ISABB Journal of Food and Agriculture Science Vol. 1(2), pp. 31-35, November 2011
Available online at http://www.isabb.academicjournals.org/JFAS
ISSN 1937-3244©2011Academic Journals

Full Length Research Paper

Mycoflora of smoke-dried fishes sold in Guilan Province, Iran

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Accepted 27 October, 2011

Fish and fish products constitute more than 60% of the total protein intake in adults especially in the rural areas. Fish flesh is one of the best sources of protein. Smoke dried fishes (common carp_big head carp_silver carp) were randomly sampled and purchased from three different marketing sites located at main markets, Astara and Anzali cities. Fifty-five samples of related species were grouped together to make eleven composite samples. Aflatoxins were extracted from the samples according to the method of Seitz and Mohr. 10 g of the fishes samples obtained from each of the markets were weighed aseptically and macerated using a Warring blender and were extracted with chloroform and concentrated. Thin layer chromatography of aflatoxin and samples extracted were performed on silica gel DG 254. Of the extracted samples 5, 10 and 15 µl were spotted on three different points on a ruled base line of the TLC plates. Also 5, 10 and 15 µl of the aflatoxin standard were spotted on another three points near the previous sample extract spotted points. Results obtained from this study showed that *Aspergillus flavus, Aspergillus tereus, Aspergillus fumigatus, Absidia* sp., *Rhizopus* sp., *Aspergillus niger, Mucor* sp., *Cladosporum* sp., *Penicillium italicum, Penicilium viridatus, Candida tropical*s and *Fusarium moniliformis* were found to be associated with smoked_dried fishes sold in different markets in Guilan Province, Iran.

Key words: Smoked_dried fish, Aflatoxin, *Aspergillus flavus*, Mycoflora.

INTRODUCTION

Fish supplies a good balance of protein, vitamins and minerals. It has a relatively 10% calories content hence its role in nutrition is recognized (Akande and Tobor, 1992). Fish and fish products constitute more than 60% of the total protein intake in adults especially in the rural areas (Adeleye, 1992). They are widely accepted on the menu cards and form a much-cherished delicacy that cut across socioeconomic, age, religious and educational barriers (Adeleye, 1992). Fish flesh is one of the best sources of protein. Its flesh is tender due to bundles of muscle fibers, which are held together by fibrous material when heated (Fagade, 1992). It is better digested than beef or other types of protein. In Iran fish is eaten fresh, preserved or processed. The percentage composition of the different methods of fish disposed for consumption in the artisanal sector according to in research are as follows live fish 37%, fresh fish 21%, smoke dried 32%, sun dried 10% salted. Smoke drying methods used in Iran requires low capital, investment and it is conducted in fishermen camps and fish processing centuries in traditional smoking kilns of clay, cement blocks, drums or iron sheets (Eyo, 1992). This results in a very short shelf life and low market value as well as inability to withstand handling and transportation by retailers (Akande and Tobor, 1992).

For long, fungi were regarded as causing only anesthetics spoilage of food. But during 1966 when the famous “Turkey X” diseases killed 10,000 turkey poultry in Great Britain, the Western world became aware that common spoilage molds could produce significant toxigenic fungi and potentially toxic compounds have been discovered. Aflatoxins, a group of toxic metabolite produced by certain *Aspergillus* species have been found to be carcinogenic, tetratogenic and mutagenic to several

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species of experiment animals (Butler and Barnes, 1968; Gopalan et al., 1972; Adamson et al., 1973). Aflatoxin occurs in a variety of crops and animal product. The conditions that contribute to fungal growth and production of aflatoxins are a hot and humid climate, moisture content of 16% and above favorable substrate characteristics and factors that decrease the host's immunity such as insect damage (Hamblin, 2000). Aflatoxins have a high melting point that is 250℃. It has been proved that food items do carry residue of the toxin. Thus, it is certain that human beings are exposed to aflatoxins through contaminated food items among which fish is an important component (Murgani, 2000). This research was embarked upon to investigate the mycoflora associated with smoke dried fishes in Guilan Province Iran and the presence of aflatoxins in the smoke-dried fishes.

MATERIALS AND METHODS

Sample collection

Smoke dried fishes (common carp_big head carp_silver carp) were randomly sampled and purchased from three different marketing sites located at main markets , Astara and Anzali cities. Fifty-five samples of related species were grouped together to make eleven composite samples, they were subsequently kept in sterile polyethylene bags, which were used for analysis.

Isolation of fungi

Attempts to isolate fungi from the smoke-dried fish's samples were made aseptically on Saboraud dextrose agar. 10 g of the fishes samples obtained from each of the markets were weighed aseptically and macerated in 90 ml sterile watery agar (0.2%) using a Warring blender. From this, subsequent tenfold dilution was made up to 10 to 5 (Clark, 1968). 1 ml of each dilution was dispensed in triplicate in sterile Petri dishes. Molten Saboraud dextrose agar to which penicillin and streptomycin had been incorporated were added to the Petri dishes, which were gently rotated to ensure even dispersion.

The plates were allowed to solidify and were incubated at 28±1℃ for 3 to 5 days. All observed colonies were subculture to obtain pure cultures which were subsequently isolated and identified using morphological characteristics, spore formation, the production of fruiting bodies and biochemical reactions (Banrnet and Hunter, 1972; Lodder and van Rijn, 1971; Raper and Fennel, 1973) and by compares with already identified cultures, which were obtained from the plant pathology laboratory of the Institute of Agricultural Research and Training, Islamic Azad University of Tabriz, Iran. The moisture content was determined by oven drying at 105℃ for 41/2 h.

Determination of pH

2 g each of the macerated fish's samples were weighed in triplicates. Water was added and mixed thoroughly to make a fish slurry. The pH readings were taken using digital pH meter equipped with a glass electrode (digital thermo pH meter mod B-E105). The electrode was rinsed and immersed into the fish slurry. The pH reading were then recorded. Determination were done in triplicates and the mean value were obtained.

Aflatoxins detection in smoke dried fish's samples

Aflatoxins were extracted from the samples according to the method of Seitz and Mohr (1977). 10 g of the fishes samples obtained from each of the markets were weighed aseptically and macerated using a Warring blender and were extracted with chloroform and concentrated. Thin layer chromatography of aflatoxin and samples extracted were performed on silica gel DG 254. Of the extracted samples 5, 10 and 15 µl were spotted on three different points on a ruled base line of the TLC plates. Also 5, 10 and 15 µl of the aflatoxin standard were spotted on another three points near the previous sample extract spotted points. The plates were developed first with diethyl ether and then with chloroform: acetone (9:1 v/v). Aflatoxin was identified on the basis of comigration with aflatoxin standards (Fluka) and by their characteristic fluorescent color under long Ultra Violet (UV) illumination was at 360 nm. The fluorescent spots of aflatoxins were scraped off the TLC and eluted by chloroform: methanol (9:1 v/v). The solvent was evaporated under nitrogen to dryness and the residue was dissolved in methanol. The concentration of aflatoxins (B_1 and G_1) in solution was determined by measuring its absorbance at 360 nm then calculated according to the method of Masri et al. (1969).

Confirmatory tests for aflatoxin

Three different derivatives were prepared by treating portions of the isolated toxin or the aflatoxin standard with formic acid thionyl chloride, acetic acid-thionyl chloride and trifluoroacetic acid. The test was then continuing according to the method of Stolof and Friedman (1976).

Statistical analysis

Duncan multiple range test was used to compare significant differences between the means (Duncan, 1956).

RESULTS AND DISCUSSION

Results obtained from this study showed that *Aspergillus flavus, Aspergillus tereus, Aspergillus fumigatus, Absidia* sp., *Rhizopus* sp., *Aspergillus niger, Mucor* sp. *Cladosporum* sp., *Penicillium italicum, Penicilium viridatus, Candida tropical*is and *Fusarium moniliformis* were found to be associated with smoked dried fish sold in different market in Astara and Anzali. As stated earlier, *A. flavus* and *A. tereus, A. fumigatus* were the dominant mycoflora in decreasing sequential order. *P. viridatus, C. tropicalis and F. moniliformis* occurred less frequently (Figure 1). The presence of *A. flavus* in the samples might probably make its consumption hazardous to health. According to Akande and Tobor (1992), in artisanal fishery, freshly caught fish are covered with damp sacks and at times, they are mixed with wet grass or water weeds to reduce the temperature. Fish treated this way is prone to contamination with microorganisms

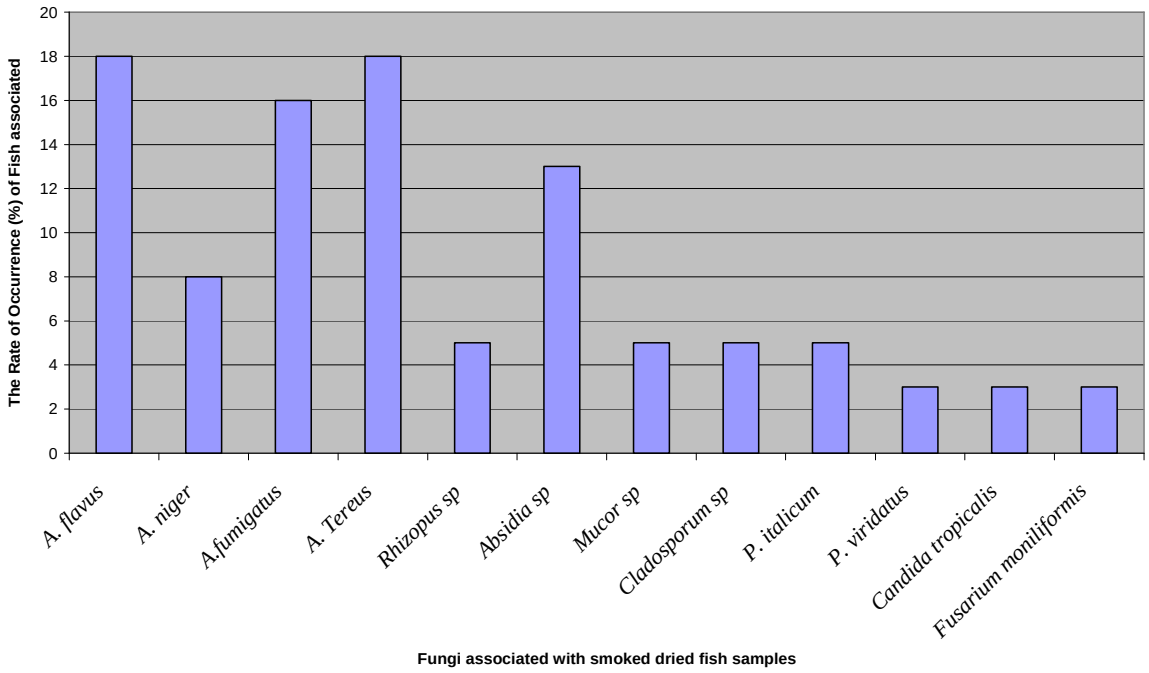

Figure 1. The rate of occurrence (%) of fungi associated with marketed smoked dried fish samples Fungi associated with smoked dried fish samples.

such as bacteria and fungi. This indicates that spoilage of fish starts right from the aquatic ecosystem. Handling fishes are also prone to microbial attack especially in artisanal fishery due to unhygienic methods of reducing temperature. During the smoke drying period, smoking kilns used in artisanal fishery and the overloading of the fishes on the trays leads to improper processing which in turn encourages fungal attack (Eyo, 1992). During storage of smoked dried fish products, good storage practices are not adhered to by wholesalers, hence stores are not well ventilated and pest can easily gain access into the stores.

The environment in which fishes are displayed in the market is not always hygienic and this is another avenue for microbial contamination. Very often, retailers display the smokedried fish samples in open trays beside the gutter on refuse heaps, this also encourages fungi attack and subsequent production of toxins. This is in agreement with the report of Akande and Tobor (1992). The result also revealed that the average mould count ranged from 3.0×10^2 to 8.4×10^4 cfu/g (Table 1). The microbial levels obtained in this report which is 10^4 could be considered hazardous to consumers because of the possibility of the presence of enterotoxigenic strains. The pH ranged between 3.0 to 6.0 and also the moisture content ranged from 22.7 to 27.6%. Specimen E had the lowest moisture content while specimen H had the highest moisture content as shown in Table 1. The spots from the extracted smoke dried fish's samples and the standard aflatoxin fluorescence produced bluish and greenish sport. Sharma (1992) reported that the two major metabolites of *Aspergillus* sp. called aflatoxins were designated B_1 and G_1 because they fluoresce blue (B_1) and green (G_1) when exposed to long-wave ultraviolet light. Aflatoxins were detected in all of the samples. The concentration of aflatoxin B_1 and G_1 ranged between 1.505^g to 8.105^a µg/kg and 1.810^j to 4.51^a µg/kg respectively. As shown in Table 2, sample E had the lowest AFB_1 and AFG concentration while sample K had the highest AFB and AFG concentration. This indicate that the smoke_dried fish samples have been contaminated by fungi especially *A. flavus* which produced the toxins.

A. flavus is known to produce aflatoxins (Fennel et al., 1973). Aflatoxins are highly carcinogenic, causing hepatoma (cancer of the liver) and have also been associated with acute hepatitis in man, mostly the developing world (Eaton and Groopman, 1994; Krogh, 1992; Prasad, 1992). Aflatoxin has been reported in grapes and musts in France (Sage et al., 2002), edible nuts and nut products, milk and milk products (Prasad, 1992). The implication of this report is that, though in Guilan Province, Iran most of the populace feed on fishes, it can be confirmed that most of the consumers would have been consuming this and other metabolites. Golbatt and Stolloff (1983) reported that aflatoxins occurred in the human diet and this could pass from feed to milk. Prolong intake of this metabolite may constitute a health hazard. It

Table 1. Fungi count, pH and moisture content (%) in different fish samples.

Samples	Fungi count	pH	Moisture content (%)
A	4.8×10^3	3.0	25.2
B	5.8×10^3	5.0	25.7
C	1.72×10^4	3.0	26.6
D	6.4×10^3	5.0	26.3
E	3.0×10^2	6.0	22.7
F	2.3×10^3	3.0	24.9
G	4.2×10^4	6.0	27.6
H	9.0×10^2	6.0	24.3
I	8.4×10^4	5.0	27.2
J	2.8×10^4	4.0	27.3
K	7.6×10^3	5.0	26.1

A: Stock fish (*Gadus morhua*), B: skipjack tuna (*Katsuworus pelamis),* C: croaker (*Pseudotolithus typhus)*, D: sting - ray (*Dasyatis margarita)*, E: catfish (*Arius hendeloti)*, F: bonga fish (*Ethalmosa fimbriota*), G: ribban fish (*Triuchurius trichurius)*, H: Stark (*Carchanas faunis)*, I: Thread - fin (*Pentanemis qumquarius)*, J: sole (*Cynoglossus browni)*, K: spade - fish (*Drepane Africana).*

Table 2. Aflatoxin B1 and G1 concentrations in the samples.

Samples	AF B1 µgkg^{-1}	AF G1 µgkg^{-1}
A	4.750^{c}	3.550^{c}
B	3.55^{d}	3.050^{d}
C	2.100^{i}	2.8200^{e}
D	3.005^{e}	2.515^{g}
E	1.505^{g}	1.8100^{j}
F	2.5150^{g}	2.205^{h}
G	3.0005^{e}	3.505^{c}
H	2.205^{h}	2.000^{l}
I	2.805^{f}	2.715^{f}
J	7.525^{b}	3.710^{b}
K	8.105^{a}	4.51^{a}

Each value represents a mean of three replicates. Means followed by the same letter are not significantly different by Duncan's multiple range tests.

is therefore important that both the artisanal fisher-men and the marketers should adapt a better method of preservation and better smoking kilns should be provided for artisanal fishermen at subsidized rates and stored fish product should be well stored. Equally complicated aflatoxin analysis procedure should be replaced with commercial kits such as veratose and Afla B^{+m} that are easy to run and health regulatory bodies such as NAFDAC should carry this out so that the toxin can be easily detected and samples containing them discarded. Improper smoking and drying of fishes may lead to insect infestation, fungal attack, fragmentation and degradation of the product (Eyo, 1992). Since most of the moulds isolated are probably contaminants rather than originating in the fishes sample, better methods of preservation (drying and storage) will reduce their incidence or eliminate them.

Conclusion

Smoked dried fishes samples stored for sale in Astara and Anzali markets were heavily contaminated with aflatoxigenic fungi and they are not acceptable for consumption due to the presence of aflatoxin B_1 and G_1 contents and prolong intake may constitute a health hazard. Since most of the moulds isolated are probably contaminants rather than originating in the fishes sample, better methods of preservation (drying and storage) will reduce their incidence or eliminate them.

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