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The Susceptibility Simulation of Ochratoxin A and Aflatoxins Contamination on Fermented and Unfermented Cocoa Beans in High Storage Humidity

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Abstract

Most of Indonesia's cocoa beans have been produced through fermentation. Various metabolite end products such as alcohol, lactic acid, and acetic acid are produced during cocoa beans fermentation. These metabolites would induce different chemical characteristic of fermented cocoa beans. In this research, ochratoxin A (OTA) and aflatoxins (AFs) was used to simulate the susceptibility of OTA and AFs production during storage. This research was conducted on storage simulation condition, which was 91% of relative humidity. It is hypothesized that metabolic end-products from the breakdown substrate during fermentation process have an effect on mycotoxin production by fungi.

This is the first report of OTA and AFs contamination susceptibility in fermented and unfermented cocoa beans. Fermented cocoa beans were more susceptible to AFs contamination. The highest AFs concentration was found in the fermented cocoa beans after 10 days storage. Unfermented cocoa beans were more susceptible to OTA production. The highest OTA concentration was found in unfermented cocoa beans after 15 days storage.

The results from the simulation indicated a promising different potential between OTA and AFs production in fermented and unfermented cocoa beans during storage, suggesting the existence of limiting factors on the accumulation of OTA and AFs in the beans by the metabolic-end products produced during fermentation.

Keywords: ochratoxin A, aflatoxins, fermented cocoa beans

Introduction

Cocoa is one of distinguished Indonesia's plantation commodities. According to the Indonesian National Standards (2002), cocoa bean quality requirements for grade I, is 97-98% of fermented cocoa beans. Fermentation is an important step of cocoa beans post-harvest processing that mostly governs ultimate product quality. Spontaneous fermentation is generally conducted by succession of indigenous species of yeast, lactic acid bacteria, and acetic acid bacteria (Schwan and Wheals, 2004).

Microbial during fermentation solubilizes the pulp material and produces a range of metabolic end-products (e.g. alcohols and organic acids), which diffuse into the

beans to cause microbes death (Copetti et al., 2012[a]). Several organic acids were produced during fermentation processes, such as acetic acid, lactic acid, and citric acid. Acetic acid was produced 5 mg/g at the end of cocoa beans fermentation (Galvez et al., 2007). Lactic acid and acetic acid are the main products of carbohydrates fermentation by lactic acid and acetic acid bacteria. Those organic acids are known as food preservatives. The previous studies reported that weak organic acids such as acetic acid, citric acid, and lactic acid have an antimicrobial activity (Dalie et al., 2010; Copetti, et al., 2012[b]; Pelaez et al., 2012) and capable to degrade aflatoxin B_1 (Albores et al., 2005; Copetti, et al., 2012[b]).

Ochratoxin A (OTA) is a secondary metabolite toxin that is produced by several species of *Aspergillus* and *Penicillium*. It has nephrotoxic, immunotoxic, teratogenic, and carcinogenic properties (Alvarez et al., 2004). Following experiments on animals (IARC, 2003) OTA is classified as carcinogenic for humans (group 2B). Aflatoxins (AFs) represent the group of the most studied mycotoxin, especially due to their widespread occurrence in foods and toxicological and carcinogenic potential associated with their consumption. Aflatoxin B_1 (AFB_1) is the most potent hepatocarcinogen known in mammals classified by the International Agency of Research on Cancer as group 1 carcinogen and also has toxic, mutagenic and teratogenic properties (IARC, 2003).

Mycotoxins contamination in cocoa beans not only has a high risk in human health but it also could take harmful effect on the international cocoa trade market. Currently, international discussions are taking place to determine the acceptable OTA and AFs levels in food products. The maximum levels of AFs set by European Commission (EC) in food for direct human consumption are 2 µg/kg for AFB_1 and 4 µg/kg for the sum of AFs, whereas for OTA, the adopted maximum levels are ranging from 2 to 10 µg/kg (Imperato et al., 2011). The EC has established maximum limits for OTA in cocoa and cocoa products: (a) 2 µg/kg in raw material for the intermediate food products (cocoa beans, peeled beans, cocoa cake, nibs and cocoa powder) and (b) 1 µg/kg for consumer's goods (chocolate powder, chocolate, chocolate beverages) (Megalhaes et al., 2011).

AFs and OTA have special interest due to their high occurrence and toxicity. Researchers have reported the presence of genus *Aspergillus*, emphasizing aflatoxins and ochratoxins-producing species in cocoa beans, as well as the presence of that mycotoxin (Copetti et al., 2010; Mounjouenpou et al., 2008 and Nugroho et al., 2013). Co-occurrence of AFs and OTA in cocoa products has been reported (Copetti et al., 2012[a]; Turcotte and Scott, 2013), so analysis for both of these mycotoxins in the same sample is desirable.

In this study we evaluated the contamination of OTA and AFs in fermented and unfermented cocoa beans in high storage humidity, aiming to correlate them in order to establish the susceptibility of fermented and unfermented cocoa beans on OTA and AFs contamination.

Materials and Methods

Experimental setup

Cocoa beans, Forastero variety (fermented and unfermented cocoa beans) were obtained from a farmer in Yogyakarta, Indonesia. The samples were taken after dried, and then divided into two parts: the natural and artificial contamination. In the first part (natural contamination), it was observed the OTA and AFs contamination from the field and during storage simulation with high relative humidity. In the second part (artificial contamination), it was observed the susceptibility of fermented and unfermented cocoa beans to OTA and AFs contamination.

An artificial inoculation with *Aspergillus ochraceus* was conducted in second part to observe the potency of the produced OTA as well as the possibility of the formed AFs on fermented and unfermented cocoa beans during storage simulation with high relative humidity. Two mL of spore suspension (4.32 x 10^8 spore/mL) was inoculated into 100 g of cocoa beans. Afterward, the samples were incubated to support fungal growth and mycotoxin production in 91% of relative humidity at room temperature. OTA and AFs analysis were conducted at 5, 10, and 15 days after storage.

pH and water activity measurement

Measurement of pH was performed according to SNI (01-2323-2002); ten g of finely ground cocoa beans were dissolved in 90 mL of hot distilled water (70-80^{0}C). It was then cooled to room temperature and pH was determined using pH meter. The water activity (a_w) of cocoa beans was determined by water activity instrument (*Decagon Pawkit*).

Ochratoxin A analysis

1. Clean-up of ochratoxin A

Five gram of finely ground cocoa beans were extracted in 100 ml $NaHCO_3$ (0.1%) and polyethylene glycol (PEG) (0.3%). Suspensions were shaken for 60 min at room temperature. The homogenized solutions were filtered through Whatman No.4 filter paper. The filtrate (20 mL) was diluted in 20 mL of phosphate buffered saline (PBS) and applied to an Ochraprep immunoaffinity column clean-up (R-Biopharm). The column was then washed with PBS (10 mL) and that continued with distilled water (10 mL). OTA was eluted with acidified methanol (methanol:acetic acid, 98:2, v/v; 3 mL) into an amber vial. After evaporation to dryness at 40ºC under a stream of N_2, the dry residue was dissolved in mobile phase (0.4 mL).

2. HPLC parameters

A Shimadzu 10 A VP HPLC system (Japan) was used with Lichocart 125-4, C18 column, 250x4.6 mm, 5 µm, and fluorescence detection set at 333 nm excitation and 445 nm emission. The mobile phase consisted of mix water: acetonitrile: acetic acid (525:450:25, v/v/v) and the flow rate was set at 1 mL/min. An ochratoxin A standard was used for the construction of a calibration curve of peak areas versus concentration (µg/L). The injection volume was 20 µL for both standard solution and sample extracts.

Aflatoxins analysis

1. Clean-up of aflatoxins

Five gram of finely ground cocoa beans was added to 0.5 g of NaCl and further extracted with 25 mL of 80% methanol. Suspensions were shaken for 60 min at room temperature. The solutions were filtered through Whatman No.4 filter paper. About 12.5 ml n-hexane was added to the filtrate and shaken. Then 10 mL of the down layer (methanol phase) was diluted in PBS (30 mL) and applied in Aflaprep immunoaffinity column clean-up (R-Biopharm). The column was then washed with PBS (10 mL) and that continued with distilled water (10 mL). Aflatoxins were eluted with methanol (1 mL) into an amber vial. After evaporation to dryness at 40ºC under a stream of N_2, the dry residue was dissolved in methanol (0.2 mL).

2. HPLC parameters

A Water e2695 HPLC system was set at 365 nm excitation and 445 nm emission. Waters symmetry C18 column (4.6x150 mm) was used. The detector was fluorescence Waters 2475 multi-wavelength coupling with UV derivatization (AURA industries). The mobile phase consisted of mix deionized water: acetonitrile: methanol (60:20:20, v/v/v) and the flow rate was 1 mL/min. An AFs standard was used for the construction of calibration curve of peak areas versus

concentration (µg/L). The injection volume was 100 µL for both standard solution and sample extracts.

Result and Discussion

Ochratoxin A contamination in fermented and unfermented cocoa beans

Mycotoxins were found as the contaminant in food products related to the presence of fungal contamination since the post-harvest handling. Profile of OTA contamination on cocoa beans samples was shown in **Fig 1**. Based on OTA profile contamination during 15 days storage, the amount of OTA concentration in the unfermented cocoa beans was higher than the fermented cocoa beans on all days. This contamination was affected by the environmental condition, wherein cocoa beans storage was conducted at 91% RH environment to stimulate mycoflora growth and mycotoxin production. Based on fungal contamination (data not shown), unfermented cocoa beans were dominated by *A. carbonarius* and *A. niger* as known as OTA-producing fungi.

Aflatoxins contamination in the fermented and unfermented cocoa beans

Aflatoxins profile contamination in the fermented and unfermented cocoa beans from the field and during storage simulation was shown in Fig 1. Fermented cocoa beans have a higher AFs contamination than unfermented cocoa beans. Beside the high humidity of the storage, diversity of fungal growth also has an effect on AFs production. In fermented cocoa beans, fungal contamination was dominated by *Aspergillus* section *flavi* (data not shown) as known as AFs-producing fungi.

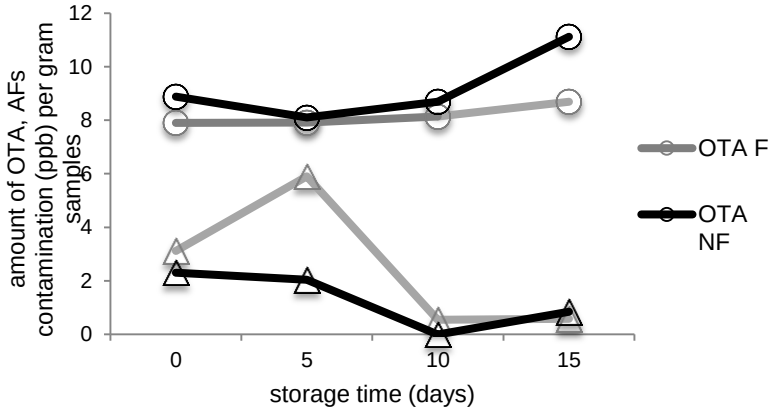

Fig. 1. Profile of OTA and AFs contamination in fermented and unfermented cocoa beans during storage (F: fermented cocoa beans; NF: unfermented cocoa beans)

Ochratoxin A contamination susceptibility in the fermented and unfermented cocoa beans in high storage humidity

The susceptibility of fermented and unfermented cocoa beans to OTA and AFs contamination by mycotoxigenic fungi in high relative humidity during storage by inoculated using *A. ochraceus* were evaluated. This study showed that the a_w of the inoculated samples were 0.955-0.969, which was suitable for fungal growth and mycotoxins production. There were differences between the

minimum a_w of a substrate to allow fungal growth and mycotoxin production. The growth of *A. flavus* and *A. parasiticus* has been verified with a minimum a_w of 0.78, but a minimum a_w of 0.82 and 0.86 were required to produce aflatoxins for *A. flavus* and *A. parasiticus*, respectively (Pitt and Hocking, 2009). The minimum a_w for *A. ochraceus* growth was 0.77 at 25^0C. The optimum a_w to grow and produce OTA was at 0.95-0.99. The ability of *A. niger* strain to grow and produce OTA was in a wide a_w range from 0.92 – 0.99 (Esteban et al., 2006).

Profile of OTA contamination on the inoculated cocoa beans was given in **Fig.2.** The levels of OTA contamination in the fermented cocoa beans were lower than in the unfermented cocoa beans. The highest amount of OTA contamination level was found in the unfermented inoculated cocoa beans after 15 days storage. Based on dominant of fungal contaminants (data not shown), the unfermented inoculated cocoa beans were dominated by *A. carbonarius* and *A. niger*, as known as OTA-producing fungi. It is suggested that higher *A. niger* population in the unfermented cocoa beans may be associated with the availability of fermentable sugar on the unfermented pulp, like glucose and sucrose, which can act as substrate growth. *Aspergillus niger* was species, which was able to ferment these sugars, so the presence of cocoa pulp could support their growth (Magnuson and Linda, 2004). The inoculated *A. ochraceus* was more dominant in the fermented inoculated cocoa beans

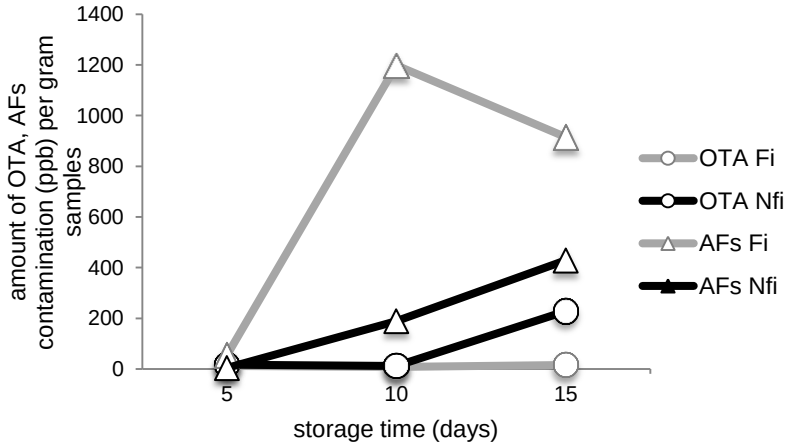

Fig. 2. Profile of OTA and AFs contaminants in the fermented and unfermented cocoa beans during storage simulation in high relative humidity (Fi: the fermented cocoa beans was inoculated with *A. ochraceus*; Nfi: the unfermented cocoa beans was inoculated with *A. ochraceus*)

(data not shown), but OTA contamination in the fermented cocoa beans was lower than in the unfermented cocoa bean. It is suggested that an inhibition of OTA production by *A.ochraceus* was due to the presence of metabolite end-products, which was produced during fermentation but they did not have an effect on *A. ochraceus* growth. From these results, it can be seen that the unfermented cocoa beans were more susceptible to OTA contamination, and black *Aspergili* as OTA-producing fungi were dominant contaminants.

Copetti et al. (2012[b]) reported the influence of the main produced weak organic acids during cocoa fermentation on fungal growth and OTA production by *A. carbonarius* and *A. niger*. In this study, fermentation that was conducted for the enhancement of acetic acid could minimize the problem of OTA contamination in cocoa. The added organic acids to the synthetic medium had an effect

on *A.carbonarius* and *A.niger* growth and OTA production. The highest inhibition was observed with the addition of acetic acid. It is suggested that this inhibition may occur because the weak acids show a dynamic equilibrium and pH-dependent between molecular acids and their respective charged ions. The undissociated state is able to freely penetrate the microbial cell membrane and once inside the cell, furthermore, neutral pH causes weak acid molecules to dissociate into anions and proton (Theron and Lues, 2011).

The inhibition mode of acetic acid is not completely clear. It is generally attributed to the release of protons, acidification of the cytoplasm and dissipation of the membrane pH gradient, which was disrupting normal cell physiology (Theron and Lues, 2011). An experiment carried out by Stratford et al. (2009) demonstrated that acetic acid caused a large and rapid fall in the internal pH of *A.niger* conidia, consistent with its action as a classic weak acid preservative.

Aside from *A. carbonarius* and *A. niger*, there was *Rhizopus* sp and *Mucor* sp, which were found as a fungal contaminant in cocoa beans (data not shown). Varga et al. (2005) reported the OTA degradation by *Rhizopus* sp and non-toxigenic *A. niger*. OTA degradation activity by *Rhizopus* sp and non-toxigenic *A. niger* was possibly due to enzyme production by the fungus. It is suggested that a carboxypeptidase A activity may be responsible for OTA decomposition in these isolates. The products of OTA degradation by carboxypeptidase A resulted in the compounds, which had a retention time close to the ochratoxin α (Abrunhosa et al., 2002).

Aflatoxins contamination susceptibility in the fermented and unfermented cocoa beans in high storage humidity

Profiles of AFs contamination in the analyzed samples of the fermented and unfermented cocoa beans are represented in Fig. 2. The highest amount of AFs was found in the fermented inoculated cocoa beans after 10 days storage. AFs production was influenced by fungal contamination that has been grown. In fermented cocoa beans, fungal contamination was dominated by the inoculated *A. ochraceus* and *Aspergillus* section *flavi* (data not shown). According to literature, about half of the isolates of *A. flavus* were aflatoxigenic and this species produced only AFB_1 and AFB_2, while about 100% of *A. parasiticus* had such ability and synthesized aflatoxins from groups B and G (Klich and Pitt, 1988; Vaamonde et al., 2003). From these results, it can be seen that the fermented cocoa beans were more susceptible to AFs production, and *flavus* group, as AFs producing fungi, were dominant contaminants.

According to Pelaez et al. (2012), mycoflora susceptibility to organic acids depended on the strains. Minimal Inhibitory Concentration (MIC) of lactic acid to *A. flavus* growth was higher than MIC to *A. fumigatus* and *A. nidulan* on the same pH, thus *A. flavus* was considered more resistant to lactic acid. This possibility caused *A. flavus* more dominant than other contaminants growth in the fermented cocoa beans during storage, while other species decreased during storage.

The previous study reported there was an inhibition of AFB_1 production by the presence of OTA (Dimitrokallis et al., 2008). OTA inhibited AFB_1 production by *A. parasiticus* in yeast extract sucrose (YES) medium and the degradation was observed after maximum AFB_1 production. As reported in the literature, one or more enzymes might be involved in aflatoxin degradation by mold mycelia. Shanta and Archana (2002) reported the aflatoxin production by *A. flavus* and *A. parasiticus* gradually increased, which started from the second day of incubation, and reached a maximum in 7-10 days, and then decreased gradually.

Alborez et al. (2005) tested the ability of acidification method using citric acid to degrade AFB_1. These studies showed that aqueous citric acid had detoxification activity in treating aflatoxin-contaminated maize. The results suggested that detoxification of AFB_1 initially involves the formation of the β-keto acid structure, catalyzed by acidic medium, followed by hydrolysis of the lactone ring yielding AFD **(Fig. 3)**.

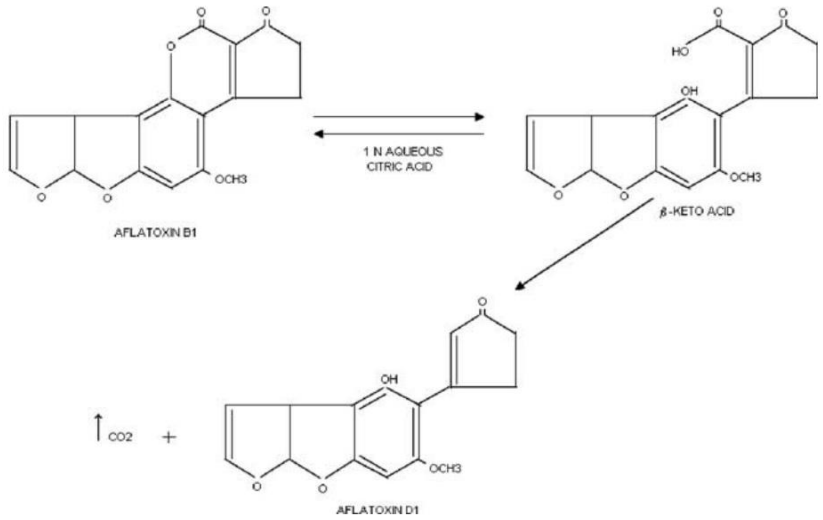

Fig. 3. Proposed mechanism for the acidification of Aflatoxin B_1 to produce Aflatoxin D_1 (Alborez et al., 2005)

Fungi are not only producing AFs, but they are able to degrade them as well. *Aspergillus flavus* is able to convert AFB_1 to aflatoxicol (AFL) by the reducing the cyclopentenone carbonyl of AFB_1. These fungi convert AFB_1 to aflatoxicol-A (AFL-A), then AFL-A is converted to aflatoxicol-B (AFL-B) by the organic acids produced from the fungi (Fig. 4) (Wu et al., 2009).

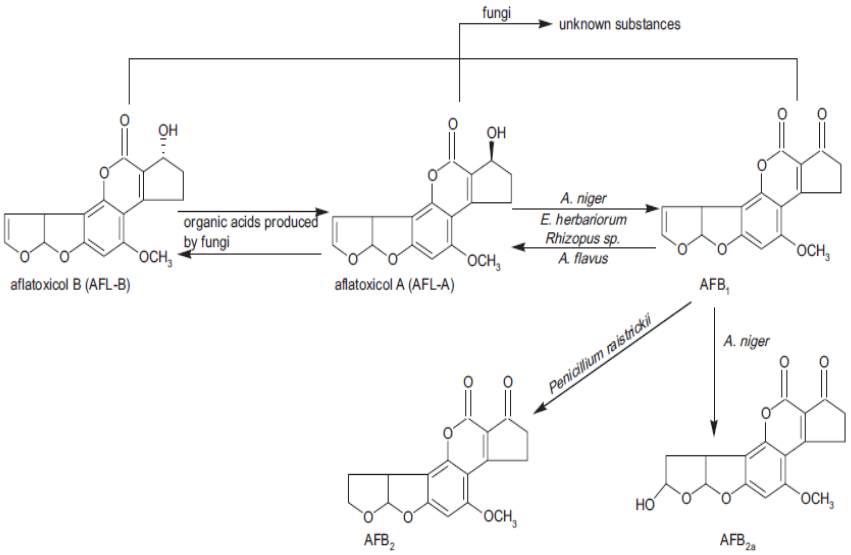

Fig. 4. Degradation of AFB_1 by fungi (Wu et al., 2009)

Conclusion

There was a different pattern between OTA and AFs contamination in the fermented and unfermented cocoa beans associated with the role of organic acids produced during fermentation as the limiting factor on the growth of certain species and their metabolites production on cocoa beans. Fermented cocoa beans were more susceptible to AFs contamination. The higher level of AFs contamination of the fermented inoculated cocoa beans was related to the fungal contamination, which was dominated by *flavus* group according to *A. flavus* resistance to the produced lactic acid during fermentation. Lower OTA contamination in the fermented inoculated cocoa beans indicated that there was an inhibition of OTA production by *A. ochraceus* due to the presence of metabolite end products, which were produced during fermentation. Unfermented cocoa beans were more susceptible to OTA contamination. The higher level of OTA contamination of the unfermented inoculated cocoa beans was related with the fungal contamination, which was dominated by black *Aspergili* associated with the availability of fermentable sugar on the unfermented pulp, like glucose and sucrose, which can act as substrate growth.

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