Analysis of mold and aflatoxins in processed fish and their control with electrolyzed water


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ABSTRACT

The purpose of this study was to look at the likelihood of mold contamination in salted feseikh, sardine, and smoked herrings that are marketed in retail settings. Additionally, the total aflatoxins (AFTs) in the examined samples were estimated and further discussion was held regarding their potential health risks associated with them. The effect of the electrolyzed water (EW) on the residual concentrations of AFTs and the overall amount of mold in the salted feseikh was also investigated. Feseikh had the highest mold contamination, followed by salted sardine and smoked herrings at 80%, 50%, and 23.33%, respectively. Total mold count was 3.05±0.15 log 10 cfu/g in feseikh, followed by salted sardine (2.68±0.09), and smoked herrings (2.26±0.04), respectively. Four mold genera were recovered from the examined samples under examination: Aspergillus spp., Penicillium spp., Cladosporium spp., and Fusarium spp. The most predominant mold species found in the analyzed samples was Aspergillus spp. The analyzed samples had recorded mean values of total aflatoxins (ppb) of 3.98±0.27 (feseikh), 3.19±0.25 (salted sardine), and 2.52±0.43 (smoked herrings). The average consumption of salted fish products resulted in estimated daily intakes (EDI) values for total AFTs of 2.75 (feseikh), 2.21 (salted sardine), and 1.74 ng/kg/BW (smoked herrings) for adults, compared to 6.44, 5.16, and 4.07 ng/kg/BW for children. For Egyptian consumers, especially younger population, the use of such products constitutes a serious source of dietary exposure to total AFTs. Children's margin of exposure (MOE) values were 38.79 for feseikh to 48.39 for salted sardine and to 61.36 ng/kg/BW for smoked herrings, whereas adults' MOE values varied from 90.65 for feseikh to 112.91 for salted sardine and to 142.97 ng/kg/BW for smoked herrings. Slightly

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INTRODUCTION

Fish is considered an important source of vitamins, trace minerals, HDL cholesterol, rich polyunsaturated fatty acids, and protein. The requirement for fish consumption is rising globally to offset the lack of red meat (Morshdy et al. 2019, 2021). One of the most perishable sources of protein is fish. Microbiological contamination and fish deterioration can occur when it is handled, processed, and transported in an unsanitary manner. This can lead to the production of a variety of chemicals (Huss 1995) and (El-Ghareeb et al. 2021). In Egypt, processed fish items including salted feseikh, salted sardine, and smoked herrings are quite popular and are especially consumed during national holidays and festivals (Morshdy 2022).

In the food industry, mold contamination of processed fish is especially concerning. Numerous factors, such as moisture content, pH, oxygen content, substrate, and interactions with other microbiological agents, can affect the growth of mold. Different pH, temperature, and water activity conditions can support the growth of mold (Pitt and Hocking, 2009).

Mycotoxins are carcinogenic that are generated by several types of mold, such as Aspergillus and Fusarium. Two of the predisposing factors that encourage the establishment of different mold genera are the high moisture content and variable storage temperatures. Moreover, one of the key factors causing greater start mold counts in processed fish is the use of fish with high initial microbial load (Darwish et al. 2014).

According to Alcaide-Molina et al. (2009), a number of fungi, including Aspergillus flavus and Aspergillus parasiticus, create dangerous secondary metabolites known as aflatoxins (AFTs). Contaminated feedstuffs are the main way that AFTs reach to fish, and subsequently human. Due to their detrimental effects on human and animal health as well as the stigma attached to contaminated agricultural crops, AFTs have the potential to cause significant economic losses on a global scale (Darwish et al. 2014). Numerous toxicological outcomes, such as teratogenicity and immunosuppression, have been linked to AFTs. Human hepatocellular carcinoma has also been connected to AFTs (Thompson and Darwish, 2019) and (Aljazzar et al. 2021, 2023).

Electrolyzed water (EW) has been reported as an emerging, recent, effective, and broad-spectrum sanitizer, (Hsu et al. 2019). Due to its main effective forms of free available chlorine compounds (FAC), such as hypochlorous acid (HOCL), which kills a wide range of pathogenic microorganisms within a short period of time, This makes EW a safe alternative to chemical sanitizers. One of EW’s contributions to the food sector is its ability to reduce and control germs without altering the food’s organoleptic characteristics (Hsu, 2003). Consequently, EW is frequently used to sterilize a variety of commodities, including fish, poultry, shrimp, beef, eggs, and other products (Hao., 2013).

Making sure that the public has access to healthy animal and fish products is one of the main duties of the food safety and public health sectors. The present investigation aimed to investigate the possibility of mold contamination in retail-sold salted feseikh, sardine, and smoked herrings. Furthermore, an estimation of the overall AFTs in the tested samples was made, and additional discussion was be done regarding their possible health risks. Additionally, the impact of the electrolyzed water on acidic EW could significantly reduce mold counts and total AFTs in an experimental trial using feseikh as a food subject. Therefore, it is highly recommended to adopt strict hygienic procedures during preparation of salted fish. Besides, the use of EW is highly advisable to reduce mold contamination and total AFTs production in salted fish.
the overall amount of mold and total AFTs in the salted feseikh was studied.

MATERIALS and METHODS

Collection of samples

Ninety processed fish samples represented by Feseikh, salted sardine and smoked herrings 30 of each were randomly collected from local markets. After being cooled, the collected samples were submitted directly to the Food Hygiene Department of the Animal Health Research Institute, where they were tested for total mold counts and checked for AFTs. To create a dilution of $10^4$, 50 mL of each sample were aseptically mixed for two minutes at 2500 rpm with 450 mL of buffered peptone water (0.1%), and then decimal serial dilutions were performed (APHA, 2001).

Estimation of the total mold count (TMC):

After five to seven days of dark incubation at 25°C, total mold counts (TMC) were measured using the pour plate technique with malt extract agar media for conventional molds and Czapek-Dox agar with 5% NaCl for xerophilic molds (Oxoid, Basingstoke, UK). Throughout the incubation period, the plates were observed daily for the development of mold. A colony counter and direct counting of cultivated plates were used to estimate TMC (APHA, 2001).

$\text{TMC/g} = \text{total number of colonies} \times \text{dilution factor}$

Identification of the recovered mold species:

The macroscopical and microscopical characteristics of the mold colonies were used to identify the molds, as stated by Pitt and Hocking (2009). The mold cultures were examined every day during the incubation period to ascertain the rate and pattern of growth. Moreover, colony borders, surface and reverse pigmentation, surface development, and folding consistency were noted. Both the front and rear surfaces of the colonies were inspected.

Estimation of the total aflatoxins:

With a few modifications, the total aflatoxins were measured using a Series-4EX Fluorometer (VICAM, Milford, USA) in compliance with traditional techniques (Abd-Elghany and Sallam, 2015; El-Ghareeb et al. 2013).

In brief, 100 mL of methanol: water (4:1) was combined with 25 g of each sample and 5 g of NaCl. The samples were then blended at a high speed for three minutes. The mixture was run through a 1.5 m glass microfiber filter after being diluted four times with double-distilled water. The AflaTest®-P affinity column was then filled with the filtrate at a flow rate of two drops per second. Using HPLC grade methanol, aflatoxin was extracted from the affinity column at a rate of one drop per second and collected in a glass cuvette (VICAM part # 34000). The cuvette was filled with 1.0 mL of AflaTest® Developer, which was then well combined with the elute. The cuvette was then put into a calibrated fluorometer. The detection range of the Afla Test was 300 ng/g (ppb) (high detection) to 0.1 ng/g (ppb) (low detection). For every matrix under investigation, the limit of detection (LOD) was set at 0.1 ng/g. Excitation was set at 360 nm and emission at 440 nm to calibrate the fluorometer.

Estimated daily intakes

The estimated daily intakes (EDI) of AFTs were determined using the Human Health Assessment Manual equation published by the US Environmental Protection Agency (USEPA, 2010). Body weight (BW) was determined to be 70 kg for adult Egyptians and 30 kg for children, respectively. The fish ingestion rate (FIR) in Egypt was set at 48.57 g/day for meat products, and C was the concentration of the food-contaminant tested (ppb for AFTs) (Morshdy et al. 2022). The European Commission's 2006 guidelines were also used to establish the maximum allowed limits (MPLs) for the AFTs for the samples. The designated benchmark MPL for AFTs in beef was 4 ng/g.

Health risk assessment

Using the margin of exposure (MOE) technique, the daily intake of AFTs from the salted
fish under examination was utilized to estimate the cancer risk among Egyptians (EFSA 2005; FAO/WHO 2006). The benchmark dosage (BMDL10) that raises the incidence of cancer in Fisher rats by 10% is compared to the average amount of total consumption in humans to determine the MOE. It was found that the BMDL10 for AFTs was 250 ng/kg body weight/day. According to Benford et al. (2010), the MOE was computed using the following algorithm:

\[ \text{MOE} = \frac{\text{BMDL}_{10}}{\text{total consumption}} \]

MOE values below 10,000 indicate no health concern (EFSA, 2020)

**Preparation of NEW disinfectant solution**

Using a portable, hand-built electrochemical cell without a diaphragm, slightly acidic electrolyzed water (EW) with a pH of 5.6 was produced. The cell is constructed of two titanium electrodes (TiO2), one for the cathode and one for the anode, that are placed in a glass container with a 0.7cm gap between them. According to Hamouda and EL-Amawy (2020), the obtained EW was labeled and kept in closed glass containers at 4°C in the refrigerator.

**Effect of the EW on the total mold count and total AFTs contents in feseikh samples**

Feseikh samples with high levels of mold and total AFTs were divided into 4 groups (n = 4 per group) and the impact of EW on the levels of mold and AFTs was investigated. The studied samples were divided into the following four groups: G1 was kept as a control and received no treatment, G2 was submerged in EW for 15 minutes, G3 was submerged in EW for 30 minutes, and G4 was submerged in EW for one hour. Following immersion in EW, samples were ready for mycological analysis and total AFT content, in accordance with APHA (2001) and as previously described. The following formula was used to get the reduction rates:

\[ \text{Reduction rate} \% = \frac{\text{Concentration before treatment} - \text{Concentration after treatment}}{\text{Concentration before treatment}} \times 100 \]

**Statistical analysis**

The mold counts were expressed as log 10 cfu/g (means ± SE). Tukey-Kramer HSD test (p < 0.05) was used for statistical testing (Gomez and Gomez, 1984).

**RESULTS**

The mold contamination rates and total counts in the processed fish samples that were analyzed are depicted in Fig. 1. Feseikh had the highest mold contamination, followed by salted sardine and smoked herrings at 80%, 50%, and 23.33%, respectively (Fig. 1A). The most likely total mold count (p<0.05) was 3.05±0.15 log 10 cfu/g in feseikh, followed by salted sardine (2.68±0.09) and smoked herrings (2.26±0.04) (Fig. 1B). According to FAO/WHO, (2003) fish products should generally be free of mold.

Four mold genera were recovered from the examined samples under examination: Aspergillus spp., Penicillium spp., Cladosporium spp., and Fusarium spp. The most common mold species found in the analyzed feseikh, salted sardine, and smoked herrings were Aspergillus spp., which were isolated at 48.3%, 44.4%, and 64.3%, respectively. While Aspergillus spp., Penicillium spp., were isolated at 29.3%, 33.3%, and 21.4% from the samples under examination, respectively. Additionally, Cladosporium spp. were found in the samples of feseikh, herrings, and sardines at 7.1%, 13.8% and 11.1% respectively. From these samples, Fusarium spp., were also found at 8.6%, 11.1%, and 7.1%, respectively (Fig. 2). Five Aspergillus spp., were retrieved after additional identification of the Aspergillus spp. recovered from the analyzed samples: A. niger, A. flavus, A. fumigatus, A. ochraceous, and A. versicolor. The most common Aspergilli were A. niger and A. flavus, which were found in 35.7% and 25% of the feseikh samples that were tested, 37.5% and 31.3% of the salted sardine samples, and 44.4% and 33.3% of the smoked herring samples, respectively. A. ochraceous was obtained from feseikh at 10.7%, and from salted sardine at 12.5%. In contrast, A. fumigatus was recovered at 14.3%, 12.5%, and 11.1% from the tested feseikh, salted sardine, and smoked herrings. In feseikh, salted sardine, and smoked herrings, A. versicolor was found at 14.3%, 6.3%, and...
and 11.1%, respectively (Fig. 3).

It is important to note that the molds identified in this study have the potential to produce secondary metabolites that have a wide range of detrimental health consequences, which raises concerns for public health. The samples of feseikh, salted sardine, and smoked herrings that were analyzed revealed 66.6%, 36.63%, and 16.65% of aflatoxins, respectively (Fig. 4A). The analyzed samples had recorded mean values of total aflatoxins (ppb) of 3.98±0.27 (feseikh), 3.19±0.25 (salted sardine), and 2.52±0.43 (smoked herrings) as shown in Fig. 4B. Comparatively, the amounts of total aflatoxins in the analyzed samples of feseikh, salted sardine, and smoked herrings were greater than the maximum allowable limits of 4 ppb (EC, 2006) at 13.33%, 3.33%, and 0%, respectively (Fig. 5). In samples of dried salted anchovies (3 out of 28), and 12.12% in samples of dried salted cotton fish (5 out of 38).

It is important to note that the molds identified in this study have the potential to produce secondary metabolites that have a wide range of detrimental health consequences, which raises concerns for public health. The samples of feseikh, salted sardine, and smoked herrings that were analyzed revealed 66.6%, 36.63%, and 16.65% of aflatoxins, respectively (Fig. 4A). The analyzed samples had recorded mean values of total aflatoxins (ppb) of 3.98±0.27 (feseikh), 3.19±0.25 (salted sardine), and 2.52±0.43 (smoked herrings) as shown in Fig. 4B. Comparatively, the amounts of total aflatoxins in the analyzed samples of feseikh, salted sardine, and smoked herrings were greater than the maximum allowable limits of 4 ppb (EC, 2006) at 13.33%, 3.33%, and 0%, respectively (Fig. 5).
Fig 1. A) Mold contamination rates (%), B) total mold counts (log 10 cfu/g) in the examined salted fish (feseikh, sardine, and smoked herrings). Columns with different letters are significantly different at p < 0.05 (n = 30/ each fish species).

Fig 2. Prevalence rates (%) of different mold genera in the examined salted fish (feseikh, sardine, and smoked herrings).
Fig 3. Prevalence rates (%) of different *Aspergillus spp.* in the examined salted fish (feseikh, sardine, and smoked herrings).

Fig 4. A) Aflatoxin occurrence rates (%), B) total aflatoxin contents (ppb) in the examined salted fish (feseikh, sardine, and smoked herrings). Columns with different letters are significantly different at $p < 0.05$ ($n = 30$/each fish species)
Fig 5. Percentage of samples containing total aflatoxins higher than the established maximum permissible limits (4 ppb) according to EC (2006).

Fig 6. Estimated daily intakes (EDI) of total aflatoxins (ppb) among adult and children in the examined salted fish (feseikh, sardine, and smoked herrings).

Fig 7. MOE values among adults and children due to consumption of salted fish (feseikh, sardine, and smoked herrings).
The data shown in Fig. 6 showed that the average consumption of salted fish products resulted in EDI values for total AFTs of 2.75 (feseikh), 2.21 (salted sardine), and 1.74 (smoked herrings) ng/kg/BW for adults, compared to 6.44, 5.16, and 4.07 ng/kg/BW for children. For Egyptian consumers, especially children, the use of such products constitutes a serious source of dietary exposure to total AFTs.

The MOE technique was used to estimate the cancer risk for AFTs among Egyptians (EFSA, 2005; FAO/WHO, 2006). MOE was thought to be the most accurate and illuminating way to assess AFTs' cancer risk. Children's MOE values were 38.79 for feseikh to 48.39 for salted sardine and to 61.36 for smoked herrings, whereas adults' MOE values varied from 90.65 for feseikh to 112.91 for salted sardine and to 142.97 for smoked herrings (Fig. 7). EW was employed in a reduction experiment for the mold contamination and overall aflatoxin generation in feseikh. As shown in Fig. 8 on an exposure time-dependent way, the acquired data showed that EW could dramatically lower mold counts and the concentrations of the produced AFTs. As feseikh submerged in EW for 15, 30, and 60 minutes, it was possible to reduce total AFT residues by 19.1%, 39.2%, and 63.8%, and mold counts by 20.1%, 29.8%, and 53.8%, respectively (Fig. 8).

**DISCUSSION**

The use of raw fish with a high microbial load, improper manufacturing processes, contaminated curing chemicals, fluctuation of temperature during storage, or post-processing contamination as a result of inadequate worker hygiene precautions are some of the ways that allow molds to get into processed fish, including salted and smoked ones. For this reason, it is crucial to check for mold contamination on processed fish that is sold in retail shops in order to ensure food safety. The mold contamination rates and total counts in the processed...
fish samples that were analyzed are depicted in Fig. 1. Feseikh had the highest mold contamination, followed by salted sardine and smoked herrings at 80%, 50%, and 23.33%, respectively (Fig. 1A). The most likely total mold count (p<0.05) was 3.05±0.15 log 10 cfu/g in feseikh, followed by salted sardine (2.68±0.09) and smoked herrings (2.26±0.04) (Fig. 1B). According to FAO/WHO, (2003) fish products should generally be free of mold. Mold contamination was found in smoked herrings in Egypt (Elmossalami et al., 1968), dried fish from Indonesia (Wheeler and Hocking, 1988), and dried fish from Sri Lanka (Atapattu and Samarajeewa, 1990), all of which are in line with documented mold contamination of processed fish and fish products. Molouha that was taken from Ismailia, Egypt, had a relatively similar mold count (2.69 log 10 cfug) (Ismail et al., 2013). Lower mold counts of 1.67±0.021 and 1.69±0.18 log 10 cfu/g were found in feseikh and salted sardine that were sold in Giza, Egypt (Edris et al., 2020).

Four mold genera were recovered from the examined samples under examination: *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., and *Fusarium* spp. The most common mold species found in the analyzed feseikh, salted sardine, and smoked herrings were *Aspergillus* spp., which were isolated at 48.3%, 44.4%, and 64.3%, respectively. While *Aspergillus* spp., *Penicillium* spp., were isolated at 29.3%, 33.3%, and 21.4% from the samples under examination, respectively. Additionally, *Cladosporium* spp. were found in the samples of feseikh, herrings and sardine at 7.1%, 13.8%, and 11.1%, respectively. From these samples, *Fusarium* spp., were also found at 8.6%, 11.1%, and 7.1%, respectively (Fig. 2). Five *Aspergillus* spp., were retrieved after additional identification of the *Aspergillus* spp. recovered from the analyzed samples: *A. niger*, *A. flavus*, *A. fumigatus*, *A. ochraceous*, and *A. versicolor*. The most common Aspergilli were *A. niger* and *A. flavus*, which were found in 35.7% and 25% of the feseikh samples that were tested, 37.5% and 31.3% of the salted sardine samples, and 44.4% and 33.3% of the smoked herring samples, respectively. *A. ochraceous* was obtained from feseikh at 10.7%, and from salted sardine at 12.5%. In contrast, *A. fumigatus* was recovered at 14.3%, 12.5%, and 11.1% from the tested feseikh, salted sardine, and smoked herrings. In feseikh, salted sardine, and smoked herrings, *A. versicolor* was found at 14.3%, 6.3%, and 11.1%, respectively (Fig. 3). *Aspergillus* and *Penicillium* were the most common mold taxa recovered from salted fish sold in Assuit City, Egypt, (Ismail et al., 1994). Furthermore, *Aspergillus* spp. (36.8% and 53.6%) was found to be the most prevalent genera in dried salted anchovy and cotton fish, followed by other genera (36.8% and 28.6%) and *Penicillium* spp. (26.3% and 17.9%) according to Kusmarwati et al., (2021). The total fungal counts of the cotton fish and dried salted anchovies from Bandung city's traditional market in Indonesia ranged from 5.00×10^2 to 1.40×10^2 cfu/g and 2.50×10^2 to 4.00×10^2 cfu/g, respectively. The incidence was 13.16% in samples of dried salted anchovies (3 out of 28), and 12.12% in samples of dried salted cotton fish (5 out of 38).

It is important to note that the molds identified in this study have the potential to produce secondary metabolites that have a wide range of detrimental health consequences, which raises concerns for public health. The samples of feseikh, salted sardine, and smoked herrings that were analyzed revealed 66.6%, 36.63%, and 16.65% of aflatoxins, respectively (Fig. 4A). The analyzed samples had recorded mean values of total aflatoxins (ppb) of 3.98±0.27 (feseikh), 3.19±0.25 (salted sardine), and 2.52±0.43 (smoked herrings) as shown in Fig. 4B. Comparatively, the amounts of total aflatoxins in the analyzed samples of feseikh, salted sardine, and smoked herrings were greater than the maximum allowable limits of 4 ppb (EC, 2006) at 13.33%, 3.33%, and 0%, respectively (Fig. 5).

The data shown in Fig. 6 showed that the average consumption of salted fish products resulted in EDI values for total AFTs of 2.75 (feseikh), 2.21 (salted sardine), and 1.74 (smoked herrings) ng/kg/BW for adults, compared to 6.44, 5.16, and 4.07 ng/kg/BW for children. For Egyptian consumers, especially children, the use of such products constitutes a
serious source of dietary exposure to total AFTs.

EFSA (2007) has classed AFTB1 as both a carcinogen and a mutagen. Consuming even trace amounts of AFTs as little as 1 ng/kg/day may result in liver cancer, especially in people with hepatitis C or B viruses (American Cancer Society, 2011). The MOE technique was used to estimate the cancer risk for AFTs among Egyptians (EFSA, 2005; FAO/WHO, 2006). MOE was thought to be the most accurate and illuminating way to assess AFTs' cancer risk. Children's MOE values were 38.79 for feseikh to 48.39 for salted sardine and to 61.36 for smoked herrings, whereas adults' MOE values varied from 90.65 for feseikh to 112.91 for salted sardine and to 142.97 for smoked herrings (Fig. 7). These results were significantly lower than the EFSA-established threshold of 10,000 (EFSA, 2020), indicating that there may not be a cancer risk associated with consuming such fish products. To preserve a long life, however, excessive use of these products should be avoided, particularly in children. According to Elzupir and Abdulkhair (2020), it is probable that 175 and 311, respectively, were the MOE values for the total AFTs in processed beef and poultry meat products among Saudi citizens.

EW was employed in a reduction experiment for the mold contamination and overall aflatoxin generation in feseikh. As shown in Fig. 8 on an exposure time-dependent way, the acquired data showed that EW could dramatically lower mold counts and the concentrations of the produced AFTs. As feseikh submerged in EW for 15, 30, and 60 minutes, it was possible to reduce total AFT residues by 19.1%, 39.2%, and 63.8%, and mold counts by 20.1%, 29.8%, and 53.8%, respectively (Fig. 8).

CONCLUSION

The current study's findings showed that retailed salted fish products have variable degrees of mold contamination and aflatoxin residues. The highest contamination rate and aflatoxin were found in feseikh. It was determined through risk analysis that eating such products might not be harmful to one health. For the ongoing monitoring of total AFTs in fish products sold in retail markets, additional investigations are still required. The use of slightly acidic EW could significantly reduce the mold contamination and AFTs production in feseikh. Thus, it is strongly advised to cut back on the daily consumption of fish, especially among children, to lower the chance of developing cancer and living longer.

Conflict of interest: The authors declare that they have no conflict of interest.

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