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Microbial and chemical contamination and salt content of smoked fishes produced in Guilan (north of Iran)

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Abstract

Background and objective: Several species of fish are commonly harvested and consumed in north of Iran and distributed elsewhere. Unfortunately, smoked fish is produced traditionally in north of Iran and may become contaminated by carcinogenic metabolites and microorganisms during the smoking process. Additionally, high amount of salt is added to the product to extend its shelf life. Therefore, we aimed to determine both microbial and chemical contaminations and salt content of the smoked fishes produced in Guilan province (north of Iran).

Materials and methods: Smoked fish samples (n=20) were collected from five processing units in Guilan. They were transferred to the laboratory under aseptic condition. Microbial tests were included to determination of mold/yeast (by plating method) and *Listeria monocytogenes* (by polymerase chain reactions). Polycyclic aromatic hydrocarbons (by high liquid chromatography), cadmium and lead (by atomic absorption spectroscopy) were detected as chemical contaminants. Amount of salt was detected by titration method.

Results and conclusion: None of the samples were contaminated by *Listeria monocytogenes*, while more than 100 CFU mold/yeast per gram were enumerated in 15% of the smoked fishes. Salt content was calculated as 8.66%. Average concentration of lead, cadmium, and polycyclic aromatic hydrocarbons was 0.082, 0.026, and 0.0036 mg/kg, respectively. Compared to national and international regulations, concentration of the chemical contaminants was within the acceptable range. Although, heavy metals are accumulated in the body and their concentration should be minimized in foods as low as possible. Moreover, more restriction is required with respect to salt because of its role as a risk factor in cardiovascular diseases. To adopt the consumers' taste, slight reduction in salt content of the products is recommended. In

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conclusion, there was no serious risk of microbial/chemical contamination in the smoked fishes produced in Guilan. However, their consumption should be controlled in hypertensive patients.

Keywords: Fungi, heavy metal, *Listeria monocytogenes*, polycyclic aromatic hydrocarbon, smoked fish, salt

1. Introduction

Marine foods have high nutritional value and contain beneficial fats and high-quality protein. They are strongly recommended by dietitians, which has led to their increasing consumption in the world [1]. Fish is a good source of iron, iodine, zinc, omega 3 fatty acids, calcium and B-group vitamins [2]. It has high protein and low fat and is easily digested in the gastrointestinal tract more than 85% [3,4]. Fish contains essential amino acids of lysine, methionine, and tryptophan, which are not found in plant sources [5].

Per capita consumption of seafood has increased significantly in Iran in recent decades [6]. Importantly, it is a perishable food due to high concentration of unsaturated fat and protein. It undergoes microbial spoilage caused by enzymatic degradation if stored under inappropriate condition and may pose the consumers at different risks [7].

Approximately, 100 million tonnes of fish are harvested in the world annually, of which more than 50% are processed by different preservative methods for edible purposes [8]. Smoking is one of preservative methods to extend shelf life of fish [9]. In addition, it develops flavor and color of the product by chemicals in the smoke such as ether, ester, phenol, hydrocarbon, alcohol, and ketone [10].

Smoked fish is popular in north of Iran, but its production method is of serious concern. on the one hand, it is produced traditionally without strict supervision by the authorities and usually not heated further before consumption. Such condition increases the risk of microbial infection and poisoning. On the other hand, inappropriate smoking may increase the concentration of some carcinogens such as benzo(a)pyrene and benzo (a)anthracene in the smoked fish or may lead to its cross-contamination by heavy metals [11].

According to national surveys, cardiovascular diseases (CVDs) are the leading cause of mortality and morbidity in the populations. CVDs are developed by several behavioral and nutritional risk factors, of which high salt intake is of concern. To prevent and control non-communicable diseases, the World Health Organization (WHO) released a global action plan by focusing on nine targets. Thirty percent reduction in salt intake was one of the targets that was greed by the countries [12]. In line with the WHO action plan, monitoring of salt and/or sodium concentration in daily diet has become important in Iran in recent years.

With regard to the high consumption of smoked fish in north of Iran and its possible contamination by microorganisms and hazardous chemicals, the aim of this study was determination of mold/yeast, *Listeria monocytogenes*, polycyclic aromatic hydrocarbons (PAHs), lead (Pb), and cadmium (Cd) in the smoked fishes distributed in Guilan. For health issues, salt concentration in the samples was also determined.

2. Materials and methods

2.1. Sampling

Smoked fish samples (n=20) were collected from five traditional processing units certified by the Ministry of Jihad in Guilan province (north of Iran) in Autumn 2020. They were transferred to the laboratory under aseptic condition and stored at -18 ºC until analysis. Bone and internal organs of the samples were removed and the muscle was grinded for analysis.

2.2. Enumeration of mold/yeast

Culture medium of Dichloran Rose Bengal Chloramphenicol agar (Merck, Germany) was used for detection of fungi by surface plating method. At first, 10 g of grinded sample was

added to 90 ml saline solution (0.85% w/v). Then, 0.1 ml of the first dilution was added on surface of the culture medium and spread homogenously. The inoculated medium was incubated at 25 ºC for 3-5 days [13].

2.3. Enumeration and detection of *L. monocytogenes*

Primary enrichment of the bacteria was done in Palcam broth (Merck, Germany) followed their selective enrichment in Listeria enrichment broth (Merck, Germany). Both media were incubated at 37 ºC for 48 h. Then, the grown bacteria in the selective enrichment broth were streaked onto Palcam agar (Merck, Germany) as selective medium for listeria growth and incubated at 37 ºC for 48 h. The suspected colonies (gray-green with black halo) were isolated for polymerase chain reaction (PCR) analysis.

For DNA extraction, the isolated colonies were added to 1.5-ml microtube containing 50 µl PCR buffer. Then, 50 µl Triton X-100 (2% w/v) was added to the mixture. The microtube was incubated at 100 ºC for 10 min. After cooling, it was centrifuged at 2000 rpm at 4 ºC for 5 sec.

PCR mixture was included to 32.5 µl water, 0.5 µl MgCl2-free PCR buffer, 4 µl $MgCl_2$ (25 mM), 20 mM dNTPs (0.5 µl of each of four nucleotides), 0.5 µl reveres primer (100 µM; hlyA-d 5'-GTATCCTCCAGAGTGATCGA-3'), 0.5 µl forward primer (100 µM; hlyA-u 5'-CATTAGT GGAAAGATGGAATG-3'), Taq DNA polymerase (0.5 units), 5 µl the extracted DNA from the sample, and 5 µl positive control (DNA of *L. monocytogenes*). Thermal cycler (Trinity, Australia) was set on denaturation at 94 ºC for 5 min, annealing at 55 ºC for 1 min, and extension at 72 ºC for 2 min. The PCR products were analyzed by agarose gel electrophoresis by staining with ethidium bromide [14]. All chemicals were purchased from Sigma-Aldrich (USA).

2.4. Determination of PAHs

For extraction, 5 g of grinded sample was mixed with 5 ml KOH, 75 ml methanol, and 1 ml 9,10-dimethylantracene as internal standard. The mixture was boiled for 4 h. Then, liquid phases were separated and extracted with 100 ml n-hexane under vigorous shaking for 3 min. Methanol KOH phase was discarded and n-hexane phase was rinsed with 50 ml methanol-water solution (8:1) and 50 ml deionized water. After rinsing, organic phase was separated and concentrated in a rotary evaporator at 42 ºC. PAHs were determined by high performance liquid chromatography (Hewlett Packard 1100 Series, Germany) equipped with C18 column (250 mm × 4.6 mm, 5 µm) and fluorescence detector (λ 250-290 nm). Mobile phase was included to solvent A (acetonitrile) and solvent B (acetonitrile/water, 50:50). Gradient elution was developed: 100% solution B for 26 min, 40% solution B and 60% solution A for 9 min, 100% solution A for 7 min, and 100% solution B for 3 min. Injection volume was 20 µl with flow rate of 1.2 ml/min. External standard solutions were used for calculation of PAHs in the analyte [15]. All chemicals were of HPLC grade and purchased from Sigma-Aldrich (USA).

2.5. Determination of heavy metals

Graphite furnace atomic absorption spectrometry (Varian, USA) at 283.3 nm and 228.8 nm for Pb and Cd, respectively, was used. At first, 10 g of the homogenized sample was dried by direct heating in the laboratory, Then, the dried sample was ashed at 500 ±50 ºC for 8 h. To help the organic compounds removal, the sample was removed from the furnace after 4 h, H2O was added to the content after cooling followed by heating on water bath, and the dried sample was returned to the furnace. After 8 h heating at 500 ºC, 50 ml hydrochloric acid 6 M and 30 ml nitric acid 0.1 M were added to solve the ash. The solution was further filtered and the volume was made up to 100 ml with nitric acid 0.1 M. Calibration curve of Pb and Cd was developed by preparation of five working standard solutions [16]. The chemicals were purchased from Sigma-Aldrich (USA).

2.6. Salt

10 g of grinded sample was mixed with 100 ml hot deionized water and the mixture was put in boiling water bath for 15 min. After cooling, 2 ml $K_4Fe(CN)_6$ solution and 2 ml $Zn(CH_3COO)_2$ solution were added to the last mixture and mixed vigorously. The mixture was left in the environment for 30 min. The supernatant was transferred to a 200-ml flask and the volume was made up with deionized water. After filtration, 20 ml of final solution was mixed with 5 ml nitric acid (4 N), 1 ml saturated ferric ammonium sulfate, 20 ml silver nitrate, and 3 ml nitrobenzene followed by titration with potassium thiocyanate until a dark red color was appeared. The procedure was repeated for black [17]. The chemicals were purchased from Sigma-Aldrich (USA).

$$Cl\ (\%\ w/w) = 58.55 \times \frac{V_2 - V_1}{m} \times C$$

Where, V_1 is volume of potassium thiocyanate (ml) used in titration of sample, V_2 is volume of potassium thiocyanate (ml) used in titration of blank, m is sample weight (g), C is concentration of 0.1 N potassium thiocyanate (M/l).

2.7. Statistical analysis

SPSS software version 19 was used for analysis. Data are presented as mean ±standard deviation. Experiments were done in duplicate.

3. Results and discussion

3.1. Microbial contamination

Listeria contamination of smoked fish is occurred by their polluted environment before harvesting or cross-contamination under processing and storage [18]. Moreover, long storage in the environment increases the rate of bacterial growth in the product [19]. Garrido et al. investigated listeria contamination of ready-to-eat foods in Spain and found that smoked fish was the most contaminated product with 25% rate of contamination [20]. Although, it depends on fish species, method of smoking, salt concentration, geography, and method of harvesting [21].

L. monocytogenes is of serious concern for pregnant women and may lead to miscarriage [22]. According to national regulation of Iran, no contamination by *L. monocytogenes* (0 CFU/g) is accepted in the products [23]. As seen in Table 1, all of our products were free of *L. monocytogenes* which shows that the fishes were harvested and processed under appropriate condition. Interestingly, salting suppresses the bacterial growth due to plasmolysis and cell death [24]. Therefore, high salt level in our products should not be ignored in this regard. On the other hand, bactericidal compounds of the smoke such as phenol and formaldehyde affect proteins' structure and integrity of bacterial cell membrane, leading to bacterial loss [25,26].

Table 1- Microbial load in the smoked fish samples collected from Guilan (north of Iran) in 2020

Sample	*L. monocytogenes* (CFU/g)	Mold/yeast (CFU/g)	Sample	*L. monocytogenes* (CFU/g)	Mold/yeast (CFU/g)
1	Neg.	Neg.	11	Neg.	Neg.
2	Neg.	Neg.	12	Neg.	100 <
3	Neg.	Neg.	13	Neg.	Neg.
4	Neg.	Neg.	14	Neg.	Neg.
5	Neg.	100 <	15	Neg.	Neg.
6	Neg.	Neg.	16	Neg.	Neg.
7	Neg.	Neg.	17	Neg.	Neg.
8	Neg.	Neg.	18	Neg.	100 <
9	Neg.	Neg.	19	Neg.	Neg.
10	Neg.	Neg.	20	Neg.	Neg.

*Neg.: negative

Our samples were cold smoked by traditional method. Incidence of mold/yeast contamination in 15% of the smoked fish samples (Table 1) might be due to cold smoking which increases

fungal contamination. However, antimicrobial effect of salt and smoke compounds in suppression of majority of fungi is of interest. In general, most of our samples were acceptable for edible use but development of hot smoking and further cooking under controlled condition is recommended to avoid the remained fungal growth by thermal process [26].

3.2. Chemical contamination

3.2.1. PAHs

These chemicals are carcinogenic to human due to their potency to from DNA- or protein-adduct. They are hydrophobic and inactive agents in nature, which are metabolized to the active hydrophilic compounds as carcinogen in the body [27]. Direct heating of foods on open flame is of main routes of PAHs contamination. Time and temperature of heating, distance from the heat source, type of fuel (coal or wood), and fat content of the food are effective factors in formation of PAHs [28]. PAHs are the product of fat pyrolysis as a result of free radical formation and development of closed-loop dehydrogenized molecules followed by polymerization under high temperatures [29]. They accumulate in fatty tissues due to their hydrophobic nature [30]. They are also produced during smoking and enter the smoked food [29]. It is clear that foods containing high fat especially unsaturated fatty acids are of great concern for PAHs contamination.

Number of 16 PAHs have been introduced by the United States Environmental Protection Agency as priority pollutants, of which benzo(a)pyrene, benzo(a)anthracene, chrysene, and benzo(b)fluoranthene (known as PAH4) are of interest in risk assessment studies [31]. For smoked fish, maximum permitted levels of 0.005 mg/kg and 0.012 mg/kg have been determined by the European Union for benzo(a)pyrene and PAH4, respectively [32]. As demonstrated in Figure 1, no contamination by benzo(a)pyrene and lower contamination by PAH4 compared to the international regulation was observed in our smoked samples. Therefore, there was no concern of carcinogenicity by consumption of the smoked fish in Guilan province.

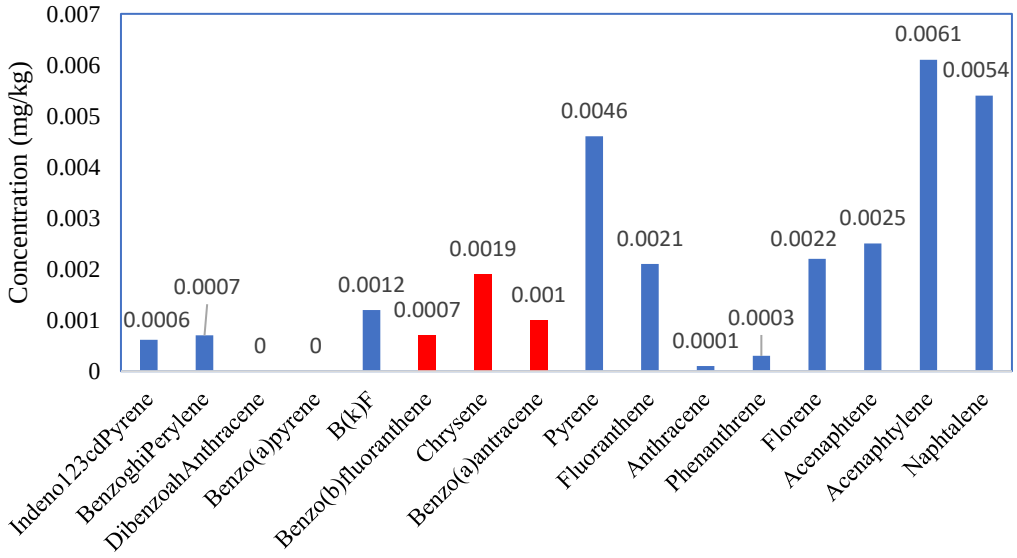

Figure 1- Average concentration of polycyclic aromatic hydrocarbons in the smoked fish samples collected from Guilan (north of Iran) in 2020

3.2.2. Heavy metals

These chemicals are of natural components found in the earth crust. Some heavy metals are beneficial for human (e.g., iron, zinc, copper, selenium) and some others are hazardous to health (e.g., cadmium, lead, arsenic). They accumulate

in the body and may pose a threat to human after chronic exposure [33]. Seafoods are of edible sources susceptible to heavy metal contamination and bioaccumulation [34].

Pb is the most widespread heavy metal in the environment and causes skeletal and renal defects in the consumers. Cd may cause skeletal and neural defects, cancer, and kidney disorder [34,35]. Studies revealed that fish mostly absorbs Pb and Cd from water rather than foods. Therefore, these chemicals accumulate in their gill or other internal organs such as kidney and liver. Furthermore, fish skin is coated with mucus layer that is a barrier against heavy metals' attachment and further absorption to fish muscle [36,37]. According to Figures 2 and 3, contamination of the smoked fish samples by Pb and Cd is significantly lower than the maximum permitted level determined by the national regulation. As defined in the method section, muscle of the smoked fish samples was analyzed in our study, and their bone and other non-edible organs were removed before analysis. Accordingly, there is no risk for the consumers after consumption of the smoked fish in term of heavy metal bioaccumulation.

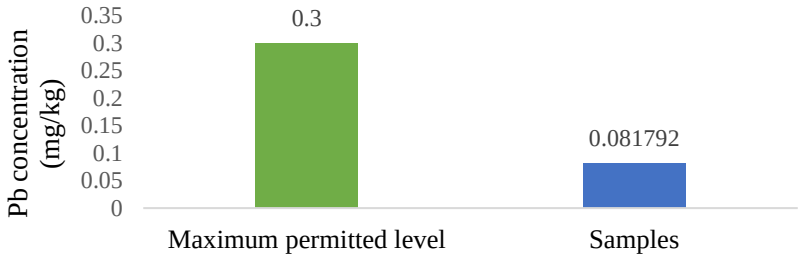

Figure 2- Average concentration of Pb in the smoked fish samples collected from Guilan (north of Iran) in 2020 compared to the maximum permitted level

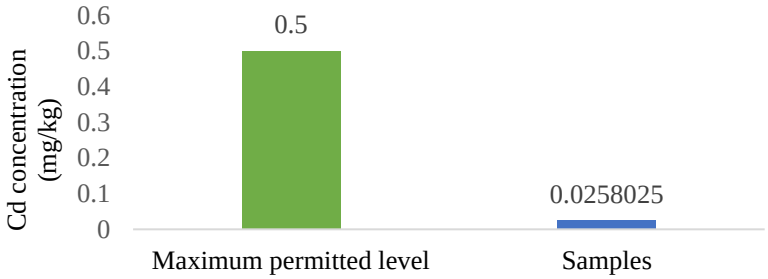

Figure 3- Average concentration of Cd in the smoked fish samples collected from Guilan (north of Iran) in 2020 compared to the maximum permitted level

3.3. Salt content

High salt intake is directly associated with hypertension, cardiovascular diseases, stroke, osteoporosis, kidney stone, gastric *Helicobacter pylori* infection, and stomach cancer [38]. In this regard, table salt and the salt added during food preparation have a significant share in daily salt intake and hypertension [12]. Following the high incidence of non-communicable diseases in the world, WHO developed a global action plan in 2013 by focusing on behavioral and nutritional risk factors. Through which, 30% reduction in daily salt intake of the population by 2025 was programmed by the countries [12].

It has been estimated that reduction of salt intake from 10 to 5 g/day (according to WHO recommendation) decreases the incidence of cardiovascular diseases and stroke by 17% and 23%, respectively [39], which significantly decreases financial burdens of the governments. Iran is a

pioneer country in the region in salt intake reduction of the population. Reduction of salt in bread and the industrial foods which have great share in daily food basket has been mandated by the national authorities [12]. As observed in Figure 4, average salt concentration in the smoked fish samples was significantly higher than the maximum permitted level determined by the national regulation. Unfortunately, smoked fish is popular in north of Iran and its high salt increases risk of the associated diseases in the habitats. Therefore, the production units producing traditional foods such as smoked fish should be supervised and monitored strictly by the regulatory authorities to reduce the added salt to the products.

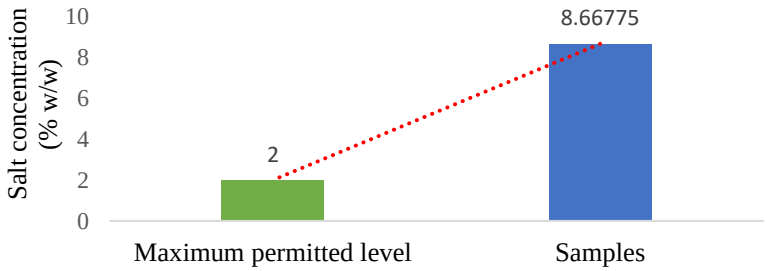

Figure 4- Average concentration of salt in the smoked fish samples collected from Guilan (north of Iran) in 2020 compared to the maximum permitted level

4. Conclusion

The current work aimed to examine chemical and microbial commination of the smoked fishes in Guilan province (north of Iran). According to the results, concentration of PAHs and heavy metals of Cd and Pb was within the acceptable ranges determined by the health agencies. However, the low concentration of these chemicals may be of concern due to their accumulation in the body, which may pose a serious risk to the consumers under chronic exposure. No contamination by *L. monocytogenes* was observed in the samples. The low rate of fungal contamination can be controlled by hot smoking and cooking of the products before consumption. In line with the WHO action plan, the high salt content of the smoked fishes should be strictly monitored by the authorities to reduce the risk of associated diseases.

5. Conflict of interest

The authors declare that they have no conflict of interests.

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