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OCCURRENCE OF MYCOTOXIGENIC FUNGI AND MULTI-MYCOTOXIN CONTAMINATION IN CORN-BASED POULTRY FEED SUPPLY CHAIN

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By

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OCCURRENCE OF MYCOTOXIGENIC FUNGI AND MULTI-MYCOTOXIN CONTAMINATION IN CORN-BASED POULTRY FEED SUPPLY CHAIN

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Corn, a main feed ingredient in the livestock industry, is one of the most susceptible crops to fungal infection and mycotoxin contamination which may occur at any point along the feed supply chain. Most mycotoxins are chemically stable and may be carried over into meat and eggs when poultry are fed with contaminated feed. As compared to individual effects, previous studies suggested that co-occurrence of mycotoxins may have potentially synergistic toxicological effects. Despite the health risks associated with the occurrence of these mycotoxins, no study on occurrence of multi-mycotoxin contamination along the corn-based poultry feed supply chain in Malaysia has been reported. The objectives of this study were therefore to examine the occurrence of mycotoxigenic fungi and aflatoxin contamination. The occurrence of multi-mycotoxin contamination along corn-based poultry feed supply chain was also determined. A total of 51 samples were collected from different points along the feed supply chain from two integrated poultry feed companies. The samples were subjected to mycological analyses (fungal isolation, enumeration, identification), and aflatoxins were quantified by high performance liquid chromatography equipped with fluorescence detector (HPLC-FLD). The samples were then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the occurrence of other mycotoxins. The results showed that samples collected from sampling point 1 (company A) and sampling point 9 (company B) yielded the highest total fungal loads (> log 4 CFU/g). The prevalent fungal genera isolated were *Aspergillus* (43.1%), *Fusarium* (52.9%), and *Penicillium* spp. (41.2%). Aflatoxin B_1 was detected in 8.3% of corn samples (0.12 – 1.51 µg/kg) and 7.4% of corn-based poultry feed (0.23 – 0.63 µg/kg) samples along the feed supply chain, whereas aflatoxins B_2, G_1, and G_2 were not detected. Results from LC-MS/MS analysis revealed that 100% of samples along the corn-based poultry feed supply chain were contaminated with more than one mycotoxin. Fumonisin B_1 (8.02 – 1,220.33

μg/kg) and fumonisin B_2 (11.05 – 1,108.67 μg/kg) were the main mycotoxins detected at all sampling points along the feed supply chain. Zearalenone (ZEA) (2.57 – 7.50 μg/kg) and HT-2 (< LOD – 32.90 μg/kg) were also detected in 17.65% (*n* = 9) and 52.94% (*n* = 27) (out of a total of 51) samples, respectively. As the supply chain progresses, reduction in mycotoxin contamination was observed. Aflatoxins, ochratoxin A (OTA), deoxynivalenol (DON) and T-2 toxin were also detected, but at very low levels (< LOD). The levels of mycotoxins detected along the supply chain were below the international regulatory limits, thus indicating that the risk level of exposure to mycotoxins along the corn-based poultry feed supply chain in Malaysia is very low. However, due to the composition of multiple ingredients in most food and feed, efforts to understand and address challenges associated with mycotoxins along the entire supply chain need to be more holistic to protect the public health.

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KEJADIAN KULAT MIKRO BERMIKOTOKSIN DAN PENCEMARAN PELBAGAI MIKOTOKSIN DALAM RANTAIAN BEKALAN MAKANAN AYAM BERASASKAN JAGUNG

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Jagung, bahan utama dalam industri ternakan, adalah salah satu tanaman yang paling mudah terdedah kepada jangkitan kulat dan pencemaran mikotoksin yang boleh berlaku di mana-mana di sepanjang rantaian bekalan makanan. Kebanyakan mikotoksin adalah stabil secara kimia dan boleh masuk ke dalam daging dan telur apabila ayam diberi makan dengan makanan yang tercemar. Berbanding dengan kesan individu, kajian sebelumnya mencadangkan bahawa kejadian mikotoksin berpotensi menyebabkan kesan toksikologi yang sinergistik. Walaupun terdapat risiko kesihatan yang berkaitan dengan kejadian mikotoksin ini, tiada kajian mengenai kejadian pencemaran pelbagai mikotoksin di sepanjang rantaian bekalan makanan ternakan berasaskan jagung di Malaysia telah dilaporkan. Oleh itu, objektif kajian ini adalah untuk mengkaji kejadian kulat mikro bermikotoksin dan pencemaran aflatoksin. Kejadian pelbagai mikotoksin di sepanjang rantaian bekalan makanan ternakan ayam juga ditentukan. Sejumlah 51 sampel dikumpulkan dari pelbagai lokasi di sepanjang rantaian bekalan makanan dari syarikat makanan ternakan bersepadu. Sampel tersebut tertakluk kepada analisis mikologi (pengasingan kulat, penghitungan, pengenalpastian), dan aflatoksin telah dinilai dengan kromatografi cecair berprestasi tinggi yang dilengkapi dengan pengesan pendarfluor (HPLC-FLD). Sampel kemudian dianalisis dengan spektrum massa kromatografi cecair (LC-MS/MS) untuk menentukan kejadian mikotoksin lain. Keputusan menunjukkan bahawa sampel yang dikumpul dari titik persampelan 1 (syarikat A) dan titik persampelan 9 (syarikat B) menghasilkan jumlah kulat yang tertinggi (> log 4 CFU / g). Genera kulat lazim yang diasingkan adalah *Aspergillus* (43.1%), *Fusarium* (52.9%), dan *Penicillium* spp. (41.2%). Aflatoksin B_1 dikesan dalam 8.3% sampel jagung (0.12 – 1.51 µg/kg) dan 7.4% daripada makanan ternakan ayam berasaskan jagung (0.23 – 0.63 µg/kg) di sepanjang rantaian bekalan makanan, manakala aflatoksin B_2, G_1, dan G_2 tidak dikesan.

Keputusan dari analisis LC-MS/MS mendedahkan bahawa 100% sampel di sepanjang rantaian bekalan makanan ternakan berasaskan jagung telah tercemar dengan lebih daripada satu mikotoksin. Fumonisin B_1 (8.02 – 1,220.33 µg/kg) dan fumonisin B_2 (11.05 – 1,108.67 µg/kg) adalah mikotoksin utama yang dikesan di semua titik persampelan di sepanjang rantaian bekalan makanan. Zearalenone (ZEA) (2.57 – 7.50 µg/kg) dan HT-2 (< LOD – 32.90 µg/kg) juga dikesan dalam 17.65% (n = 9) dan 52.94% (n = 27) (daripada sebanyak 51) sampel. Pengurangan pencemaran mikotoksin dapat dilihat di sepanjang rantaian bekalan makanan ternakan. Aflatoksin, okratoksin A (OTA), deoxynivalenol (DON) dan toksin T-2 juga dikesan, tetapi pada tahap yang sangat rendah (< LOD). Tahap mikotoksin yang dikesan di sepanjang rantaian bekalan adalah di bawah had pengawalseliaan antarabangsa, yang menunjukkan bahawa tahap risiko pendedahan kepada mikotoksin di sepanjang rantaian bekalan makanan ternakan berasaskan jagung di Malaysia adalah sangat rendah. Walau bagaimanapun, kerana komposisi pelbagai bahan dalam kebanyakan makanan dan makanan ternakan, usaha untuk memahami dan menangani cabaran yang berkaitan dengan mikotoksin di sepanjang rantaian bekalan perlu lebih holistik untuk melindungi kesihatan awam.

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

°	Degree
°C	Degree Celcius
Ø	Diameter
>	Greater than
<	Less than
L	Liter
µm	Micrometer
%	Percentage
±	Plus-minus
–	To
ACN	Acetonitrile
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFs	Aflatoxins
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemist
BEN	Balkan endemic nephropathy
CIN	Chronic interstitial nephropathy
CFU/g	Colony-forming unit per gram

cm	Centimeter
Codex Alimentarius	Codex Alimentarius International Food Standards
DON	Deoxynivalenol
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Services
DG-18	Dichloran 18% glycerol agar
DRBC	Dichloran rose Bengal chloramphenicol agar
drop/sec	Drop per second
EC	European Commission
EU	European Union
ELEM	Equine leukoencephalomalacia
FAO	Food and Agriculture Organization of the United Nations
FLD	Fluorescence detector
FUM	Fumonisin
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
g	Gram
HPLC	High-performance liquid chromatography
HPLC-FLD	High-performance liquid chromatography with a multi λ fluorescence detector
HSD	Honest significant difference
IAC	Immunoaffinity chromatography
IARC	International Agency for Research on Cancer
Fr	Isolation frequency

kg	Kilogram
Log CFU/g	Log colony-forming unit per gram
LOD	Limit of detection
LOQ	Limit of quantification
LC-MS/MS	Liquid chromatography tandem mass spectrometry
µg/L	Microgram per liter
µg/kg	Microgram per kilogram
min	Minute
mM	Millimolar
mL/min	Milliliter per minute
mm	Millimeter
MRM	Multiple reaction monitoring
n	Number of samples
ESI^-	Negative electrospray ionization
OTA	Ochratoxin A
ESI^+	Positive electrospray ionization
PDA	Potato dextrose agar
NaCl	Sodium chloride
sp.	Species (singular)
spp.	Species (plural)
NA	Not applicable
ND	Not detected
nm	Nanometer
NY	New York

NIV	Nivalenol
PA	Pennsylvania
PHRED	Photochemical reactor for enhanced detection
R^2	Coefficient of regression
rpm	Revolutions per minute
UK	United Kingdom
USFDA	United States of Food and Drug Administration
US	The United States of America
UV	Ultraviolet
v/v	Volume per volume
v/v/v	Volume per volume per volume
a_w	Water activity
ZEA	Zearalenone

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Poultry industry has emerged as an essential component of the agricultural sector worldwide (Bahri et al., 2019). The global poultry production for 2019 is 130 million tons and is expected to reach 137 million tons in 2020. In Malaysia, production of poultry meat showed an upward trend with production at 1,716.7 million metric tons in 2019, compared to 2018 which is 1,653.7 million metric tons (DVS, 2019). Poultry consumption in Malaysia also increase from 1,589.7 million metric tons in 2018 to 1,651.5 million tons in 2019. Moreover, Malaysia has one of the highest per capita consumption of poultry in 2019 with approximately 49 kilograms and forecasted to reach around 51 kilograms by 2025. Although the industry has been self-sufficient since 1984, factors such as rising cost of feeds and food safety issue related to poultry consumption can threatened the poultry industry (Benalywa et al., 2019). The feed supply chain plays a pivotal role in all livestock production systems. Feed supply chain typically involved several stages including feed production, processing, feed compounding and preparation at the farm, which altogether linked by transport and trade activities (FAO, 2014). The presence of any factors that could risk the feed supply chain will bring significant constraint on the livestock's production (Bryden, 2012). Among these factors, mycotoxins are one of the major threats for the security of feed supply chain and feed industry. Mycotoxins are toxic secondary metabolites produced mainly by fungal species belonging to the following genera: *Aspergillus*, *Penicillium*, *Fusarium* under favorable conditions (Kosicki et al., 2016). These fungi are ubiquitous in nature and can be commonly found in many agricultural commodities and foodstuffs (Kongkapan et al., 2015). Currently, more than 500 mycotoxins have been discovered, with aflatoxins (AFs), ochratoxin A (OTA), trichothecenes, zearalenone (ZEA), fumonisins (FUM) having the most significant impact on agro-economic and public health (Haque et al., 2020). Among the mycotoxins commonly occurred in food and feedstuffs, aflatoxin B_1 (AFB_1) is classified as group 1 (carcinogenic to humans and animals), FUM and OTA as group 2B (probably carcinogenic to humans), while the rest are not classified as human carcinogens (Group 3) (IARC, 2012, 2002, 1993).

Corn and corn-based products are known as the most susceptible cereals to fungal infection and subsequent contamination of mycotoxins (Park et al., 2018). In Malaysia, previous studies indicated that AFs, particularly AFB_1 and FUM were the most prevalent mycotoxins in corn and corn-based products (Soleimany et al., 2012; Reddy & Salleh, 2011; Hong et al., 2010). Low levels of OTA and deoxynivalenol (DON) were also reported (Soleimany et al., 2012; Rahmani et al., 2010). In addition, since corn has been commonly used as the

main ingredient in poultry ration, the occurrence of mycotoxin contamination in poultry feed has also been reported worldwide. For example, previous studies in Brazil found that the most prevalent mycotoxins in commercial poultry feed were AFB_1 and fumonisin B_1 (FB_1) followed by OTA and ZEA (Bordini et al., 2017; Oliveira et al., 2006). Similar results were reported in Argentina where FUM was the most frequently occurring mycotoxins followed by AFs, DON and OTA (Greco et al., 2014). Moreover, AFs were also detected in chicken feeds from poultry farms in Malaysia (Hussain et al., 2017).

Ingestion of mycotoxin contaminated feed by animals will cause symptoms known as mycotoxicoses. Compared to the individual effects, the combined toxicity of multiple mycotoxins has been reported to exert a greater impact on health and productivity, particularly in livestock (Grenier et al., 2016). Among the animal species, poultry is highly vulnerable to mycotoxicoses caused by major classes of mycotoxins (Poornima & Palanisamy, 2013). The negative effects of mycotoxins on poultry performance have been demonstrated in numerous studies. For example, AFs cause a reduction in body weight and an increase in liver and kidney weight of chickens (Zain, 2011). Meanwhile, prolonged intake of OTA decreases the performance, body weight and egg production of poultry (Hassan et al., 2012). Exposure to *Fusarium* mycotoxins such as T-2 toxin may cause necrosis and plaque formation (Zain, 2011) and weakened the immune system and body resistance (Bertero et al., 2018). The adverse effects of mycotoxins prompt various international institutions and organizations such as the European Commission (EC) and the US Food and Drug Administration (FDA) to set regulatory limits for several classes of mycotoxins in feedstuffs and finished feed (Krska & Crews, 2008).

1.2 Problem Statements

Infection of pathogenic fungi and subsequent mycotoxin production may occur at any point along the feed supply chain (Bryden, 2009). Most mycotoxins are chemically stable and hence, once present in the feedstuffs, there is a high risk of carry-over to feeds manufactured from it (Bryden, 2012). Ingestion of contaminated feed by poultry can lead to the accumulation of mycotoxins in the organs and tissues (Kongkapan et al., 2015), thus contributing to mycotoxin intake in human through consumption of animal-based products such as eggs, meat or milk (Pulina et al., 2014). This implies that mycotoxin contamination of feedstuffs and feed does not only threaten animal health but also represents a hazard for human health. Although studies on mycotoxin occurrence in feedstuffs and feed have been conducted in Malaysia (Hussain et al., 2017; Reddy & Salleh, 2011; Hong et al., 2010), no recent data on the occurrence of mycotoxins along the feed supply chain have been reported.

1.3 Significance of Study

Due to the ubiquitous nature of mycotoxin producing fungi, regular monitoring is important for ensuring the safety of poultry feeds or animals in general (Fareed et al., 2014). The finding of this study is helpful to indicate the occurrence of mycotoxin contamination along the corn-based poultry feed supply chain. Besides, this study will also provide baseline data for feed manufacturers and other parties involved in the feed industry on critical points along the corn-based poultry feed supply chain so that future mitigation strategies could be designed. Finally, the results of this study could provide data for the concerned regulatory authorities on the level of mycotoxin contamination along the corn-based poultry feed supply chain.

1.4 Objectives of Study

Therefore, the objectives of the present study were:

i. To identify the occurrence of mycotoxigenic fungi and aflatoxin contamination along corn-based poultry feed supply chain.
ii. To determine the occurrence of multi-mycotoxins along corn-based poultry feed supply chain.

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