Prevalence of Aflatoxigenic *Aspergillus* sp. in Dried Salted Fish from Traditional Market in Bandung City, Indonesia

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Abstract. Salted fish is an important source of protein and income in Bandung City, Indonesia. Meanwhile, salted fish products that are contaminated with aflatoxin-producing molds can pose a considerable hazard to consumers’ health. This study aims to determine the presence of aflatoxigenic *Aspergillus* sp. that contaminates salted fish products. A total of 8 samples of dried salted anchovy and 7 samples of dried salted cotton fish from 8 retailers in traditional market were analyzed for fungal contamination, sample physicochemical factors, and prevalence of aflatoxigenic *Aspergillus* sp.. This research was divided into 4 stages, namely, total mold calculation, identification of molds through morphological observations, isolation, screening, and molecular identification of aflatoxin-producing molds. Using internal transcribed spacer (ITS), *Nor-1*, and *Ord1* primers, 8 out of 66 mold isolates from salted fish samples were isolated and identified. The results showed that the dominant genera in dried salted anchovy and cotton fish are *Aspergillus* sp. (36.8% and 53.6%), followed by other genera (36.8% and 28.6%) and *Penicillium* sp. (26.3% and 17.9%). The total fungal counts in the dried salted anchovies and cotton fish ranged from $2.50 \times 10^2$ to $4.00 \times 10^2$ cfu/g, and $5.00 \times 10^0$ to $1.40 \times 10^2$ cfu/g. The prevalence of dried salted anchovy samples was 13.16% (3 of 28) and in the dried salted cotton fish sample was 12.12% (5/38). In general, the characteristics of positive aflatoxigenic samples have a temperature of 26.3 to 38.0°C, relative humidity of 44% to 59%, salt content of 3.06% to 16.06%, aw 0.71 to 0.79, and pH 6.13 to 8.75. The presence of aflatoxigenic *Aspergillus* sp. in salted fish sold in the Bandung market poses a potential hazard to consumer health.

1. Introduction

Fish is an important dietary component for the world's people and the most traded, also a very valuable source of protein [1,2]. Several traditional fish processing techniques such as salting, drying and smoking have been used to preserve and increase the availability of fish for local communities [3]. Likewise in Indonesia, there are known traditional processed products such as salted fish, *pindang* fish and fermented fish. Specifically for fish salting activities, in West Java, it is reported that there are 676 Fish Processing Units (UPI), spread over the areas of Cirebon (197 UPI), Sukabumi (56 UPI), Bogor (17 UPI) and Bandung (9 UPI) [4]. Fish production in West Java Province in 2017 was around 1.4 million tons [5]. Meanwhile, the fish consumption rate in the city of Bandung reached 37.90 kg per
capita in 2019, which is higher than the fish consumption rate in West Java, which is 29.6 kg per capita [6].

Salted fish is one of the processed fishery products that are widely consumed by the people of Indonesia, both from the bottom until the top. Likewise in the city of Bandung, consumption of salted fish is quite high which can be seen from distribution of fish in this area. Indriati et al [7] report that the speed of distribution of salted fish in West Java is higher than East Java. This means that the level of consumption of salted fish in the people of West Java is higher than the people of East Java. Most of the fish in the Bandung area comes from Andir Market which is the center of salted fish sales in Bandung, but a small portion comes from Medan, Pekalongan, Central Java, Indramayu and East Java. Therefore the Bandung area can be said as Indonesia’s largest salted fish market.

Previous research reported that salted fish products at the retail level were contaminated by several types of pathogenic fungi that produce aflatoxin B1 (AFB1), including *Aspergillus flavus* (*A. flavus*), *A. parasiticus*, *A. niger* and *A. tamari* [8]. Aflatoxin is one of five types of mycotoxins that are often found in food products. Fungi that produce mycotoxins are divided into field fungi (invading before harvest) and storage fungi (occurring after harvest) [9]. Aflatoxin are considered as carcinogenic, mutagenic, teratogenic, hepatotoxic and immunosuppressive substances that cause acute liver damage, liver cirrhosis, tumors and teratogenic effects [10,11].

Salted fish samples from India were positively contaminated by *A. niger*, *A. flavus*, *A. fumigatus*, *Absidia*, *Aerobasidium*, *Alternaria*, *Cladosporium*. While the salted fish from Sudan were contaminated by *A. niger*, *Alternaria*, and *Penicillium* [12,13]. Later, Ayotunde, Ada, Udeh & Otu [14] reported contamination of *A. flavus*, *Penicillium* and *Mucor* in salted catfish, cod and lemurin originating from Nigeria. Hassan et al. [15] stated that the most dominant contaminant in salted fish was *Aspergillus* (83.33%). *Aspergillus* and *Eurotium* are the main contaminants in dry food products. *Aspergillus* usually grows faster than *Penicillium* in tropical and subtropical regions [16]. This research was conducted to determine the presence of aflatoxigenic *Aspergillus* sp. which contaminates salted fish products in traditional markets in Bandung city.

2. Material and Methods

2.1. Sampling collection and preparation

A total of 15 samples including eight dried salted anchovy and seven dried salted cotton fish were randomly sampled from eight retailers in traditional market in Bandung city, Indonesia. Salted fish samples were put into sterile polythene bags, sealed, labeled and then placed in a cool box, and transported to microbiology laboratory, Research Center for Marine and Fisheries Product Processing and Biotechnology (RCMFPPB) for further analysis. The samples were stored at ambient temperature for future analysis. The environmental conditions around the market were recorded as well as the social conditions of the surrounding community. In addition, measurements of air temperature and in situ air humidity were also carried out. Physical properties determination (Aw, salt content, and pH) were carried out according to methodology Asurmendi et al., [10]. For aflatoxin analyses, the samples were stored in refrigerator at 4°C.

2.2. Quantitative enumeration

Quantitative enumeration of *Aspergillus* spp. carried out using pour plate method. Ten gram of each dried salted fish sample was homogenized in 90 ml sterile butterfield peptone water (0.1%) and diluted from 10^-4 to 10^-1. Aliquots of 0.1 mL were inoculated in triplicate onto dichloran 18% glycerol agar (DG18) [14]. Plates were incubated for 7 until 8 days at 25 °C and the results were expressed as CFU/g of sample. In the early stages, isolates were selected based on the color and texture of the different colonies that were seen morphologically on the petri dish according to Pitt and Hocking [16].

2.3. Phenotypic morphology observation and identification of the aflatoxigenic *Aspergillus*

Observation of phenotypic morphology of fungi refers to Hermana et al. [17] using a morphological approach (macroscopic and microscopic). Fungal isolates that showed different colors and textures
(point 2.2), were then transferred to MEA (Malt Extract Agar) media and CYA media (Czapeks Yeast Agar). With an ose needle, the spores were inserted into tween 80, stirred, and inoculated on the MEA and CYA plates each at 3 points, then incubated for 5-7 days at 25°C. Macroscopic observations consist of colony surface color, reverse side color, diameter and texture of fungi colonies. Microscopic observations were carried out using a glass object that was dripped with lactophenol blue, the fungi were observed with a magnification of 100-1000x. Fungal morphology was seen based on the shape of the fruiting bodies (conidiophores, vesicles, metulae, phialids and conidia).

Afterwards, *Aspergillus* spp. isolates identified with AFPA media according to Variane *et al.*, [18] to determine its potential as a producer of aflatoxins. Identification with AFPA is as follows: *Aspergillus* spp. isolates were inoculated into a Petri dish of diameter 10 cm (in triplicate) containing *A. flavus* and *parasiticus* agar (AFPA). Aseptically inserted into the medium 256.4 g/mL of streptomycin to inhibit bacterial growth. Supplements are added after autoclaving and cooling to 60°C. The culture was incubated at 25°C for five days.

2.4. Fungal DNA extraction

The genomic DNA of the *A. flavus* was isolated using fungal DNA extraction kit according to protocol The DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Isolated fungi that have been identified (point 2.3) were inoculated aseptically into MEA media and incubated at 25°C for 4 days. Isolate was crushed in sterile plastic using a mortar and transferred to a tube of ±0.25 g. A total of 180 µL of ATL buffer and 20 µL of proteinase-K were added and then vortexed and incubated at 56°C for 20 to 30 minutes with every 5 minutes shaking until the sample was completely dissolved. AL buffer was added as much as 200 µL and vortexed. A total of 200 µL of absolute ethanol was added and vortexed again.

The solution in the microtube was transferred to a spin column, then centrifuged for 1 minute at 8,000 rpm at 25°C. The supernatant was discarded and added 500 µL of AW1 buffer and mix by pipetting. The mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube and then centrifuge for 1 min at 8000 rpm. The flowthrough was discarded. Then, this step was repeated with the remaining sample. The spin column was placed into a new 2 ml collection tube and was Added 500 µL Buffer AW2, centrifuged for 1 min at 8000 rpm. The flowthrough was discarded. Another 500 µL Buffer AW2 was added into the mixture and was centrifuged for 2 min at 20,000 rpm. Later, the spin column was transferred to a new 1.5 ml microcentrifuge tube. Add 100 µL Buffer AE for elution, Incubate for 5 min at room temperature (15–25°C), then centrifuge for 1 min at 8000 rpm. Subsequently, add another 100 µL Buffer AE for elution, Incubate for 5 min at room temperature (15–25°C), then Centrifuge for 1 min at 8000 rpm. Furthermore, the DNA product was stored in a freezer at -20°C.

2.5. Identification of *Aspergillus*

Identification of *Aspergillus* DNA used universal mold primers namely, ITS'1 (5'-TCC-GTA-GGT-GA-CCT-GCG-G-3'), ITS'4 (5'-TCC-TCC-GCT-TAT- TGA-TAT-GC-3') [19].

2.6. PCR reactions

Individual PCR reactions were carried out by making a premix solution consisting of 7.5 µL master mix, 1 µL primer, and 2.5 µL buffer TE. Each 1 µL of sample DNA was inserted into a microtube containing 14 µL of premix solution, vortexted and spinned. The DNA amplification process was carried out in a thermocycler (Bio-Rad, USA). The conditions for PCR were as follows: pre denaturation 94°C for 3 minutes, final denaturation 94 C for 1 minute, annealing 55°C for 1-minute, initial extension 72°C for 1 minute, and final extension 72°C for 7 minutes. The time required for one run is about 2 hours with 30 cycles.

2.7. Detection of genes encoding aflatoxin production

Two target genes used in this study are genes involved in aflatoxin biosynthesis such as nor-1 [20] and Ord-1 [21]. The gene primers used were Nor1-F (5'-ACC GCT ACG CCG GCA TCT GCT-GCA-3'),
Nor1-R (5’-GTT GGC CGC CAG CTT CGA CAC TCC G-3’), Ord1-F (5’CGACTGTGTTGGCCTTTTCATT-3’), and Ord1-R (5’ATAGCGAGGTTCCAGGTAA-3’).

2.8. Gel electrophoresis
The amplified DNA fragments and 100 bp DNA marker ladder of 100 bp (Qiagen) were separated using 1.5% agarose gel. Electrophoresis was carried out at a voltage of 100 V and a current of 50 A for 25 minutes. Visualization was performed under UV light using a UV transilluminator, the Geldoc™ MP imaging system (Bio-Rad Laboratories, California, USA) after staining with Tris Boric acid EDTA (TBE) buffer solution and 3µl SYBR gold [22].

3. Results and discussion
3.1. Isolation and contamination of fungi in salted fish
A total of 66 isolates of fungi have been isolated from 8 samples of anchovy and 7 salted cotton fish. The morphology of Aspergillus colonies grown on CYA and MEA media was clearly visible on the 7th day with an incubation temperature of 25 degrees. Morphological appearance of Aspergillus spp. green like grass, has a white stripe edge, velvety texture, colony diameter 3.5-5 cm (Figure 1).

![Figure 1. Aspergillus spp. (a) colonies on CYA and MEA, 7 days, 25°C; (b) Aspergillus niger; (c) Penicillium; (d) Aspergillus spp.; (e) Aspergillus flavus](image)
**Figure 2.** Microscopic mold morphology (a) conidia head; (b) vesicle; (c) conidia (spore)

**Figure 3.** Contamination frequency (%) of fungal genera in dried salted anchovies

| Fungal Genera         | Frequency (%) |
|-----------------------|---------------|
| Aspergillus sp. (%)   | 53.6          |
| Penicillium sp. (%)   | 17.9          |
| Other genera (%)      | 28.6          |

**Figure 4.** Contamination frequency (%) of fungal genera in dried salted cotton fish

| Fungal Genera         | Frequency (%) |
|-----------------------|---------------|
| Aspergillus sp. (%)   | 36.8          |
| Penicillium sp. (%)   | 26.3          |
| Other genera (%)      | 36.8          |
In this study, the dominant genera in dried salted anchovy and cotton fish are *Aspergillus* sp. (36.8% and 53.6%), followed by other genera (36.8% and 28.6%) and *Penicillium* sp. (26.3% and 17.9%) as presented in Figures 3 and 4. Other molds found were of the *Rhizopus* and *Mucor* species, as well as other types. Nadhira et al. [23] revealed that *Mucor, Rhizopus* and *Curvularia* are the most common types of molds that produce mycotoxins and often contaminate human food and animal feed.

The results of this study are similar to Rafli et al. [24] which stated that *Aspergillus tamarii* and *A. flavus* were the main contaminants in salted fish, followed by *A. sydowii, A. niger, A. versicolor, Penicillium citrinum,* and *P. chrysogenum*. In addition, the presence of *Rhizopus* was also found. Hidayah, Hermana and Kusmarwati [25] said that from 10 samples of salted fish obtained at the market in Bandung, 5 samples of salted fish were detected as *A. flavus*. The five samples were Anchovies (*Stolephorus* sp.), Whipfin silverbiddy (*Gerres filamentosus*), Commerson's anchovies (*Gerres filamentosus*), Medan anchovy (*Stolephorus commersonii*), Snakehead fish (*Trichogaster microlepis*), and Moonlight gouramy (*Channa striata*). The results are similar to Nyamwaka, Nyamache, Maingi [26] who reported that the most of fungi grow in dried salted fish sold in market. Among the fungi that grow, *Aspergillus* is the most commonly found.

The Fungi from *Aspergillus, Fusarium* and *Penicillium* species are mostly environmental contaminants. Consequently, the consumption of salted fish contaminated with these fungi becomes a very important health issue [26]. *Penicillium citrinum* was detected in the dried fish samples and also reported in dried fish samples sold in Giza, Egypt [15]. *Aspergillus tamarii* belongs to the *Aspergillus* section Flavi and occurs widely in tropical and subtropical foods. It is closely related to *Aspergillus flavus* which produces aflatoxins, potent carcinogens [28].

### Table 1. Prevalence of aflatoxigenic *Aspergillus* sp.

| Dried salted fish | No. Of samples | N. Of isolates | AFPA (%) | ITS (%) | aflD (%) | aflQ (%) |
|-------------------|----------------|---------------|----------|---------|----------|---------|
| Anchovies         | 8              | 28            | 3 (10.71)| 3 (10.71)| 3 (10.71)| 3 (10.71)|
| Cotton fish       | 7              | 38            | 5 (13.16)| 5 (13.16)| 5 (13.16)| 5 (13.16)|
| Total             | 15             | 66            | 8 (12.12)| 8 (12.12)| 8 (12.12)| 8 (12.12)|

Based on Table 1, both anchovy and cottonwood samples have the opportunity to produce aflatoxigenic *Aspergillus* sp., which is the prevalence of these samples 10.71% and 13.16%, respectively. Therefore, it can more or less pose a threat to human health. As reported that *A. flavus* is widely known to be capable of producing mycotoxins that are carcinogenic so that it poses a serious threat to agriculture, industry, human and animal health [29,30]. Aflatoxin is a secondary metabolite produced by fungi, where the prevalence of aflatoxin from *A. flavus* with hepatocarcinogenic compounds in it ranks sixth of compounds that poses a health risk [30]. *Aspergillus flavus* is one of the most typical spoilage fungal species [32,33]. Aflatoxin-producing fungi have the potential to produce mycotoxins [32]. High aflatoxin contamination at market indicates a lack of control or supervision over the contamination of *A. flavus* [34]. There are many factors that influence safety of the dried fish product. Firstly, unhygienic processing environment and undesirable storage conditions such as high temperature and humidity. Secondly, storage condition such as oxygen concentration and also, consumers inadvertently transfer some fungi to the products when they select them by hand [33].
### Table 2. The water activity, salt content, and pH in the salted fish samples (salted anchovies)

| No | Sample code of salted anchovies | Water activity (%) | Salt content (%) | pH | Temperature °C | RH (%) | Total of mold cfu/g | Market origin |
|----|---------------------------------|--------------------|------------------|----|----------------|--------|---------------------|--------------|
| 1  | SD1T                            | 0.79±0.00          | 5.21±0.07        | 8.03 | 37.0          | 44.0   | 65*                 | Sederhana    |
| 2  | CR2T                            | 0.80±0.00          | 7.80±0.18        | 8.32 | 30.8          | 49.0   | 15*                 | Caringin     |
| 3  | UB2T                            | 0.75±0.00          | 9.00±0.08        | 6.96 | 27.0          | 60.0   | 5*                  | Ujung Berung |
| 4  | CR3T                            | 0.75±0.00          | 12.75±0.18       | 6.09 | 29.5          | 54.0   | 30*                 | Caringin     |
| 5  | AN1T                            | 0.78±0.00          | 12.89±0.26       | 7.98 | 30.5          | 42.0   | 45*                 | Andir        |
| 6  | AN2T                            | 0.78±0.00          | 14.78±0.45       | 8.75 | 38.0          | 43.0   | 65*                 | Andir        |
| 7  | UB1T                            | 0.76±0.02          | 3.06±0.28        | 7.01 | 26.2          | 58.0   | 10*                 | Ujung Berung |
| 8  | KC1T                            | 0.78±0.00          | 6.93±0.04        | 7.53 | 29.1          | 49.0   | 140*                | Kiara Condong|

* mean beyond the number of colonies 25-250

### Table 3. The water activity, salt content, and pH in the salted fish samples (salted cotton fish)

| No | Sample code of salted cotton fish | Water activity (%) | Salt content (%) | pH | Suhu °C | RH (%) | Total of mold cfu/g | Market origin |
|----|----------------------------------|--------------------|------------------|----|---------|--------|---------------------|--------------|
| 1  | AN1K                             | 0.71±0.00          | 10.91±0.40       | 6.33 | 30.6    | 42.0   | 105*                | Andir        |
| 2  | UB1K                             | 0.72±0.00          | 9.40±0.59        | 6.27 | 26.5    | 58.0   | 40*                 | Ujung Berung |
| 3  | SD1K                             | 0.73±0.00          | 16.06±0.22       | 6.62 | 38.0    | 44.0   | 75*                 | Sederhana    |
| 4  | CR2K                             | 0.71±0.00          | 7.63±0.26        | 6.36 | 30.5    | 49.0   | 25*                 | Caringin     |
| 5  | CR3K                             | 0.73±0.02          | 13.37±0.76       | 6.13 | 29.9    | 55.0   | 80*                 | Caringin     |
| 6  | KC1K                             | 0.71±0.01          | 5.43±0.39        | 7.04 | 29.4    | 48.0   | 175*                | Kiara Condong|
| 7  | AN2K                             | 0.79±0.01          | 3.23±0.15        | 7.37 | 26.3    | 59.0   | 400*                | Andir        |

* mean beyond the number of colonies 25-250
Water activity (aw) is one of the parameters used to know the existence of free water in a foodstuff. Microorganisms can grow with different aw values [16], where bacteria can grow on media with a value of aw 0.9, khamir 0.85, while the fungi 0.65 – 0.95 [17]. Salted anchovies water activity in this study ranged from 0.75-0.80, and in salted cotton fish was slightly lower which ranged from 0.71-0.79. It is reported that in the aw value range less than 0.85, the serophilic mold can grow, resulting in Aspergillus spp. was easier to grow on salted fish samples. Other studies mention aw levels in salted fish range from 0.69 – 0.71 [35]. In this study, the minimum aw for growth (0.71) occurred at 29.4°C lower than the minimum of 0.85 aw at 30°C reported by [28]. The effects of water activity (aw) and temperature on germination and growth of A. flavus reported by Wheeler et al [35] and Mohamed et al. [28].

Salt content in salted anchovies ranges from 3.06-14.78 and in salted cotton fish is slightly higher with a range of 3.23-16.06. Other studies have reported salt content of mackerel-salted fish ranging from 9.73% - 16.31% [37]. Indonesian National Standard (SNI) requires that salt content in salted fish should not exceed 20%. Based on these results, the salted anchovies and cotton from the city of Bandung is still classified as safe [37].

| Isolate code | Gene expression | Origin |
|--------------|-----------------|--------|
| SD1T         | SD1T            | salted anchovies |
| CR2T         | CR2T            | salted anchovies |
| UB2T         | UB2T            | salted anchovies |
| CR3T         | CR3T            | salted anchovies |
| AN1T         | AN1T            | salted anchovies |
| AN2T         | AN2T            | salted anchovies |
| UB1T         | NG, NA          | salted anchovies |
| KC1T         | KC1T            | salted anchovies |
| AN1K         | AN1K            | salted anchovies |
| UB1K         | UB1K            | salted cotton fish |
| SD1K         | SD1K            | salted cotton fish |
| CR2K         | CR2K            | salted cotton fish |
| CR3K         | CR3K            | salted cotton fish |
| KC1K         | KC1K            | salted cotton fish |
| AN2K         | AN2K            | salted cotton fish |

NG: no growth
NA: not analyzed

Tabel 4. Presence of Genes ITS, aflD, and aflQ PCR gene Expression in Aspergillus spp. isolat

pH value is one of the indicators used to determine the freshness of fish. A good pH for fish preservation is 2.0 – 5.5, while for the growth of microorganisms it takes pH media 6.0 – 8.0 [38]. The pH value in this study was 6.09-8.32 in salted anchovies and 6.13-7.37 in salted cotton fish, which allows for the growth of fungi in the products tested. In previous research, Since et al. [39] revealed that pH value ranges from 5.92 – 6.23. Omeiza et al. [40] reported that the high presence of A. flavus in the product indicates the suitability of environmental conditions for growth and proliferation. In current study, almost all green Aspergillus spp. isolates showed positive results for the presence of aflD and aflQ genes. Both genes are target genes associated with aflatoxin biosynthesis. Rafli et al. [24]
mentioned that the existing of three target genes involved in aflatoxin biosynthesis such as aflR, nor-1 (aflD), and omkB (aflO) may represent that the isolate was able to produce aflatoxin. PCR analysis was able to amplify aflatoxin biosynthetic genes, i.e aflR, nor-1, and omkB in almost green Aspergillus group. aflR is a positive regulatory gene which is required for transcriptional activation of most of the structural genes such as nor-1 and omkB, where the presence of the target gene in green Aspergillus is related to the ability of cells to produce aflatoxins [24]. The presence of aflatoxigenic Aspergillus spp. in salted fish products provides information to the competent authorities to be more concerned and provides instructions on good methods of distribution and storage of salted fish to prevent fungal growth and produce aflatoxins.

4. Conclusion

Result showed the dominant fungal genera and prevalence of aflatoxigenic Aspergillus in dried salted anchovy and cotton fish sold in Bandung market. The dominant genera in dried salted anchovy and cotton fish are Aspergillus sp. (36.8% and 53.6%), followed by other genera (36.8% and 28.6%) and Penicillium sp. (26.3% and 17.9%). The prevalence of aflatoxigenic Aspergillus in dried salted anchovy samples was 13.16% (3 of 28) and in the dried salted cotton fish sample was 12.12% (5/38). The presence of aflatoxigenic Aspergillus in salted fish products indicates a potential risk of Aspergillus contamination that could endanger health.

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