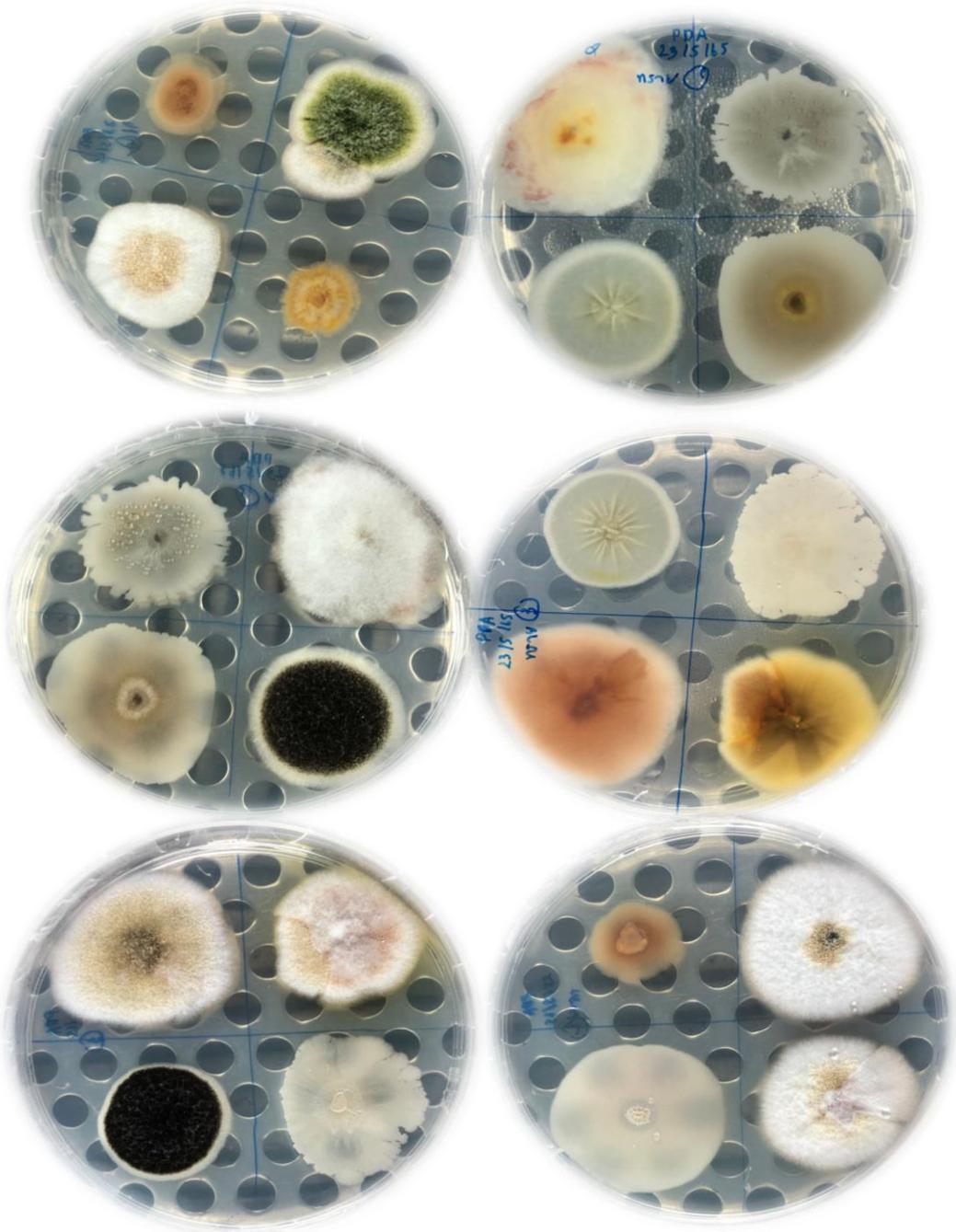
+ 100 µl Lysis Buffer
+ 2 µl Enzyme Mix
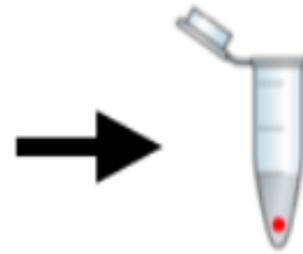
1. 65°C, 20 min
2. 95°C, 5 min
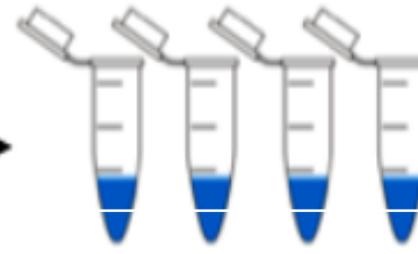
EUDirect 2X PCR Master Mix

The amplification:
95°C……..10 min
45 cycles of
95°C…….15 sec
53°C…….15 sec
72°C…….30 sec

Primers for Detection: 18S rRNA, ITS1/ITS4, and β-tubulin

Sequencing

SANGER SEQUENCING

Electrophoresis

Chain termination method of DNA sequencing
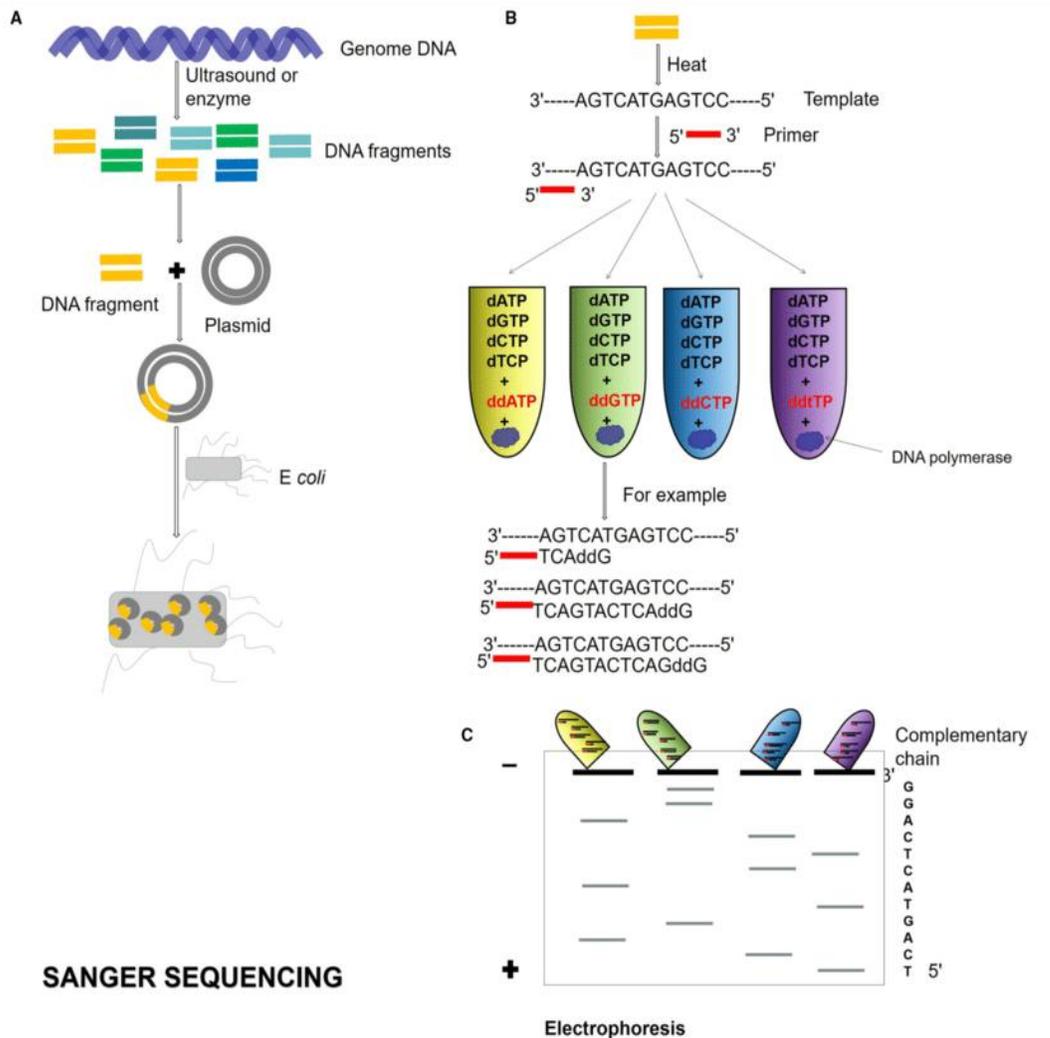
➢ **It involves following components:**

- Primers (18sRNA, ITS1/ITS4 and β-tubulin)
- DNA template
- DNA polymerase
- dNTPs (A, T,G, C)
- ddNTPs

➢ **4 steps:**

- Denaturation
- Primer attachment and extension of bases
- Termination
- Poly acrylamide gel electrophoresis

Phylogenetic analyses

Selection of organisms or a gene family

↓

Choosing appropriate molecular markers

↓

Amplification, sequencing, assembly

↓

Alignment

↓

Evolutionary model

↓

Phylogenetic analysis

↓

Tree construction

↓

Evaluation of phylogenetic tree
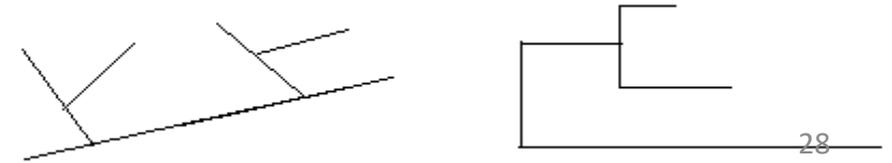
Part II: Development and Method validation

LC/MS/MS

Method validation by LC/MS/MS

❖ **Linearity and range**

❖ **Accuracy & precision**

❖ **Limits of detection (LOD)**

❖ **Limits of quantification (LOQ)**

❖ **matrix effect** ➡ **matrix-matched calibration curve**

The validation criteria are based on the guidelines by the European Union, specifically SANTE 2016 and SANTE 2021

Results of Part I
Fungal isolation and molecular characteristics of fungi
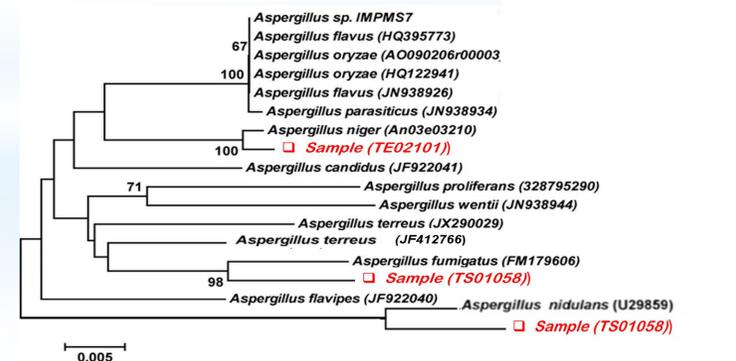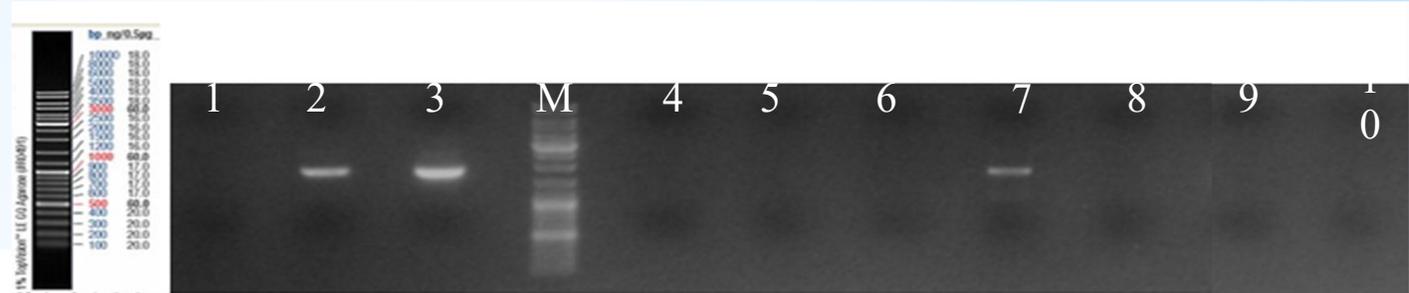
Isolation of different fungi from complete feeds and corn husks (n = 40)

Samples	Fungal species		
	Aspergillus spp.	*Monascus* spp.	*Rhizopus* spp.
complete feeds	9/10	5/10	4/10
corn husks	10/10	3/10	5/10

20 Others; Non-fungal species

Isolation of *Aspergillus* spp. from complete feeds and corn husks using 18S rRNA primer
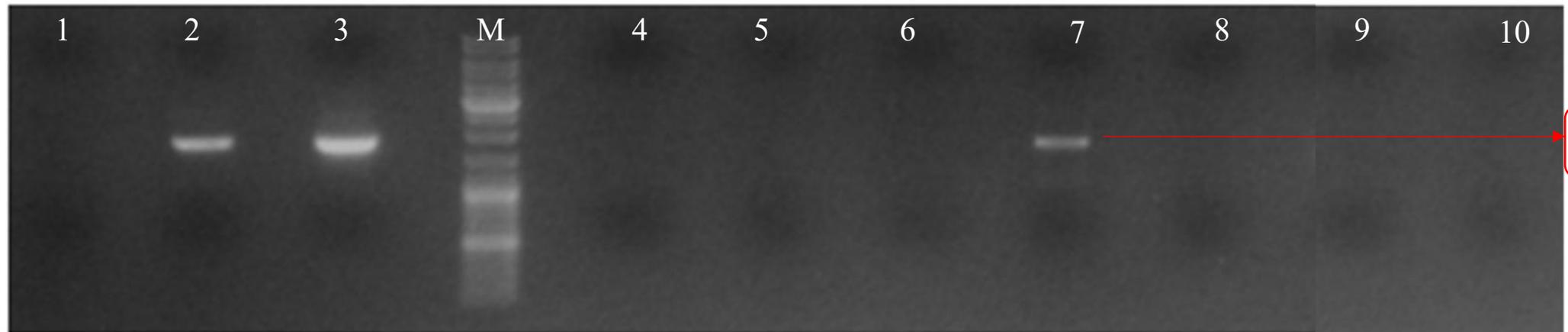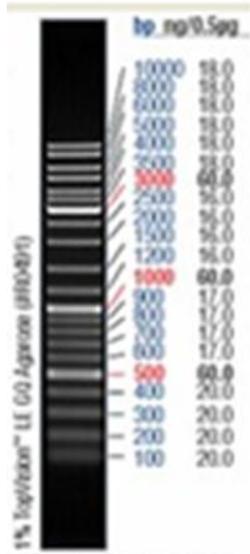
1800 bp

Isolation of *Aspergillus* spp. from complete feeds and corn husks

Samples	Fungal Species		
	Aspergillus fumigatus	*Aspergillus nidulans*	*Aspergillus niger*
complete feeds	7/9	3/9	6/9
corn husks	6/10	4/10	3/10
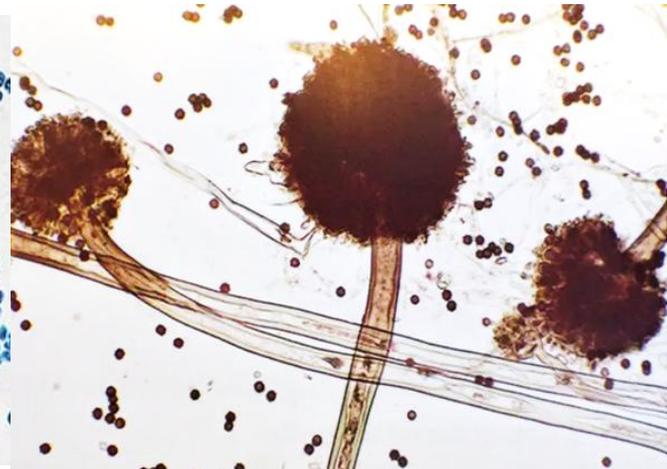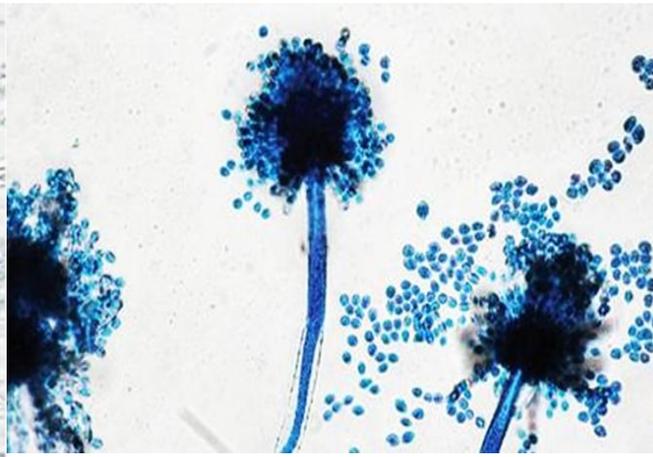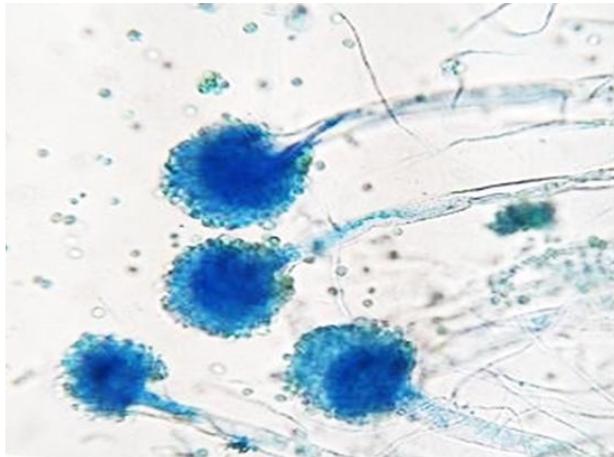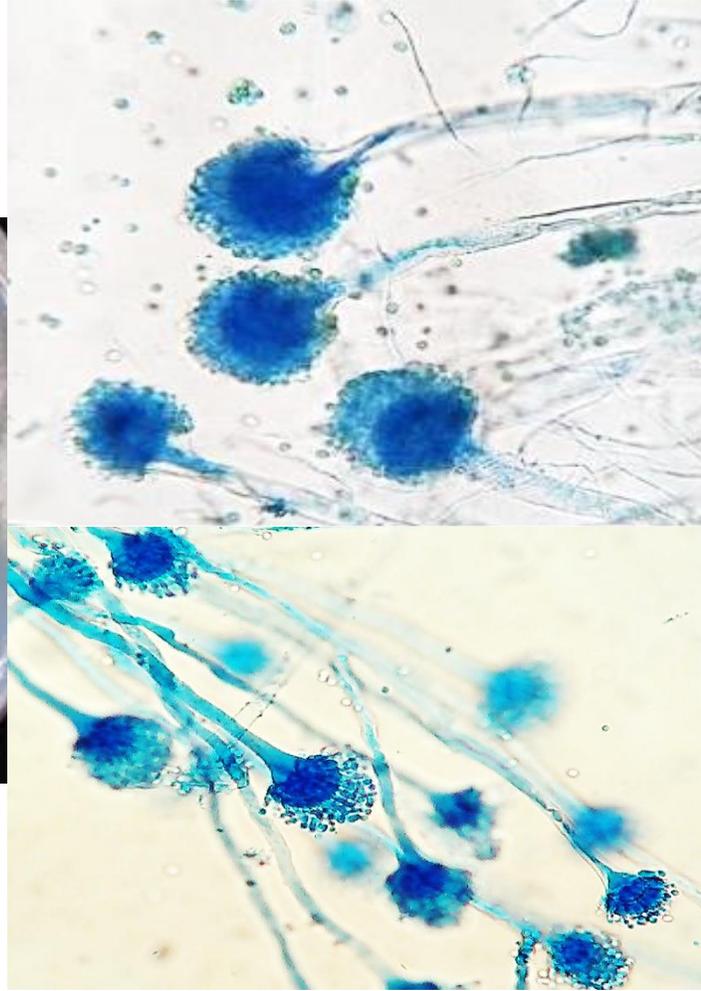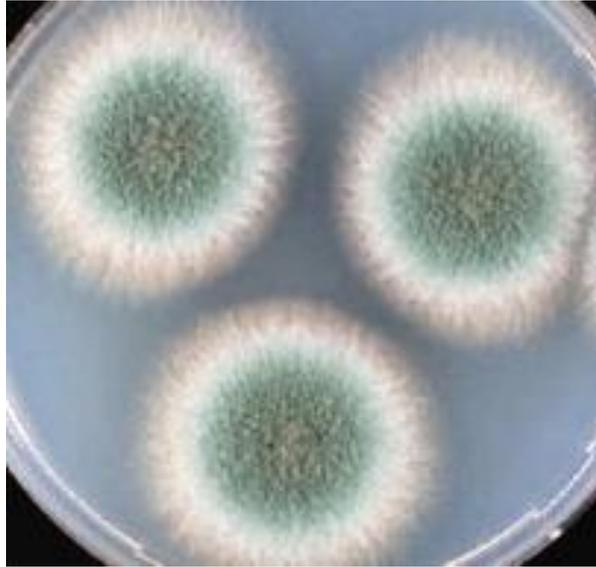
Aspergillus fumigatus
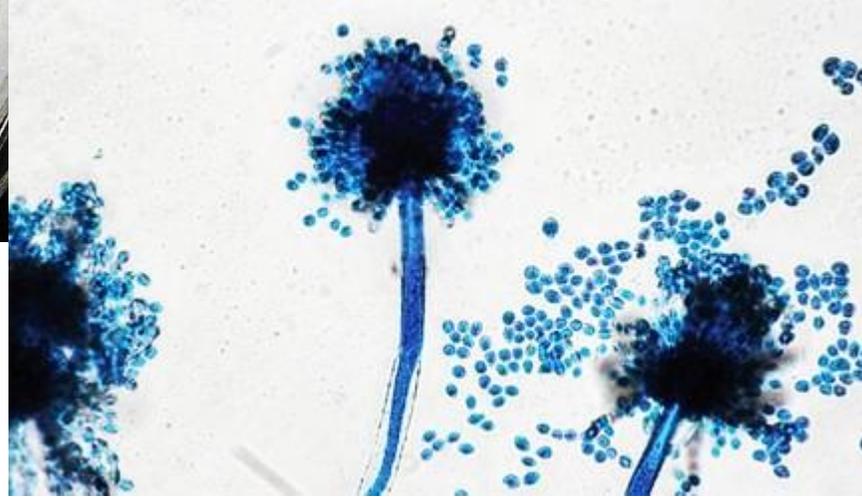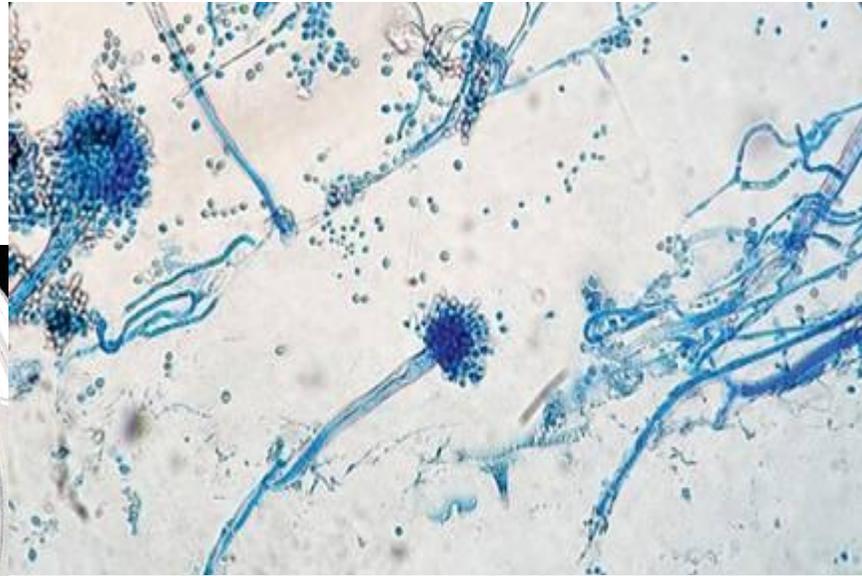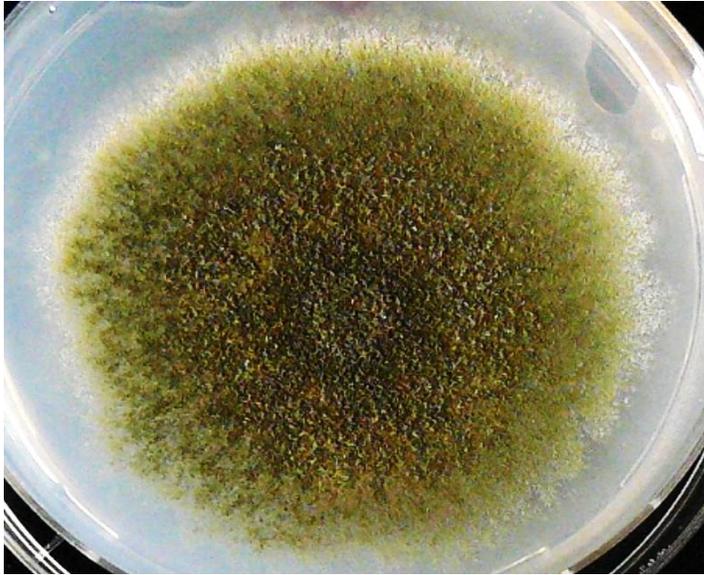
Aspergillus nidulans
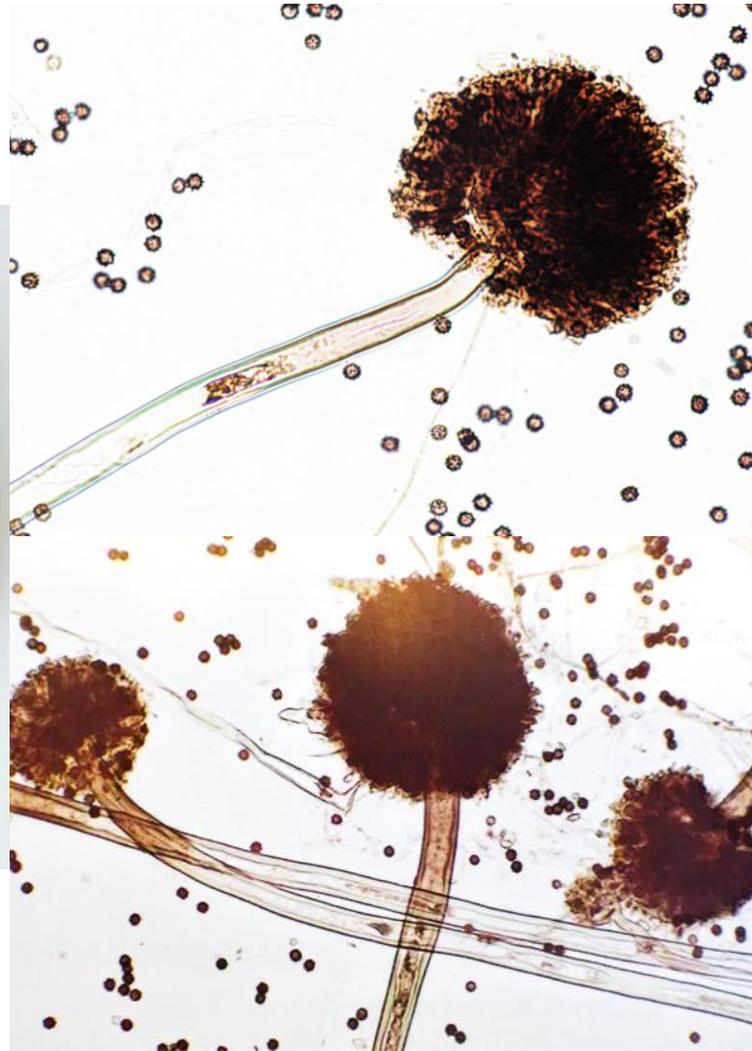
Aspergillus niger

Phylogenetic tree of *Aspergillus* spp.
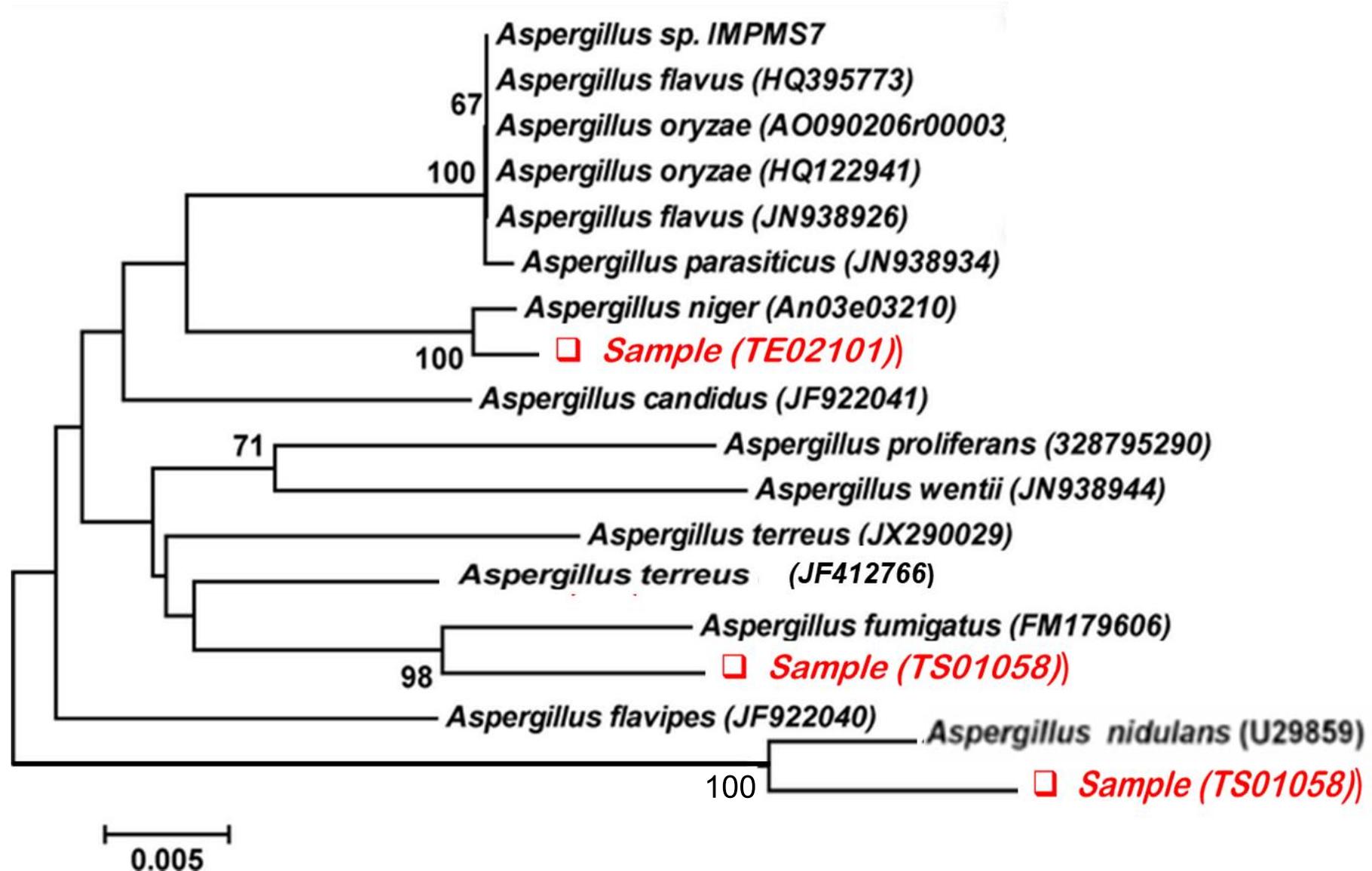
Agarose gel electrophoresis from *Aspergillus fumigatus* of gliotoxin gene (*gliP*) producing fungi

1320 bp
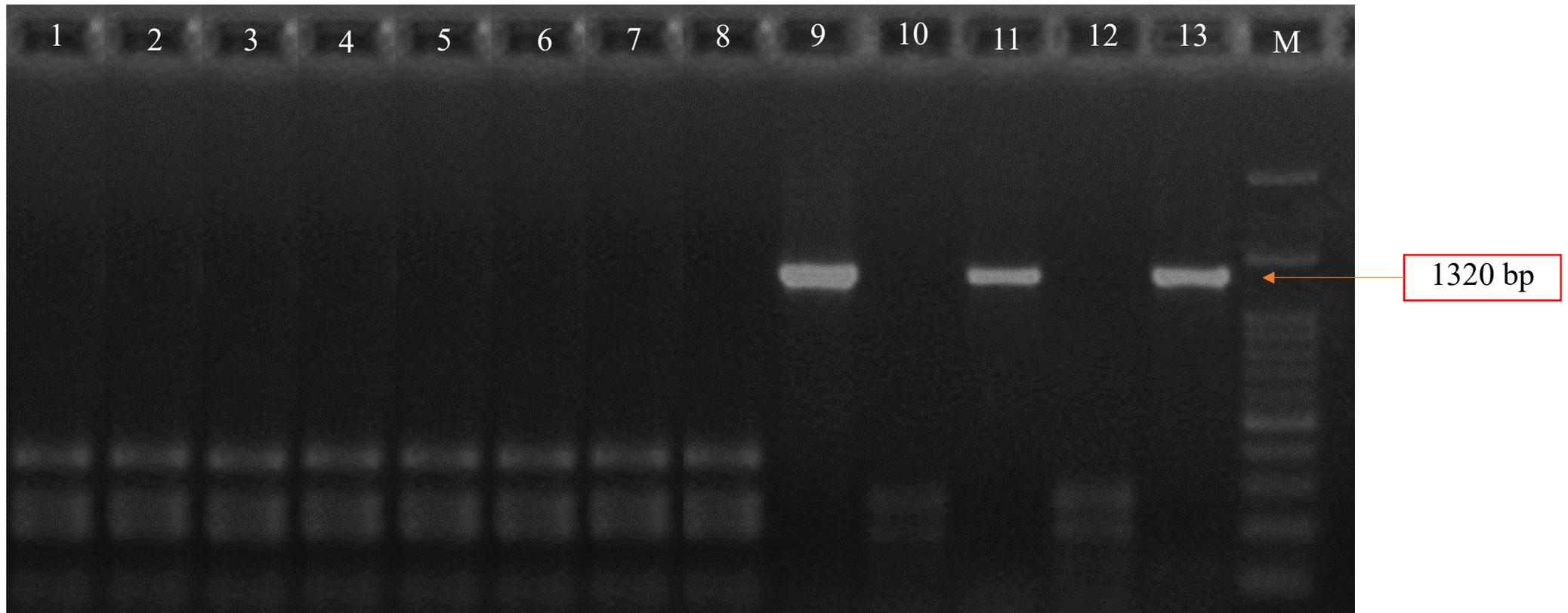
Results of Part II LC/MS/MS
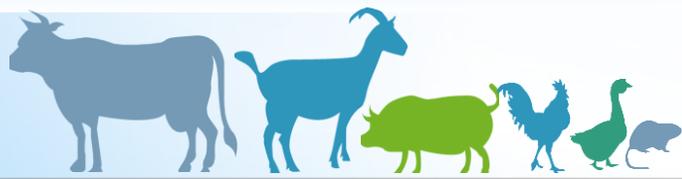
Mycotoxin analysis

Table 1 HPLC condition for AFB_1 AFB_2 AFG_1 and AFG_2 analysis

Parameters	Information
HPLC Column	Zorbax Eclipse Plus C18 column (50 x 4.6 mm, 1.8 μm)
Column temperature	40° C
Mobile phase	Phase A: 5 mM ammonium formate in 0.2% formic acid in millli-Q
	Phase B: 0.2% formic acid in ACN
Injection volume	5 μL
Elution mode	Gradient elution
Flow rate	0.4 mL/min
Run time	12 minutes

Table 2 HPLC condition for gliotoxin analysis

Parameters	Information
HPLC Column	Zorbax Eclipse Plus C18 column (50 x 4.6 mm, 1.8 μm)
Column temperature	40° C
Mobile phase	Phase A: 0.1% formic acid in Milli-Q
	Phase B: 0.1% formic acid in ACN
Injection volume	5 μL
Elution mode	Gradient elution
Flow rate	0.4 mL/min
Run time	12 minutes

Mass analyzer (MS/MS) condition for AFB_1 AFB_2 AFG_1 AFG_2 and gliotoxin analysis

Parameters	Information
Ion source	Electrospray ionization (ESI), positive mode
Gas temp (ºC)	325
Gas flow (l/min)	10
Nebulizer (psi)	50
Sheath gas heater	350
Sheath gas flow	11
Capillary (V)	4,000

Mass analyzer (MS/MS) condition for AFB_1 AFB_2 AFG_1 AFG_2 and gliotoxin analysis

Mycotoxins	Precursor ion (m/z)	Product ion (m/z)	Fragmenter (V)	CE (eV)	Polarity	RT (min)
AFB_1	313.1	285.1*	160	21	Positive	7.34
		241	160	35		
AFB_2	315.1	287.1*	160	25	Positive	6.92
		259	160	29		
AFG_1	329.1	311	160	21	Positive	6.90
		243*	160	25		
AFG_2	331.1	313*	160	25	Positive	6.50
		245	160	29		
Gliotoxin	327.1	263.2*	100	0	Positive	6.87
		245.3	100	11		

Remarks : RT = retention time   * = quantifier ion   CE= Collision energy

The results of the <u>linearity and range</u> of the analytical method and the matrix effect (SSE%) test as determined from tests conducted on complete feed and corn husks.

Mycotoxins	Calibration range (µg/kg)		Correlation coefficient (r)		SSE %	
	Complete feed	Corn husks	Complete feed	Corn husks	Complete feed	Corn husks
AFB$_1$	2.0-20.0	2.0-20.0	0.995	0.997	-21.73	-12.03
AFB$_2$	2.0-20.0	2.0-20.0	0.998	0.996	-25.18	-14.15
AFG$_1$	2.0-20.0	2.0-20.0	0.995	0.999	-18.45	-11.64
AFG$_2$	2.0-20.0	2.0-20.0	0.993	0.997	-22.37	-12.21
Gliotoxin	10.0-300	10.0-300	0.996	0.998	-28.65	-15.57

r ≥ 0.990 (SANTE, 2016; AOAC, 2019)

<u>A positive</u> %SSE indicates signal enhancement due to the matrix.
<u>A negative</u> %SSE indicates signal suppression caused by the matrix.

Accuracy and precision of aflatoxins and gliotoxin in complete feed

mycotoxins	Spiked concentration (µg/kg)	accuracy Recovery (%)	Precision (%RSD)	
			Intra-day	Inter-day
AFB$_1$	2.0	104.6	5.2	6.4
	10.0	93.7	4.8	7.3
	20.0	90.5	5.1	6.8
AFB$_2$	2.0	101.8	7.8	4.6
	10.0	92.7	4.5	8.9
	20.0	91.5	3.1	4.8
AFG$_1$	2.0	90.8	5.6	6.1
	10.0	91.5	3.9	4.7
	20.0	96.4	4.0	8.8
AFG$_2$	2.0	88.7	6.7	4.6
	10.0	90.2	4.3	5.1
	20.0	89.4	5.1	5.8
Gliotoxin	10	84.5	7.4	8.2
	50	89.9	6.6	7.4
	200	90.6	4.8	6.3

Recovery = 70-120% / %RSD < or = 20% (SANTE, 2021)

Accuracy and precision of aflatoxins and gliotoxin in corn husks

mycotoxins	Spiked concentration (μg/kg)	accuracy Recovery (%)	Precision (%RSD)	
			Intra-day	Inter-day
AFB$_1$	2.0	97.5	5.6	8.2
	10.0	95.8	6.1	5.9
	20.0	100.8	4.5	6.3
AFB$_2$	2.0	103.3	3.8	5.7
	10.0	105.7	5.5	4.2
	20.0	97.8	2.9	5.5
AFG$_1$	2.0	97.5	4.6	6.1
	10.0	101.7	3.0	6.5
	20.0	96.4	4.9	6.7
AFG$_2$	2.0	89.8	4.2	4.3
	10.0	90.5	3.3	6.8
	20.0	93.4	6.7	6.4
Gliotoxin	10	90.7	6.4	8.6
	50	92.5	5.6	4.9
	200	90.4	4.4	7.5

Recovery = 70-120% / %RSD < or = 20% (SANTE, 2021)

The limits of detection (LOD) and limits of quantification (LOQ) for the fungal toxins AFB₁, AFB₂, AFG₁, AFG₂, and gliotoxin in complete feed and corn husk samples.

Mycotoxins	complete feed		corn husks	
	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
AFB_1	0.65	2.0	0.65	2.0
AFB_2	0.65	2.0	0.65	2.0
AFG_1	0.65	2.0	0.65	2.0
AFG_2	0.65	2.0	0.65	2.0
Gliotoxin	3.2	10.0	3.2	10.0

n = 10, cal. SD
LOD = 3SD
LOQ = 10SD

Concentrations of aflatoxins and gliotoxin in complete feeds (n=9) and corn husks (n=10)

Mycotoxin	Positive sample (%)	complete feeds Concentration (µg/kg) Range	complete feeds Concentration (µg/kg) Mean	Positive sample (%)	corn husks Concentration (µg/kg) Range	corn husks Concentration (µg/kg) Mean
AFB_1	66.67	4.11-27.89	15.15	50.0	8.45–24.68	14.07
AFB_2	ND	-	-	ND	-	-
AFG_1	ND	-	-	ND	-	-
AFG_2	ND	-	-	ND	-	-
Gliotoxin	ND	-	-	ND	-	-

ND = Not detected (lower than LOD)

Conclusion (*cont.*)

❖ **Fungal Contamination in Samples**

- ✓ Detected *A. fumigatus*, *A. nidulans*, and *A. niger* in complete feeds and corn husks

- ✓ Only *A. fumigatus* is relevant due to its ability to produce gliotoxin

- ✓ Detection rate of *A. fumigatus* : 77.8% (7/9) in complete feed, 60% (6/10) in corn husks

❖ **Aflatoxigenic Fungi**

- ✓ No detection of *A. flavus* and *A. parasiticus* from Total samples tested: 19 (complete feed + corn husks)

Conclusion (*cont.*)

Mycotoxin Detection Results

❖ **AFB$_1$ detected:**

✓ **Complete feed: 4.11–27.89 ppb**

✓ **Corn husks: 8.45–24.68 ppb**

❖ **AFB$_2$, AFG$_1$, AFG$_2$, and GT: Not detected**

Conclusion (*cont.*)

Possible Explanation for Toxin–Fungus Mismatch

❖ **AFB$_1$ found in complete feed and corn husks despite absence of *A. flavus / A. parasiticus***

❖ **Possible reason:**

✓ **The fungus is dead.**

✓ **AFB$_1$ remains stable:**

❑ **Resistant to heat >250°C**

❑ **Stable at pH 3–10**

Conclusion (*cont.*)

Absence of Gliotoxin Despite *A. fumigatus*

❖ **No GT detected in samples**

❖ **Possible environmental limitations:**

    ✓ **Temperature**

    ✓ **Humidity**

    ✓ **Growth phase**

Detection of GT Biosynthesis Gene (*gliP*)

    ✓ ***A. fumigatus* isolates tested by PCR 13 samples**

    ✓ ***gliP* gene found in 3 isolates:**

        ❑ **2 from complete feed**

        ❑ **1 from corn husks**

    ✓ **Presence of *gliP* suggests potential genetic capacity for GT biosynthesis**

Conclusion (*cont.*)

❖ **Isolates positive for the *gliP* gene are genetically capable of GT biosynthesis.**

❖ **If environmental conditions become optimal (suitable temperature, humidity, nutrient availability, and appropriate growth stage), these isolates could potentially produce GT and contaminate feed.**

Conclusion (*cont.*)

Sample (n)	*Aspergillus flavus, A. parasiticus*	AFB_1 level Concentration (µg/kg)
Complete feed (9)	ND	4.11-27.89
Corn husks (10)	ND	8.45–24.68

ND = Not detected

❖ AFB_2, AFG_1, & AFG_2: Not detected

All aflatoxin contaminations were lower than the acceptable level of Animal Feed Control Act. B.E.2018.

Conclusion (*cont.*)

Sample (n)	*Aspergillus fumigatus*	GT level Concentration (µg/kg)	*gliP*
Complete feed (9)	7	ND	2
Corn husks (10)	6	ND	1

ND = Not detected (lower than LOD)

Conclusion (*cont.*)

Analytical Method Validation

❖ **LC/MS/MS method**

❖ **Method validation confirmed: High accuracy and precision**

❖ **High sensitivity for low-level detection (ppb range)**

❖ **Suitable for quantifying: Aflatoxins (AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$), Gliotoxin (GT)**

Conclusion (*cont.*)

Aflatoxins and Gliotoxin Contamination in Dairy Cattle Feed in Bangkok

❖ **Low levels of Aflatoxins (AFB1, AFB2, AFG1, AFG2) were found**

❖ **All AFs levels were below national safety limits**

❖ **Gliotoxin (GT) was not detected in any sample**

❖ **Indicates good safety of feed in the Bangkok dairy farming area**

❖ **Likely due to effective farm management practices**

Recommendations

❖ **Continuous surveillance of AFB1 and GT is essential**

❖ **Expand the study to other provinces with significant dairy farming**

❖ **Support a larger-scale survey for a more representative data set**

Limitations

❖ **The current study is preliminary**

❖ **Limited sample size = Not sufficient for national risk assessment**

❖ **Cannot be used to set regulatory limits**

Contributions of the Study

❖ **Provides a validated LC-MS/MS method**

❖ **Can be used as a reliable tool for AFs and GT detection**

❖ **Lays a foundation for future monitoring and research**