Mold Contamination and Aflatoxin B₁ Levels in Salted Fish Commodities from Traditional Market in Yogyakarta and Surabaya, Indonesia

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ABSTRACT

Aims: To identify mold contaminant on salted fish, from two different market locations (Kenjeran market, Surabaya and Beringharjo market, Yogyakarta). Furthermore, levels of AFB1 (aflatoxin B1) in salted fish samples were assayed.

Methodology and results: The samples were cultivated on DRBC (Dichloran Rose Bengal Chloramphenicol Agar) and DG-18 (Dichloran (18%) Glycerol Agar) medium for enumeration, then transferred on MEA (Malt Extract Agar) medium for isolation and identification, followed by ELISA test to measure the AFB1 level. Meanwhile aflatoxin biosynthesis correlated genes (i.e. afIR, nor-1 and omtB genes) were identified using Polymerase Chain Reaction (PCR) method. The results showed that Aspergillus tamarii and Aspergillus flavus being contaminant on salted fish along with Aspergillus sydowii, Aspergillus niger, Aspergillus versicolor, Penicillium citrinum, and Penicillium chrysogenum. Rhizopus sp. contamination was also found. AFB1 was positively detected in all of samples with the highest concentration measured was 75.81 µg/kg which belong to Lidah salted fish and the lowest concentration measured was 4.33 µg/kg which belong to Rese salted fish. The suspected A. flavus and A. tamarii isolated from salted fish was positively detected in the presence of afIR, nor-1 and omtB genes.

Conclusion, significance and impact of study: Mold contamination was detected in salted fish from two different markets and all of those samples were contaminated by AFB1. These can be important information related to food safety aspect for salted fish.

Keywords: Salted fish, mold contamination, Aflatoxin B1

INTRODUCTION

Indonesia is an archipelago country with the abundant of fish commodities, which are non-perishable due to high moisture and nutrient content of fish. According to the statistical data on Indonesia’s fisheries year of 2015, fish production in 2014 reached 6.50 million tons. Furthermore, in Indonesia, the most common method to prolong the self-life of fish is fish salting, which are done by adding salt and drying. In Indonesia, salted fish is the second preference after fresh fish to be consumed. It is also often used as an ingredient or condiment in several Indonesian foods.

According to Sutarni (2013), the variation of salt concentration used in the salting process affects the salt penetration into the fish’s body. Although salting the fish may extend the self-life of fish, due to moisture content reduction, the product is often sold uncovered. This can lead to contamination of the product either from dust or insect, such as flies. Sources of contamination also come from poor personal hygiene from both the sellers and the buyers. Based on fact that mentioned before, mold contamination may occur in salted fish product. Moreover, Indonesia’s tropical climate which has a high relative humidity also gives a contribution to mold contamination. In addition, based on Indonesia National Standard of salted fish (SNI 01-2721-1992), which mentioned that mold must not be detected.

The mold can grow at temperatures of 20-30 °C, with a relatively low aw at 0.85 (Rahayu et al., 2014) and affects the appearance of salted fish e.g. color and smell. Furthermore, the most negative effect from mold contamination is mycotoxins, because it cannot be easily detected by the eyes. However, the growths of mold and mycotoxins production are dependent on several factors such as the mold strain and competitor, substrates, temperature and relative humidity.

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The occurrence of mold contamination has been reported in dried salted fish, dried fish and smoked fish. Smoked fish from tropical area has the potential for contamination by toxigenic molds, such as A. flavus (Adebayo-Tayo et al., 2008). A. flavus is also the dominant species identified on smoked fish from Sierra Leone area, followed by contamination of A. ochraceus, A. niger and A. tamarindus (Jonsyn & Lahai, 1992). In addition, A. flavus is also isolated from dried fish in Sri Lanka and Indonesia (Atapattu & Samarajeewa, 1990). Furthermore, A. tamarindus is found to be the predominant contaminant in Maldives fish (Mohamed, 2013). A. sydowii is also isolated from dried fish sold in traditional markets in Jakarta (Santoso et al., 1999).

Some mold can produce mycotoxins as their second metabolite. Aflatoxin, which majorly produced by A. flavus and A. paraciticus, has hepatotoxic, mutagenic, teratogenic, and carcinogenic effect for both humans and animals (Alberts et al., 2006). An outbreak of aflatoxin was first noticed in the early 1960s in the UK (turkey X disease) (Do & Choi, 2007). Furthermore, according to The International Agency for Research on Cancer (IARC, 2002), AFB1 is classified as a group 1 carcinogen which means carcinogenic to humans. The occurrence of aflatoxin is very common in developing countries (Samuel et al., 2009). Moreover, aflatoxin is very heat stable, therefore, they are not easily removed from food, even after processing by high temperature (Rahayu et al., 2003). According to Firsad et al. (2005), there are other molds that have the ability to generate aflatoxin. There are A. nomius, A. pseudotamarius, A. parvisclerotigenus, and A. bombycis of section Flavi, A. ochraceoroseus and A. rambelli from section Ochraceoroseae and Emericella astellata and E. venezuelensis from Nidulatans section.

Because of those reasons, detection and identification of mold contamination on salted fish from traditional market in Indonesia is required as food safety and good manufacturing processes information. The aim of this study was to identify the mold that can contaminate salted fish, and to quantify the AFB1 level in salted fish.

**MATERIALS AND METHOD**

Sample Collection and Preparation

A total of 20 different salted fish samples were used in this study and purchased from two different traditional markets to illustrate mold domination from different supply chain. Salted fish from Kenjeran market, Surabaya were sold in the open-air stalls near the production site, while salted fish from Beringharjo market, Yogyakarta were supplied from another city with semi-permanent stalls. All salted fish were produced traditionally by adding salt followed by sun-drying. Each sample was stored in polyethylene plastic with specific code. Samples were stored in a cool room in the microbiology laboratory, Center Study of Food and Nutrition, Universitas Gadjah Mada. Before performing the tests, the salted fish was allowed to heat up to room temperature (±25 °C).

**Mold Enumeration**

All salted fish samples were inoculated on DRBC media (Oxoid) and DG-18 (Oxoid) directly, large-sized samples were cut with sterile scissors before inoculated. Samples were then incubated at room temperature (±25 °C) for 5 days. Colonies formed were differentiated by appearance and color (i.e. bluish grey, green, brownish green, black, yellow, bluish green and velvet blue colony) for enumeration. Enumeration shows percentage of samples contaminated.

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\text{% of samples contaminated = } \frac{\text{samples contaminated by mold}}{\text{total pieces of analyzed samples}} \times 100\%
\]

The total pieces of analyzed samples can be seen in Table 1.

**Identification of mold contamination**

Colonies that grew on the surface of the sample were then isolated based on the colour differences on MEA media (Oxoid). Then the incubation was done for 5 days at room temperature (±25 °C). Isolates obtained were identified using macro-morphology and micro-morphology according to Rahayu et al. (2014).

**Determination of Aflatoxin B1 Contamination**

Detection of AFB1 in salted fish was assayed using ELISA. Twenty-five grams of crushed samples was extracted using 75 mL of 70% methanol and shook for 3 min. There was no pre-treatment to reduce the salt content in salted fish samples. The extract was then separated from the cake using Whatmann paper No.1. AFB1 content was tested in accordance to the manual on RIDASCREEN Aflatoxin B1 ELISA kit (Bioo Scientific) then absorbance at 450 nm wavelength was measured. AFB1 standard solutions concentrations of 0, 2, 5, 20, and 50 ng/mL were used as the calibration curve. The calculated AFB1 in extract was then converted to AFB1 in weight of samples.

**Detection of aflatoxigenic genes in Aspergillus sp**

Molecular detection of aflatoxigenic genes were performed based on PCR method for amplification of several genes correlated to aflatoxin production, such as aflR, nor-1, and omtB genes. Reaction of PCR amplification was performed in 25 μL of mix PCR using Ready To Go PCR kit mixed with 21 μL ddH2O, 1 μL of each primer, and 1 μL of DNA template. The PCR program of aflR gene based on the procedure operated by Rahimi et al. (2008), amplification was started with 1 cycle initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 2 min, as well as 1 cycle of final elongation at 72 °C for 10 min. In addition,
nor-1 gene amplification was performed according to Geisen (1996), which consisted of 1 cycle initial denaturation at 94 °C for 5 min, 30 cycles of denaturation, annealing, and extension at 95 °C, 1 min; 65 °C, at 2 min; and 72 °C, at 4 min, respectively. While, The PCR program for omTB gene was based on Rodrigues et al., (2007) comprising of 1 step initial denaturation at 94 °C for 3 min continued by 35 cycles denaturation, annealing, extension for 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, respectively and 1 cycle final extension for 10 min at 72 °C.

RESULTS AND DISCUSSION

Mold Contamination of Salted Fish

From observation of the incubated samples, it became clear that all of the samples on DRBC and DG 18 media are contaminated by mold as seen on Figure 1. Yeast colonies were also found on the salted fish, but was not identified. Contamination on examined salted fish was dominated by Aspergillus, followed by Penicillium. Hence, suspected aflatoxin producer was also successfully isolated (Figure 1) and identified as A. flavus and A. tamarii. This was in alignment with result from Hassan et al., (2011) that the most dominant contamination of salted fish which is randomly collected from different shops and retail markets at Giza Governorate was by Aspergillus sp members, at about 83.3% with Aspergillus flavus contamination being at 66.6%. According to Pitt & Hocking (2009), Aspergillus and Eurotium were the most dominant contamination species found on dried food in tropical and subtropical regions. Aspergillus and Eurotium were also dominant on smoked dried fish from warm water regions (Adebayo-Tayo et al., 2008). This was caused by Aspergillus which usually grows faster than Penicillium but takes longer to sporulate (Rahayu et al., 2014).

The samples obtained from Kenjeran market Surabaya had different dominant mold from those obtained in Beringharjo market, Yogyakarta. This was due to differences in the supply chain and the handling. Samples from Kenjeran market, Surabaya were sold in coastal and open roadside stalls, in addition the production sites and the dried fish stalls were close to each other. While the samples from Beringharjo market, Yogyakarta consisted of salted fish supplied from Pati, Central Java and the stalls were all in one room, with not only salted fish stalls but also another staple food (i.e. vegetables, fruits, tubers, and beef). According to Sharma (2012), A. niger is commonly found in indoor environment, fruit and vegetable. This explains that mold domination in Beringharjo market comes from the environment of the stalls. On the other hand, Aspergillus sp are closely associated with the soil (Owaga et al., 2009). Therefore, it is possible that the sun-drying of salted fish on the roadside of Kenjeran market could have exposed with Aspergillus sp, particularly from A. flavus.

The frequency of mold contamination on salted fish is shown in Table 2. Samples from Kenjeran market, Surabaya showed that all samples were found to have mold contamination. The dominant mold was 100% A. tamarii (9/9 samples) and 89% A. flavus (8/9 samples). Lidah salted fish had the highest frequency of A. flavus contamination which was about 92%. Spores of A. flavus were found in the tropical air. In Indonesia itself, which is a tropical country, the drying of salted fish still takes the advantage of sunshine, thus increasing the likelihood of contamination by fungal spores. The samples from Beringharjo market, Yogyakarta were also contaminated by mold. The dominant molds were A. niger, A. flavus, A. tamarii, and Rhizopus sp with the frequency of each contamination approximately 45% (5/11 samples). The highest A. flavus contamination was found on Pethek salted fish at the level of 50%. By the presence of A. flavus on salted fish, it is possible to find aflatoxin contamination.

Figure 1: (a) Mold diversity contaminating on salted fish (upper-side is on DG-18 medium and down-side is on DRBC medium). (b) Suspected aflatoxin producer, identified as A. flavus (upper-side) and A. tamarii (down-side).
Another important genus which was identified is *Penicillium*, although it had a lower frequency than the *Aspergillus* genus. *P. citrinum* and *P. chrysogenum* were also identified on some salted fish samples. The highest *P. citrinum* contamination was found on Ebi salted fish, at about 50% and the highest *P. chrysogenum* contamination was found on Lidah salted fish, at about 33%. *P. citrinum* produces citrinin toxin which is nephrotic and carcinogenic for animals (Flajs & Peraice, 2009).

Seven isolates identified had halotolerant and xerophilic properties. *A. sydowii* is a mold mostly found on marine organisms. According to Prakash et al. (2011) *A. flavus* and *A. niger*, which originated from dried seafood products in India showed salt resistance of up to 18%. *A. flavus* was also found to be the dominant contaminant on smoked dried fish (Adebayo-Tayo et al., 2008). In the case of Maldives fish, which is a salted fish from the Maldives region, *A. tamarii* contamination was higher than *A. flavus* contamination, because Maldives fish has a low salinity at an average 2.08% (Mohamed, 2013). It is similar to the total mold contaminant in samples from Kenjeran market, Surabaya.

### Aflatoxin B1 contamination in Salted Fish

The presence of *A. flavus* indicates the possibility of contamination by AFB1 in salted fish. Out of the samples analyzed, all were positively contaminated by AFB1. The highest AFB1 contamination was on the Lidah salted fish taken from Kenjeran market, Surabaya which had 75.81 µg/kg. And the lowest AFB1 contamination was on Rese salted fish taken from Beningharjo market, Yogyakarta which had 4.38 µg/kg. The AFB1 levels of the salted fish are shown in Table 2.

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### Table 1. Macro- and micro-morphology of identified mold.

| Isolate code | Macro morphology | Micro morphology | Mold identified |
|--------------|------------------|------------------|-----------------|
| HAF1         | Green colony, ±50 mm colony diameter, reverse colony creamish-yellow | Biseriate, spherical vesicle, hyaline, long conidiophores | *A. flavus* |
| BAAS1        | Bluish gray colony, ±15 mm colony diameter, produce exudate, reverse colony brown | Biseriate, circular vesicle, hyaline, cylindrical phialides, small conidia | *A. sydowii* |
| CAT2         | Solid brownish green colony, ±50 mm colony diameter, reverse colony creamish-yellow | Biseriate, long conidia, spherical vesicle, hyaline, cylindrical phialides | *A. tamarii* |
| KAV2         | White colony at the beginning and turn to yellow, velvet-like appearance, ±10 mm colony diameter, reverse colony cream | Biseriate, oval (spoon-shaped) vesicle, spreading conidia, hyaline | *A. versicolor* |
| HAN1         | Black colony, ±60 mm colony diameter, reverse colony dark creamish-yellow | Biseriate, black spherical vesicle, long conidiophores, circular conidia, cylindrical phialides | *A. niger* |
| HBPC1        | Velvet blue colony, ±25 mm colony diameter, reverse colony pale yellow, produce exudate | Tervertisilata, hyaline, cylindrical phialides | *P. chrysogenum* |
| HAPC2        | Bluish green colony, ±25 mm colony diameter, produce exudate, slow growth, reverse colony pale yellow | Bivertisilata, hyaline, cylindrical phialides | *P. citrinum* |

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The concentration of aflatoxin in samples was determined from the standard calibration curve plotted in the range of 0, 2, 5, 20, and 50 ng/mL using ELISA. Linear regression analysis was further used for the quantification of aflatoxin present within the samples. The standard calibration graph showed excellent linearity with R² value of 0.9967. The limit of detection and quantification of ELISA method were 0.34 and 1.02 ng/mL respectively.

If the result of ELISA test is correlated with the frequency of *A. flavus* contamination on the samples (Figure 2), it can be fully understood that the amount of AFB1 in the samples was not affected by the frequency of *A. flavus* contamination. Hence, if the ELISA result compared to mold species contaminated on samples, not all samples were contaminated by *A. flavus* which is an AFB1 producer. Meanwhile, all of samples were positively contaminated by AFB1 in different amounts.

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**Figure 2. Correlation Frequence of Aspergillus flavus and Aflatoxin B1 level.**
Table 2: Frequency of mold contamination and Aflatoxin B1 levels in salted fish samples.

| Salted Fish | Species                  | Analyzed sample | Source                          | Aflatoxin B1 (µg/kg) | Frequency of Mold Contamination (%)* |
|-------------|--------------------------|-----------------|---------------------------------|----------------------|-------------------------------------|
| Bulu bebek  | Thryssa mystax           | 8               |                                 | 8.52                 |                                     |
| Campur      | Johnius coitor           | 18              |                                 | 9.63                 | 22 28 39 - 25 - 38 25 - 25          |
| Glomo       | Anguilla rostrata        | 12              | Kenjeran market, Surabaya (9 samples) | 16.14               | 42 25 17 33 - 25 -                  |
| Jannan      | Mystus planiceps         | 12              |                                 | 9.63                 | 50 25 8 50 25 8                    |
| Kindo       | Unknown                  | 11              |                                 | 13.29                | 18 36 - 27 - 33 - 55                |
| Lidah       | Cynoglossus lingua      | 12              |                                 | 75.81                | 92 25 8 25 25 33 -                  |
| Talang      | Chorinemus tala          | 12              |                                 | 13.77                | 42 50 - 8 - -                       |
| Udang       | Litopenaeus vannamei     | 12              |                                 | 27.42                | 25 25 8 17 - -                      |
|             |                          |                 |                                 |                      |                                     |
| Banyar peda | Rastrelliger             | 12              |                                 | 26.37                | 8 8 - - - -                         |
| Belek       | Unknown                  | 12              |                                 | 7.59                 | - - - - 8 -                        |
| Ebi         | Litopenaeus vannamei     | 12              |                                 | 6.24                 | - - - - - -                         |
| Kacangan    | Charangidae sp           | 12              |                                 | 9.39                 | - - 17 - - - - 25 -                |
| Layur       | Trichurus savala         | 12              | Beringharjo market, Yogyakarta (11 samples) | 33.72               | - 8 - 17 - 33 8 - 17 8 -          |
| Pedho alit  | Rastrelliger             | 12              |                                 | 7.98                 | 33 8 - - - - - - 17 -              |
|             | scombridae              |                 |                                 |                      |                                     |
|             | Leiognathus equulus      | 8               |                                 | 5.61                 | 50 - - - - 63 - 100 -              |
| Pethek      | Acetes indicus           | 30              |                                 | 4.38                 | 33 7 7 - - - 30 -                  |
| Reise       | Unknown                  | 12              |                                 | 10.80                | - - - - - - - - - - 23             |
| Teri        | Stolephorus trifasciatus | 10              |                                 | 6.48                 | 10 - 20 - - 10 20 - -             |
| Teri Nasi   | Stolephorus citrinus     | 22              |                                 | 11.19                | - 9 - - - 14 -                    |

*The number expressed as the percentage was calculated in relation to the amount of analyzed samples.

Aflatoxin contamination has no correlation to the frequency of A. flavus on samples because it does not grow well. The growth of A. flavus was resisted by other more dominant molds, so there were some samples that were not contaminated by A. flavus. In addition, according to Abidin, et al. (2010) A. tamarii also can produce AFB1 and AFB2, probably the presence of aflatoxin was produced by A. tamarii instead of A. flavus. Moreover, the ELISA method has low specificity to detect other components which have a similar structure to AFB1, such as AFB2, AFG1 and AFG2 (Leszczynska, et al., 2001). The salt content in the samples caused a matrix effect that can affect the absorbance measurement. It is also possible a cross-linking component to occur in the sample, thus affecting the selectivity and specificity of ELISA measurement (Rachmawati, et al., 2004). Because of those reasons, ELISA method has a limitation for analyzing AFB1 in salted fish samples, regarding there was no pre-treatment to reduce the salt content in samples.

Molecular detection of aflatoxin biosynthesis genes in Aspergillus strain

Fungal group of Aspergillus (green Aspergilli and black Aspergilli) found in salted fish were then selected for aflatoxicogenic detection based on PCR amplification method. Three types of genes involved in aflatoxin biosynthesis namely aflR, nor-1, and omtB were used in detecting aflatoxin production ability. Based on amplification result, primer aflR, nor-1, and omtB were able to amplify an expected for approximately 600 bp, 400
bp, and 1000 bp fragment in aflatoxigenic isolates, respectively (Figure 3). Aflatoxigenic and non-aflatoxigenic strains could be determined on the basis of amplification of these three target DNA fragments.

Based on Table 3, it demonstrated that almost all isolates of green Aspergilli showed positive results in the presence of aflR, nor-1, and omtB genes. The positive results against those primer were A. flavus and A. tamari. Meanwhile, none of black Aspergilli have those three genes detected. Two isolates only have both regulatory genes, aflR and nor-1, while the other two isolates have none of three target genes.

The existing of three genes target involved in aflatoxin biosynthesis such as aflR, nor-1, and omtB may indicate that the isolate was able to produce aflatoxin. PCR analysis was able to amplify aflatoxin biosynthetic genes, i.e aflR, nor-1, and omtB in almost green Aspergilli group, while none of omtB gene detected in black Aspergilli. Aflatoxin, a secondary metabolites which is a polyketide-derived, is produced via the following conversion path: acetate polyketide → anthraquinones → xanthones → aflatoxin (Bhatnagar, Ehrlich & Cleveland, 2003; Yu, 2012). However, aflatoxin formation depends on the final step of aflatoxin biosynthesis pathway. In this case, Norsolorinic Acid (NOR) is the earliest step and the first stable aflatoxin precursor in the aflatoxin biosynthetic pathway; thus it play a role in polyketide synthase (Yu et al., 2004 and Yu, 2012). In contrast, O-methyltransferase (omtB) involve in the later step of aflatoxin formation. Meanwhile, aflR is a positive regulatory gene which is required for transcriptional activation of most of the structural genes such as nor-1 and omtB (Yu et al., 2004). Thus, the absence of omtB gene in such black Aspergilli isolate might indicate that those are unable to produce aflatoxin, while the presence of those three target genes in green Aspergilli are possible to support the correlation of ability in aflatoxin production.

**Table 3. Detection of Aflatoxigenic and non-aflatoxigenic Aspergillus group based on PCR amplified product.**

| Code | Mold species | Aspergillus group | Primer AFB1 nor-1 aflR omtB |
|------|--------------|-------------------|---------------------------|
| IC1  | A. tamarii   | Green Aspergilli  | - - -                     |
| IC2  | A. tamarii   | White Aspergilli  | + + +                     |
| IHK1 | A. flavus    | Black Aspergilli  | + + +                     |
| IHK2 | A. flavus    | Black Aspergilli  | + + +                     |
| IB   | A. niger     | Black Aspergilli  | - - -                     |
| IH   | A. niger     | Black Aspergilli  | + + +                     |
| IHIT | A. niger     | Black Aspergilli  | + + +                     |

**Figure 3.** Amplified product of five target genes in Aspergillus. (A) Amplified product of aflR gene in Aspergillus; (B) Amplified product of nor-1 gene in Aspergillus. (C). Amplified product of omtB gene in Aspergillus.
CONCLUSIONS

A. tamarindii, A. flavus, A. sydowi, A. niger, A. versicolor, P. citrinum, P. chrysogenum and Rhizopus sp are identified being contaminant on salted fish. Salted fish samples from Kenjeran market were dominated by A. tamarindii and A. flavus, while salted fish samples from Beringharjo market were dominated by A. flavus and A. niger. The difference of supply chain from both sources of samples affects the mold domination. In addition, AFB1 was detected positively in all of samples with highest level belong to Lidah salted fish (75.81 µg/kg) and the lowest AFB1 level belong to Rese salted fish (4.33 µg/kg). Besides, ELISA test has a limitation for quantify AFB 1 level from salted fish, due to the high salt content that can be interference for absorbance measurement. The suspected A. flavus and A. tamarindii isolated from salted fish were positively detected in the presence of aflR, nor-1 and omtB genes.

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