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**Original Research Article**

# Identification of Fungi and Mycotoxin in Layer Feed Sold in Traditional Markets of Bogor, Indonesia

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**Abstract**

Fungal contamination into layer feed during storage period is one of the cause of feed damage. Such contamination can lead to economic loss, i.e. the decrease in feed selling price, in addition to health problems, e.g. mycosis and mycotoxicosis in human and animal. The present study aimed to determine fungal species and mycotoxin in layer feed sold in traditional markets of Bogor. Fungal identification was conducted using slide culture following Riddell's method, while mycotoxin detection using *High Performance Liquid Chromatography* (HPLC). Out of 100 samples, 13 fungal species were found as potential contaminants for layer feed, i.e. *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus, A. tamarii, A. terreus, Cladosporium cladosporioides, C. herbarum,Endomyces fibuliger, Eupenicillium ochrosalmonecum, Eurotium chevalieri, Fusarium verticillioides,* and *Penicillium citrinum* with *A. flavus* as dominant species. Therefore, mycotoxin tested was aflatoxin. The fungal population density of 8 out of 100 samples was higher than $10^4$ cfu/g, indicating that dominant aflatoxin was $AFB_1$ with 13.4 ppb as average concentration. The aflatoxin in layer feed does not exceed the level permitted by Indonesian National Standard (SNI).

**Keywords**

Contamination

Feed

Fungi

Mycotoxin

## Introduction

Fungus is one of micro-organisms potential as contaminant source, *Mucor, Rhizopus, Aspergillus, Cladosporium, Dictyostelium,* and *Saccharomyces* genus are the examples (Susilowati and Listyawati 2001). According to Ahmad (2009), fungi from *Aspergillus* spp. are storage fungi and often found in feed. Tyasningsih (2010) reported that number of *Aspergillus* spp. exceeding threshold can lead to aspergillosis in consuming poultry. Feed is the main nutritional source for livestock and supports their growth and productivity. According to Jahan et al. (2006), there are three types of chicken feed, i.e. mash, pellet, and crumble. Mash feeds are more vulnerable to fungal contamination than pellet (Ahmad 2009). One of the causal factors of feed quality

degradation is climate (Rachmawati et al. 1999). Indonesia's tropical climate can support fungal growth during storage period (Tyasningsih 2010; Ahmad 2009). Pitt and Hocking (1997) stated that *Aspergillus* spp. and *Eurotium* spp. are the main contaminant fungi in tropical regions.

Mycotoxin is a fungal metabolite product and often found in livestock's feed, one of which is aflatoxin. According to Lewis et al. (2005), two fungi capable of producing aflatoxin are *A. flavus* and *A. parasiticus*. Bryden (2012) reported that mycotoxin can affect feed safety, health and economic aspects. Health aspect is such as acute mycotoxitosis, chronic liver cancer in human and animal, while economic aspect is such as the changes physically and chemically in feed materials, leading to degrading quality and selling value of the feed (Hastiono, 1983).
Rocha et al. (2014) stated that mycotoxin is carcinogenic cause in animal and human. Bahri (1998) reported that more than 80% of commercial poultry feeds are contaminated with aflatoxin B1 ($AFB_1$) where 13.5% out of 193 samples tested positively contains $AFB_1$ up to 200 ppb in concentration. Ahmad (2009) reported that aflatoxin content in corn kernels in Indonesia ranges from 10-300 ppb. Maximum aflatoxin content based on National Standard Agency (BSN) (2006) in Indonesian National Standard (SNI) No 01-3929-2006 is 50 ppb.

The present study aimed to determine contaminant fungal species in feeds from traditional markets in Bogor and the most potentially mycotoxin-producing fungi. This study is expected to bring about awareness to traders and farmers on the importance of good feed storage to control the contamination of fungi and their metabolite products.

## Materials and methods

### Sampling method and sample size

Samples used were layer feed from traders in each traditional market in Bogor. Sample size was determined based on 90% confidence level and calculated following Thrusfield (2006) sample size calculation formula as follows:

$$n = \frac{4PQ}{(L^2)}$$

Where, n = Sample amount;
P = Prevalence assumption;
Q = 1 – P;
L = Error desired.

Based on 50% prevalence assumption, 10% error, and 90% confidence level, sample size obtained was 100 samples. Table 1 shows the size of samples from each traditional market in Bogor.

**Table 1. The size of layer feed samples obtained from traditional markets in Bogor**

| Market | Number of traders of layer 105 M feed | Number of sample |
|---|---|---|
| Kebon Kembang Market | 3 | 30 |
| Bogor Baru Market | 2 | 20 |
| Merdeka Market | 3 | 30 |
| Gunung Batu Market | 1 | 10 |
| Teknik Bogor Central Market | 1 | 10 |
| Total | | 100 |

### Fungal isolation

Fungal isolation was conducted using serial dilution from $10^{-1}$up to$10^{-5}$, followed with pour plate method for each dilution and incubation in room temperature for 5-7 days (Dharmaputra et al., 2013). Each fungi species/g was counted following Dharmaputra et al. (1999) formula as follows:

$$\text{Population of each fungi species/g} = \frac{1}{XY} Z$$

Where,
X = volume of layer feed suspension moved onto each Petri dish;
Y = dilution providing separated fungal colony; and
Z = the average of colony number of each fungal species from the three Petri dishes.

### Fungal identification

### Fungal macroscopic observation

The colonies obtained were purified in CYA medium prior to incubation for 7 days and macroscopic observation consisting of colony colour, colony diameter, radial lines from the centre to the edge of the colony, and concentric rings. Fungal identification was

conducted based on identification book by Pitt and Hocking (1997) and Gandjar et al. (1999).

### Fungal microscopic observation

Fungal identification was conducted using Riddell's slide culture method prior to incubation for 48 hours and microscopic observation under microscope. Variables observed were spore shape, conidia shape, and conidiophore (Dorry, 1980).

### Mycotoxin concentration analysis

Mycotoxin tested in the present study was aflatoxin. The determination of which was based on prior discovery on the dominant fungi. Aflatoxin analysis ($B_1$, $B_2$, $G_1$ and $G_2$) was carried out using *High Performance Liquid Chromatography* (HPLC) method following AOAC (2005).

### Data analysis

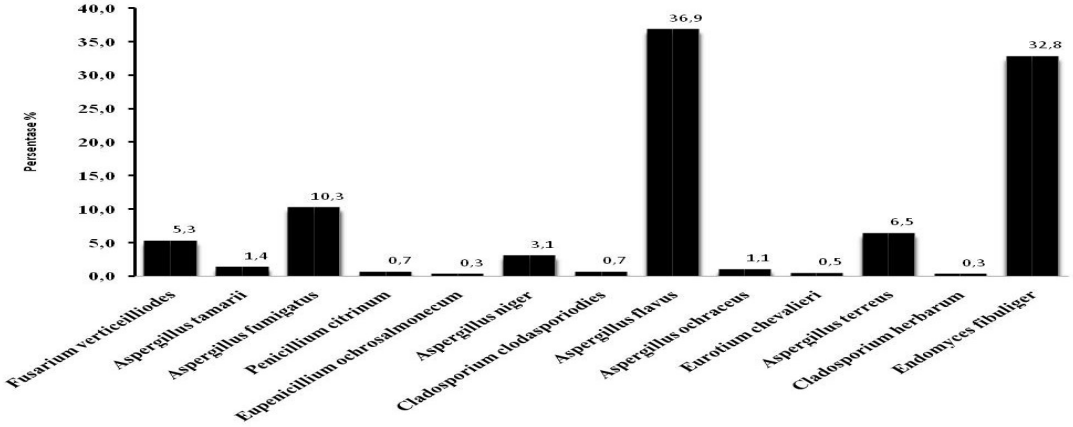
Descriptive data analysis was used. The result of fungal identification and mycotoxin test are presented in figures and tables.

## Results and discussion

### Fungal species

The result showed that from five markets consisted of 10 traders, 13 fungal species were found, i.e. *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus, A. tamarii, A. terreus, Cladosporium cladosporioides, C. herbarum, Endomyces fibuliger, Eupenicillium ochrosalmoneum, Eurotium chevalieri, Fusarium verticillioides* and *Penicillium citrinum*. Fig. 1 shows the percentage of the 13 fungal species.

**Fig. 1: The percentage of fungal species in layer feed.**




Based on the presented figure, *Aspergillus flavus* was the dominant fungus (36.9%) with $10.3\times10^5$ population density per species. Ahmad (2009) and Djaenudin et al. (2004) also found that *A. flavus* is the dominant fungus in feed. This is due to its nature as storage fungus and capability to produce aflatoxin during storage period at 11-41$^o$C (Pitt and Hocking, 1997; Ahmad, 2009).

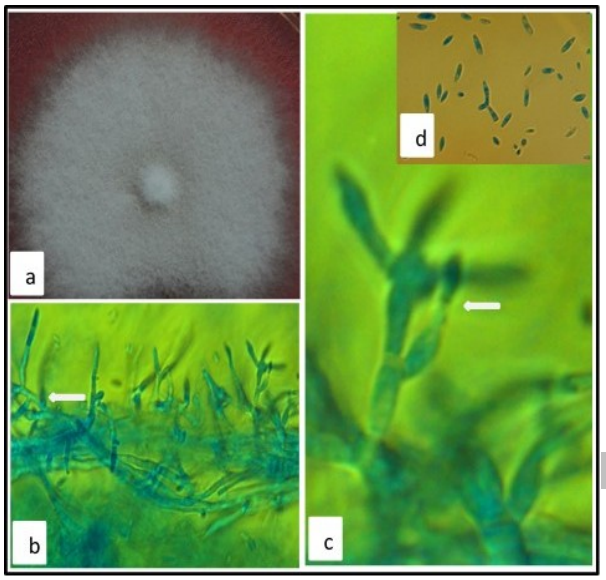
The high quantity of fungi found in layer feed at traders can be due to several factors, one of which is environmental factor during storage period. Retnani et al. (2011) reported that time-span for feed storage can significantly affects ($p$<0.01) feed product due to the presence of contaminant fungi, and therefore the feed quality is decreasing.Handayani and Sulistyo (2000) reported that environment with low temperature and humidity can trigger the growth of fungi which then act as feed contaminants. This is supported by Bryden (2012) where contaminant fungi are capable of damaging feed commodity structure in physical, chemical, and biological manners during storage.

Storage period of feed can affect its physical nature where it can be in lower quality after several particular time. According to Ratnani et al. (2009), that is because relation between temperature and air humidity in storage room. High level of both aspects leads to the increase in water content. When the humidity is low, water in feed evaporates. Retnani et al. (2010) added that low humidity during storage period supports the growth of lipid-hydrolysing fungi. The longer the storage period, water content increases, and the higher the attack of lipase-producing microorganisms capable of breaking lipids is.

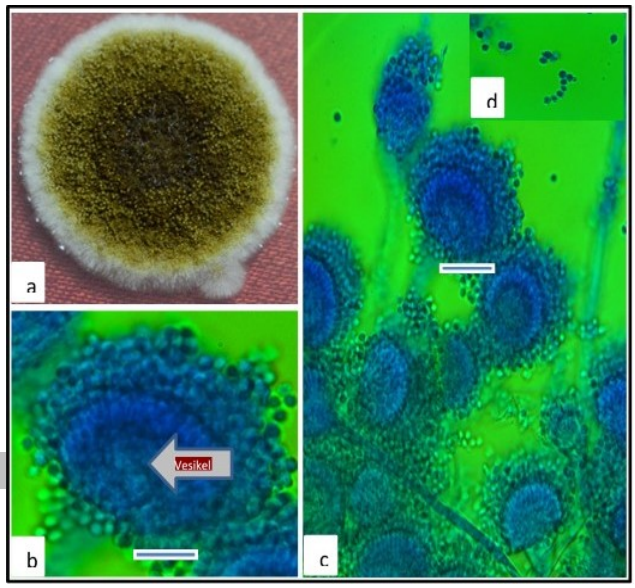
## Fungal characteristics

Based on the data, there were total 13 fungal species, i.e. *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus, A. tamarii, A. terreus, Cladosporium cladosporioides, C. herbarum, Endomyces fibuliger, Eupenicillium ochrosalmoneum, Eurotium chevalieri, Fusarium verticillioides,* and *Penicillium citrinum*. Each fungal species has different macroscopic and microscopic characteristics. Fig. 2 below describes the result of isolation and fungal identification based on the keys by Pitt and Hocking (1997) and Gandjar et al. (1999).

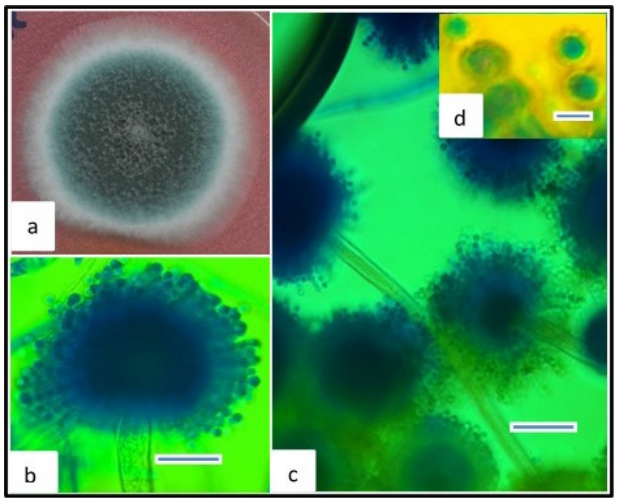
**Fig. 2: Characteristics of fungal species isolated from layer feed.**



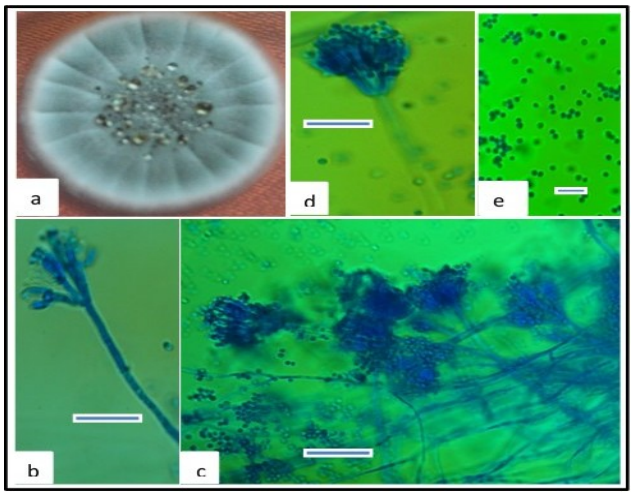

Isolate 1: *Fusarium verticillioides* (10x100 magnification) (a) Colony on CYA medium, diameter 3.5 cm, white in colour and filamentous, concentric ring and radial lines are present (b) Conidiophore short and bottle-shaped (c) Phyalid in chain (d) Microconidia with septa, straight, and without chlamydospores. Microconidia are formed by phyalids in long chain.



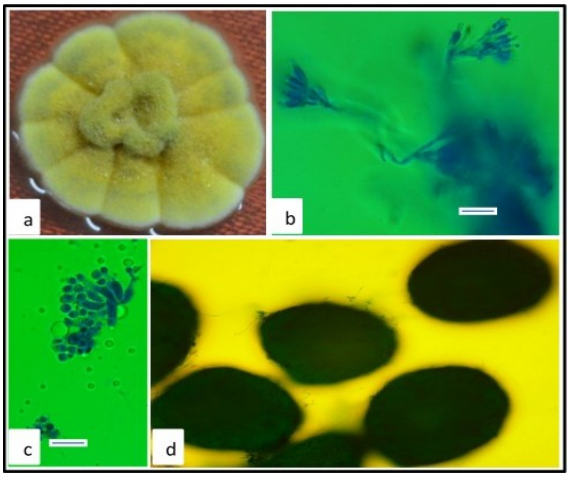

Isolate 2: *Aspergillus tamarii* (10x100 magnification)(a) Colony on CYA medium, diameter 4 cm, yellowish brown, concentric ring and radial lines present (b,c) Vesicle round and semi-round (d) conidia round and semi-round.



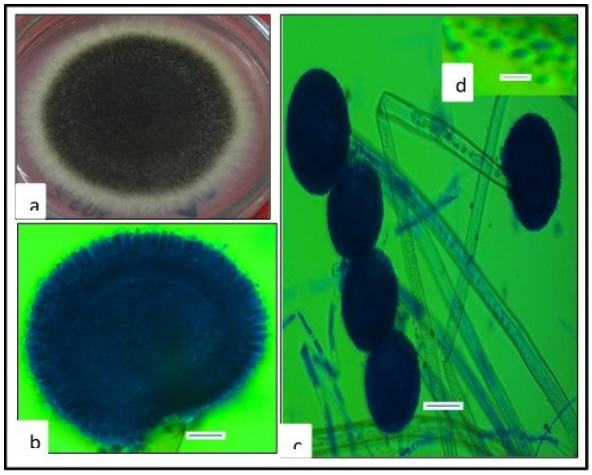

Isolate 3: *Aspergillus fumigatus* (10x100 magnification) (a) Colony on CYA medium, diameter 3 cm, dark green , concentric ring and radial lines present (b,c) broad vesicle (d) conidia round and semi-round.



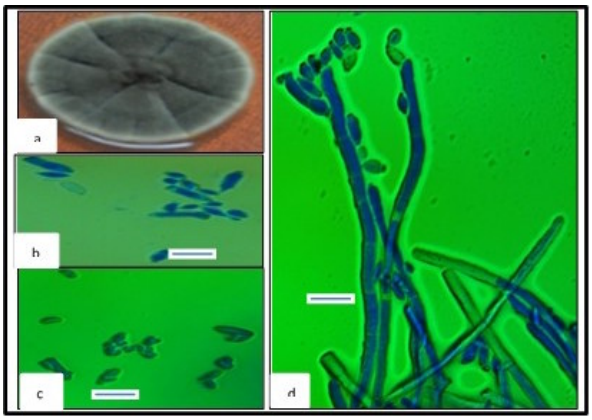

Isolate 4: *Penicillium citrinum* (10x100 magnification) (a)Colony on CYA medium, diameter 1.5 cm, green to grey, concentric ring and radial lines present (b,c,d) conidiophores long and straight (d) Conidia round and semi-round.



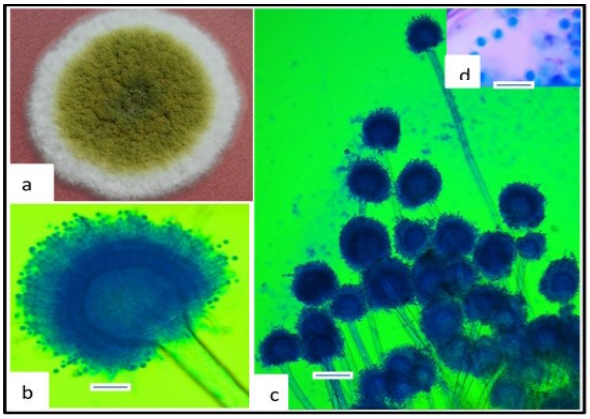

Isolate 5: *Eupenicillium ochrosalmoneeum,* (10x100 magnification) (a) Colony on CYA medium, diameter 2.5 cm, yellow in colour, concentric ring and radial lines present (b) conidiophore long and bent (c) Conidia round (d) Ascus round and ascospores present within ascus.



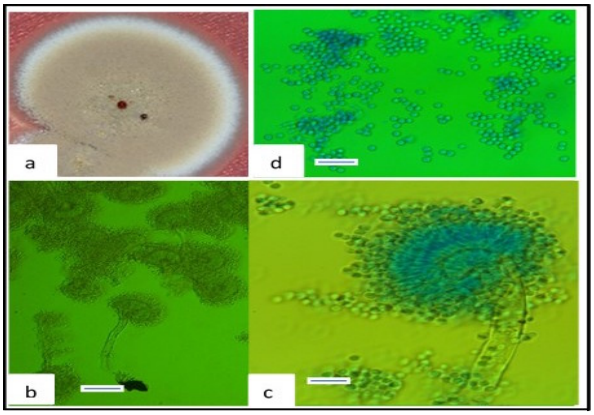

Isolate 6: *Aspergillus niger* (10x100 magnification)(a) Colony on CYA medium, diameter 4 cm, mycelium black, concentric ring and radial lines present (b) Vesicle round, (c) Conidiophore long and bent (d) Conidia round.



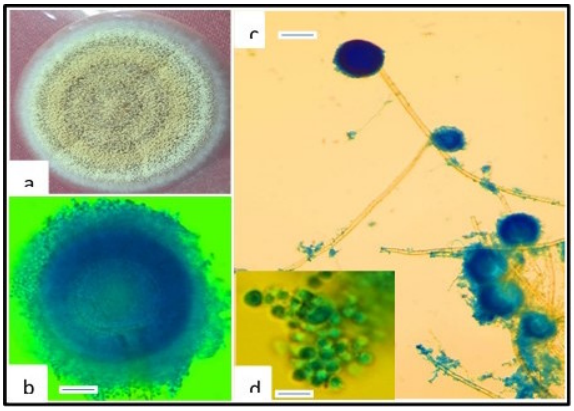

Isolate 7: *Cladosporium cladosporioides* (10x100 magnification) (a) Colony on CYA medium, diameter 2.8 cm, wrinkled surface, grey, concentric ring and radial lines present (b, c) Conidia in chain (d) Conidiophore long without septate.



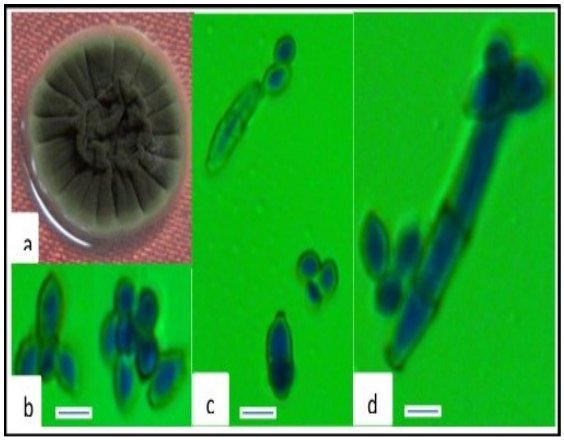

Isolate 8: *Aspergillus flavus* (10x100 magnification) (a) Colony in CYA medium, diameter 5 cm, yellow to green , concentric ring and radial lines present (b,c) Vesicle round and semi-round (d) Conidia round.



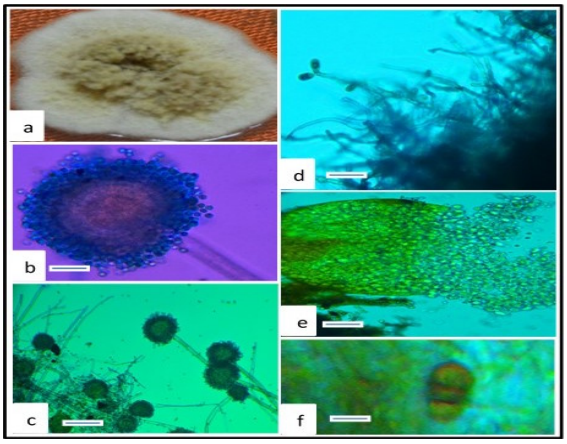

Isolate 9: *Aspergillus terreus* (10x100 magnification) (a) Colony on CYA medium, diameter 4.5 cm, brown, radial lines and concentric ring absent (b,c) Vesicle semi-round (d) conidia columnar.



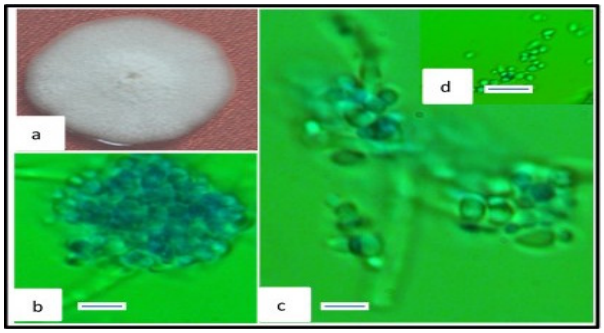

Isolate 10: *Aspergillus ochraceus* (10x100 magnification)(a) Colony on CYA medium, diameter 2.5 cm, yellow, radial lines and concentric ring present (b,c) Vesicle round (d) Conidia round and semi-round.




Isolate 11: *Cladosporium herbarum* (10x100magnification) (a) Colony on CYA medium, diameter 3 cm, dark grey in colour, concentric ring and radial lines present (b, c) conidia ellipse and cylindrical(d) Conidiophore long and has septate.




Isolate 12: *Eurotium chevalieri* (10x100 magnification) (a)Colony on CYA medium, diameter 4 cm, bright yellow to brownish dark yellow, concentric ring absent, radial lines present (b,c) Vesicle round (d) Conidia round (e) Ascoma round (f) Ascospore round to ufo-like oval.




Isolate 13: *Endomyces fibuliger* (10x100 magnification)(a) Colony on CYA medium, diameter 6 cm, concentric ring and radial lines present (b,c) Conidiophore short, two-branched, and with septate (d) Conidia round and ellipse.

Based on the identification result, 9 out of 13 fungal species are capable of producing mycotoxin. They are *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus, A. terreus, Eupenicillium ochrosalmoneum, Eurotium chevalieri, Fusarium verticillioides* and *Penicillium citrinum.*

Mycotoxins produced by the nine fungal species, according to Pitt and Hocking (1997) are as follow. *Fusarium verticillioides* produces fumonisin, the cause of nephrotoxic, hepatotoxic, and carcinogenic. *A. fumigates* produces fumitremorgen where spores of which are the cause of avian lung disease (aspergilosis). *P. citrinum* produces citrinin, the cause of kidney damage. *Eupenicillium ochrosalmoneum* produces citreovirid in toxin. The toxin in Indonesia, however, has yet to be assessed so that there is no report aboutthe effect on livestock health. *A. niger* and *A. ochraceus* produce ochratoxin causing carcinogenic, immunosuppressive, and nephrotoxic. *A. flavus* produce $B_1$ and $B_2$aflatoxin causing hepatotoxic, carcinogenic, mutagenic, and immunosuppressive. *A. terreus* produces territrem as metabolite compound which has a significant toxicity, albeit not much reported so that there is no information about its effect on health. *Eurotium chevalieri* is reported produces toxic compounds, identified as echinulin and neo-echinulin in swine feed. There are also not much reports on its toxins related to health.

## Aflatoxin content in layer feed

The result indicated that fungal population density exceeding $10^4$ cfu/g was found in eight out of 100 layer feed samples. Indonesia National Agency of Drug and Food Control (BPOM) (2009) stated that the maximum fungal threshold for cerealia products is $<10^4$ cfu/g. Fungal species found in the eight samples are potentially aflatoxin producers (Table 2), therefore the samples were then further examined for their mycotoxin content,

particularly aflatoxin. Aflatoxin is a type of mycotoxin produced by *A. flavus* (Pitt and Hocking, 1997). Based on prior data, it is known that *A. flavus* is a dominant fungus found in layer feed. Table 2 shows the concentration of aflatoxin in layer feed in traditional markets of Bogor.

**Table 2. The average of aflatoxin concentration in layer feed in traditional markets of Bogor**

| Aflatoxin | n (positive) | Concentration (ppb) | | | |
|---|---|---|---|---|---|
| | | Average | Max | Min | STDEV |
| $B_1$ | 8 | 13.43 | 40.13 | 0.11 | 1.452 |
| $B_2$ | 6 | 1.48 | 3.79 | 0.59 | 1.209 |
| $G_1$ | 3 | 2.05 | 4.06 | 0.84 | 1.750 |
| $G_2$ | 6 | 0.24 | 0.76 | 0.11 | 0.249 |

Table 2 showed that layer feed sold in traditional markets of Bogor has aflatoxin $B_1$, $B_2$, $G_1$, and $G_2$, with $B_1$ as the dominant aflatoxin (average concentration of 13.43 ppb).The maximum aflatoxin in layer feed, according to BSN (2006) in SNI no. 01-3929-2006 is 50 ppb. The result of this study showed that the content of aflatoxin in layer feed in the traditional markets of Bogor does not exceed SNI value. Even though, according to Handajani and Setyaningsih (2006), the low aflatoxin level still needs to be aware of due to its nature to accumulate in body and difficult to be degraded, and lead to chronic health impairment.

According to Stuver (1998), $B_1$ is the most toxic and available aflatoxin in nature. Widiyanti and Sagi (2001) added that aflatoxin $B_1$ is highly dangerous for human and animal due to its mutagenic and teratogenic natures, in addition to its toxic nature. Aflatoxin contamination in layer feed can also bring about economic loss, such as drop in fertility (Ibeh et al., 2000), decrease in livestock weight (Mani et al., 2001), and decrease in egg production (Exarhos and Gentry, 1982). Alberts et al. (2009) reported that aflatoxin contamination in feed in USA brought about significant economic loss, i.e. up to US $85-100 million.

## Conclusion

There were 13 fungal species found in layer egg, and *A. flavus* was the dominant. Mycotoxin produced by *A. flavus* is aflatoxin so that the tested mycotoxin was aflatoxin. Aflatoxin content found were $G_1$, $G_2$, $B_1$ dan $B_2$. Dominant aflatoxin found were $B_1$with the average of 13.43 ppb. Aflatoxin content in layer feed does not exceed the SNI.

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