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Analysis of microbiological and chemical risks in fresh and smoked fishes sold in Togo

Ekpetsi Chantal Bouka[1,2], Povi Lawson-Evi[2], Kokou Idoh[1], Essodolom Paka[1], Kwashie Eklu-Gadegbeku[2]

[1]Togolese Institute of Agronomic Research (ITRA), Lomé Togo, PO Box: 02 BP 20214 Lomé 02, Togo Tel: +228 90 07 26 80,
[2]University of Lomé-Togo, Faculty of Sciences, Department of Animal Physiology/Pharmacology

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Abstract**—***The diseases due to fish contamination are an important public health concern worldwide particularly histamine fish poisoning. Histamine is produced during bacterial decarboxylation of the histidine present in fish muscles. The aim of this study is to analyze microbiological and chemical factors of degradation of fresh fish at Lomé fishery port and assess histamine content in smoked fish sold in local market. The method of European Regulation has been used to determine Total volatile base nitrogen (TVBN) and the content of histamine was performed by Agilent 1290 Infinity II HPLC. The standard NF V08-051: 1999 was used for the count of microbial colonies expressed in colony forming units (CFU/ml). The results show that 100% of fresh fishes were highly contaminated by total aerobic flora (225 0000 CFU/g), Thermotolerant coliforms (88750 CFU/g) and E. coli (103 920 CFU/g). The content of Total volatile base nitrogen (TVBN) of anchovies and sardinella was ranged from 23.34 ± 0.23 to 103.14 ± 0.27 mg/100g. Smoked fishes from three markets have shown higher histamine mean levels varying from 295.06 to 746.54 mg/kg. These results show that Togolese are exposed to high concentrations of histamine when consuming smoked fish. Determination of histamine is very important due to its toxicity for humans and as an indicator of the freshness of fish and fish products.*

Keywords**— *Fish, TVBN, Histamine, amine biogenic, Togo.***

I. INTRODUCTION

In Sub-Saharan Africa, fish represents 22% of animal protein for population nutritional needs and occupies an important place in local economy of coastal regions of West Africa. Fish consumption is extremely high and can reach 47% in Senegal, 62% in Gambia and 63% in Sierra Leone and Ghana (Béné & Heck S., 2005; FAO, 2009). Fish is most often consumed in smoked form (70%) and dried form than fresh form (FAO, 2000). There are two types of fishery in West Africa: industrial fishery and small-scale or artisanal fishery (Dieng et al., 2013). Small-scale fishery is an important source of economic activities and foreign exchange and provides around 40% of animal protein for people who live on 6500 km of coastal states from Mauritania to Nigeria (Belhabib et al., 2015; Moehl et al., 2005). However, in artisanal fishery, basic hygiene good practices are not properly applied resulting in bacterial contamination and proliferation. Inadequate application of good hygiene practices of fishermen and sellers, deficiency of refrigerate system among fish processors are the main sources of fish contamination in small-scale fishery (Bediang et al., 2020).

Diseases due to fish contamination are an important public health concern worldwide particularly histamine fish poisoning (HFP) frequently observed among people with high risk of allergy (Butt et al., 2004; Gonzaga et al., 2009). The highest levels of biogenic amines are observed in fish products. Histamine is produced during bacterial decarboxylation of the histidine present in fish muscles Histamine poisoning is the leading cause of food-borne illness linked to fish consumption worldwide (Dalgaard et al., 2008; Durak-Dados et al., 2020). HFP is caused by

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ingestion of fish containing naturally high levels of tissue heterocyclic amines or histidine and other biogenic amines. Fish in the Scombridae family (tuna, mackerel, skipjack, and bonito) are the most common sources of illness. But consumption of other pelagic fishes such as sardinella and anchovies can also lead to histamine poisoning (Durak-Dados et al., 2020; Podeur, 2014). When harvest and post-harvest temperature conditions are favourable (0 - 25°C) bacterial decarboxylation of histidine leads to histamine formation (DUFLOS, 2009). The bacteria involved in histamine production frequently belong to Enterobacter genus which multiply fast when preservation temperature is more than 8°C (Halász et al., 1994; Prester, 2011). The effect of histamine can be increased by the presence of other biogenic amines produced during bacterial growth in fish (FAO/WHO, 2013; Pawul-Gruba et al., 2014). Biogenic amines are markers of poor storage conditions and putrefaction. They are formed from free amino acids present in living tissues by enzymatic mechanisms (Giorgio et al., 1993).

The determination of total volatile base nitrogen (TVBN) allows to evaluate the level of decomposition of food products containing free amino acids. TVBN is one of the criteria widely used as fish spoilage indicator (Castro et al., 2012). It results from the degradation of proteins by the action of bacteria or enzymes present in fish. Histamine is the most toxic of biogenic amine and can be found in high content in fishery products (Giorgio et al., 1993). Intoxication of HFP can reach a wide range of organs as vascular, heart, gastrointestinal and neurological with symptoms as oedema, urticarial, local inflammation, nausea, vomiting, diarrhoea, cramping, hypotension, headache, palpitation, and oral burning (Castro et al., 2012; FAO/WHO, 2013). In developing countries, research works on foodborne diseases risks associated with seafood products are uncommon. Recent studies showed that prevalence of food allergy is increasing in both Western and developing countries, particularly among younger children within the last two decades (Loh & Tang, 2018).

In Togo, smoked fish is widely consumed. 45% of smoked fishes made of anchovies and sardinella sold in Togo come from small-scale fishery (GIZ, 2018). About 200 tons of fishes come from Ghana small-scale fishery system every year to complete national production (Lare, 2016) Smoked fishes mainly composed of sardinella and anchovies are widely used in households' food and highly recommended in children's nutrition as protein source. Studies on the risk identification of histamine content in fish remain very rare in Togo. This study has been designed to analyse microbiological and chemical factors of degradation of fresh fish at Lomé fishery port and assess histamine content in smoked fish sold in local market.

II. MATERIAL AND METHODS

1. Samples collection

Fresh anchovies *(Engraulus encrasicolus)* and sardinella (*Sardinella maderensis*, *Sardinella aurita*) were collected by random sampling from seining fishermen and wholesalers at Lomé fishery port located at 6.14 latitude and 1.28 longitude on GPS. Processors or smoking site fishes sampling were made on their site at Katanga close to fishery port. The sampling was collected in August 2019 when anchovies and sardinella harvest were optimal. The fishes contained in vessels were sampled randomly with hands protected by latex gloves after mixing. 12 samples of 1-2 kg of anchovies and sardinella were constituted and directly introduced into a sterile freezer bags kept cold into the cooler. Samples are sent to the microbiology laboratory of foodstuffs (LAMICODA) and food analysis laboratory of Togolese institute of agronomic research (ITRA).

Smoked fish (*Engraulus encrasicolus, Sardinella maderensis, Sardinella aurita*) samples were collected on November 2019, from three different markets in Togo. Five samples were collected from Atakpamé market located at 7°31 latitude and 1°70 longitude; Six samples from Adidogomé located at 6°12 latitude and 1°08 longitude and nine samples from Adawlato located at 6°07 latitude and 1°13 longitude. A total of 20 samples were then purchased from the three local markets. The samples were collected from wholesalers in each market. Samples were stored in carrier bags with seals and were transported to Laboratoire Central d'Analyses d'Essais (LCAE) of Tunis in Tunisia where the analyses were performed. In laboratory, samples from each market were mixed and then, a composite of nine samples by market was constituted according to the recommendations of the European Commission Regulation No. 2073/2005 on the microbiological criteria for foodstuffs (KYPRIANOU, 2005).

2. Chemical reagents

Histamine dihydrochloride, 1,1-dimethylbiguanide hydrochloride, Sodium 1-decane sulfonate, hydrochloric acid were purchased from Sigma-Aldrich (Sigma, St Louis, USA), anhydrous potassium dihydrophosphate, anhydrous dipotassium hydrophosphate, Acetonitrile, methanol, orthophosphoric acid, perchloric acid (70%), hydrochloric acid were purchased from Acros organics at Belgium. All chemical reagents were analytical grade.

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3. Microbiological analysis

Microbial analysis was done on fresh fishes collected at Lome fishery port and at Katanga from processors. The analysis was performed according to AFNOR methods used by Soncy et al (2015). 90 ml Tryptone Salt (TS) were added to 10 g of the fish sample in a sterile vial. The mixture was homogenized with a ROBOR MAJOR Moulinex grinder. The stock solution of $1/10^{th}$ obtained was kept at laboratory temperature during 45 minutes for germs regrowth. This stock solution was used for a series of decimal dilutions 1:10 with TS. The count of colonies was expressed by colony forming units (CFU/ml) according to the standard NF V08-051: 1999

4. Total volatile base nitrogen (TVBN)

Total volatile base nitrogen (TVBN) was done on fresh fishes from Lome fishery port and Katanga smoking site. The analysis was performed according to European Commission Regulation (KYPRIANOU Markos, 2007). TVBN was determined by titration of the bases absorbed with hydrochloric acid (HCl) 0,01 N. Volatile nitrogenous bases were extracted from 10 g of fish ground using a solution of perchloric acid at 0.6 mol/l. After alkalization, the extract was submitted to steam distillation with Nitrogen Distiller-Protein BUCHI K 355. The volatiles base components were absorbed by an acid receiver.

5. Histamine

5.1 Histamine standard solutions preparation

Histamine stock standard solution (10 mg/ml) was prepared by dissolving exactly 165.7 ± 0.1 mg of histamine dihydrochloride in 10 ml of hydrochloric acid 0.1 mol/l. On the day of analysis, working solutions (2.5, 5, 10, 20, and 40 µg/ml) were prepared in HCl 0.1 mol/l and were used to generate a calibration curve.

5.2 Samples preparation and analysis

Histamine analysis was performed on smoked fishes collected in three markets. Skin and head of smoked fish were removed to comply with the common use of fishes in Togo. Sample preparation and extraction were carried out according to the protocol of AFSCA (2012). 100 ± 0.1 g of each sample were grounded and 5 ± 0.1 g were weighed into 50 ml polypropylene centrifuge tubes. (15 ml) of perchloric acid 1 mol/l were added and samples were homogenized for 30 minutes at room temperature. Samples were then centrifuged for 10 minutes at 4°C.

A histamine-free sample was doped with histamine (1 mg/ml) and was included in the set of analyses as control. This doped sample was processed as the samples. (250 µl) of 1,1-dimethylbiguanide hydrochloride (DBG) working solution (1 mg/ml) was added to each sample as well as the blank reagent, the doped sample, and standard working solutions for derivatization. Samples were filtered using 0.45 µm PTFE filters into amber vials of 2 ml for HPLC injection.

High pressure liquid chromatographic (HPLC) analysis was performed using Agilent 1290 Infinity II HPLC (Agilent Technologies, Santa Clara, CA, USA). The injection volume was 20 µl. The mobile phase consisted of 14% acetonitrile - 86% phosphate buffer in methanol (85:15; v/v) was applied in isocratic conditions. The column oven temperature was maintained constant at 30°C and a flow rate of 1 ml/min was used. The UV-DAD detection was monitored at 214 nm.

III. RESULTS

1. Microbial contamination

All the samples were 100% contaminated by total aerobic flora (TAF), Thermotolerant coliforms (TTCs) and E. coli (Table 1). The mean count of TAF was 2 250 000 CFU/g showing contamination level 45 times higher than the EC standard (50 000 CFU/g of product). The count of Thermotolerant coliforms (TTCs) and *E. coli* showed that the lower levels of contamination were respectively 700 and 92 times higher than EC standard (10 CFU/g of product for TTCs and E. coli). The count of Staphylococcus aureus was satisfactory according to EU standard in 100 % of the fishery port samples. The processors samples (table 2) are highly contaminated by all germs (TAFs, TTCs, *E. coli*, ASR). The count of *Staphylococcus aureus* was 5 - 10 times higher than EC standard (100 CFU/g of product). Salmonella (Sa) were not found in any sample.

2. Chemical contamination

2.1 Total volatile base nitrogen (TVBN)

The content of total volatile base nitrogen (TVBN) in anchovies and sardinella (table 3) were ranged from 23.34 ± 0.23 to 58.00 ± 0.67 mg/100g. The lowest value was found in anchovy samples from fishermen and it was in compliance to European commission regulation (25 mg/100g). The highest content was observed in anchovies taken from wholesalers and was 2.3 times higher than the

EC standard. The average of TVBN content of anchovies (40, 67 ± 18,98 mg/100g) and sardinella (32, 64 ± 5,75 mg/100g) samples from fishery port was greater than EC standard (25 mg/100g). The concentration of Total volatile base nitrogen (TVBN) in anchovies and sardinella from processors or fish smokers (tables 4) was not compliance

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with EC standard. The values were 3–4 times higher than the threshold value.

Table 1: Microbial contamination of fresh fishes from fishery port

Fresh fishes	**TAF CFU/g**	**TTC CFU/g**	***E. Coli* CFU/g**	**ASR CFU/g**	**Staph CFU/g**	**Sa**
Anch. F	300 10^3	92 10^3	34 10^3	100	<100	-
Anch. W	5 400 10^3	560 10^3	54 10^3	500	<100	-
Sard. F	2 200 10^3	96 10^3	15 10^3	<10	<100	-
Sard. W	1 100 10^3	7 10^3	920	<10	<100	-
ECS	**50 10^3**	**10**	**10**	**-**	**100**	**TA/ 25g**

Anch.: anchovies; Sard.: sardinella; F: fisherman; W: wholesalers; TAF: Total aerobic flora; TTC: Thermotolerant coliforms; E. coli: Escherichia coli; ASR anaerobic sulphite reducing; Staph: Staphylococcus aureus; Sa: Salmonella spp.; TA./25g: total absence/25g; ECS: European Commission Standard (KYPRIANOU Markos, 2007)

Table 2: Microbial contamination of fresh fishes from processors or smoking site

Fresh fishes	**TAF CFU/g**	**TTC CFU/g**	**E. Coli CFU/g**	**ASR CFU/g**	**Staph CFU/g**	**Sa**
Anch	16 530 10^3	1 271 400	299 200	67 100	543	-
Sard	7 820 10^3	2 472 10^3	41 392	98 610	1034	-
ECS	**50 10^3**	**10**	**10**	**-**	**100**	**TA/25g**

Anch.: anchovies; Sard.: sardinella; TAF: Total aerobic flora; TTC: Thermotolerant coliforms; E. coli: Escherichia coli; ASR anaerobic sulphite reducing; Staph: Staphylococcus aureus; Sa: Salmonella spp.; TA./25g: total absence/25g ECS: European Commission Standard (KYPRIANOU Markos, 2007).

2.2 Histamine

Table 5 showed that 100% of smoked fish samples collected from wholesalers in three different markets in Togo contains high amounts of histamine. Histamine contents are different from one market to another. The highest histamine contents were found in samples collected from Adawlato market (Table 5) with a mean value of 746.54 mg/kg and a maximum value of 881.87

mg/kg. Samples collected from Atakpame market also show high content of histamine with a mean value of 472.37 mg/kg and a maximum amount of 552.75 mg/kg. The lowest contents were found at Adidogome market with a mean value of 295.06 mg/kg. The smoked fish samples analyzed were not conform to the food safety criteria for histamine according to EC standard (100 – 200 mg/kg).

Table 3: Total volatile base nitrogen (TVBN) in fresh fishes from fishery port

Fresh fishes	**TVBN mg/100g**	**Mean mg/100g**
Anchovies F	23.34 ± 0.23	40.67 ± 18,98
Anchovies W	58.00 ± 0.67	
Sardinellas F	27.47 ± 0.96	32.64 ± 5,75
Sardinella W	37.81 ± 1.35	
EC Standard	**25**	

Data are expressed as means ± SD (n = 3), (mg TVBN/100 g of fish); F: fishermen; W: wholesalers; TVBN: total volatile base nitrogen; EC: European Commission (KYPRIANOU Markos, 2005).

Table 4: Total volatile base nitrogen (TVBN) in fresh fishes from processors or smoking site

Fresh fishes	**TVBN mg/100g**
Anchovies	92.67 ± 0.33
Sardinella	103.14 ± 0.27
EC Standard	**25**

Data are expressed as means ± SD (n = 3), (mg TVBN/100 g of fish); TVBN: total volatile base nitrogen; EC: European Commission, 2005

IV. DISCUSSION

Microbial germs are responsible of decarboxylation and transamination of amino acids in foods(Kim et al., 2009). Microbial analysis has shown massive contamination of fresh fishes by total aerobic flora (TAF), Thermotolerant coliforms (TTCs) and *E. coli*. The count has revealed a level of contamination 45 to 700 times higher than threshold limit of European commission regulation. This result is in line with the observation of Bediang et al. (2020) who found high contamination of sardinella and anchovies by the same germs in the fishery harbor of Lome. The processors samples were more contaminated than the samples from fishery port. High levels of contamination in processors are due to lack of refrigeration system during the transport and poor hygienic conditions on the smoking site. TAF, TTCs and *E. coli* belong to the group of Enterobacter bacteria,

very widely distributed in soil and water and hosted by human and animal intestines (Halász et al., 1994; DUFLOS, 2009;). These bacteria are involved in spoilage of fish and fish products (Gram & Huss, 1996) and produced histidine decarboxylase during their growth. Histidine decarboxylase reacts with free histidine and produce histamine a decarboxylation by-product (FAO, 2018).

Table 5: Histamine content in samples collected from Adawlato, Atakpamé and Adidogomé market

Smoked fishes	Histamine content (mg/kg)		
	Adawlato	Atakpamé	Adidogomé
1	800.28	466.17	329.39
2	716.45	492.25	172.15
3	631.41	490.69	244.39
4	768.65	430.55	306.68
5	685.80	443.46	250.17
6	782.19	552.75	239.33
7	657.53	461.85	360.72
8	881.87	439.30	348.48
9	794.81	474.38	404.25
Mean	**746.54**	**472.37**	**295.06**
EC standard	**100 - 200**	**100 - 200**	**100 - 200**

EC: European Commission (BARROSO, 2013).

Total volatile base nitrogen (TVBN) is a chemical method used to measure fish spoilage. It is a good indicator of fish freshness and used by official authorities for quality control of fish (Castro et al., 2012). Fishermen anchovies were fresher than those from processors that presented the highest values of TVBN (92.67 ± 0.33 - 103.14 ± 0.27 mg/100g). TVBN results from the degradation of proteins by the action of bacteria or enzymes present in fish. Increasing of TVBN content from fishery port to processor is due to an increasing of microbial flora in tropical condition during the transport. There is a very high correlation between TVBN production and bacterial multiplication (Podeur, 2014). Biogenic amines are basic nitrogenous compounds found in fish and fishery products that can be arise with decarboxylation of amino acids by microorganisms bacterial activities (Durak-Dados et al., 2020). Microbial contamination and TVBN content of fresh samples of anchovy and sardinella show

the presence of accelerated degradation factors before smoking. The formation of histamine in fish containing large amounts of endogenous histidine is more related to the intensive activity of microorganisms (Durak-Dados et al., 2020). Sardinella and anchovies are the product of small-scale fishing and are mainly sold in smoked form in Togo. The contents of histamine were very higher in all the samples collected from the three markets of Togo. No sample is less than 100 mg/kg of histamine content and the amount of two samples is more than 200 mg/kg as recommended by European commission regulation (BARROSO, 2013). According to this regulation, the histamine content in fish and fish-derived products is considered safe for consumption, if among nine samples analyzed, the mean observed concentration is ≤ 100 mg/kg. A maximum of two samples must have a concentration between 100 and 200 mg/kg and no concentration exceeds the limit of 200 mg/kg. The concentrations of histamine found in smoked fishes in Togo were 20-30 times higher than the concentration found in smoked fishes in Poland (24,1 mg/kg) by Pawul-Gruba et al. (2014) and in fresh fish in Ivory Coast (18,01 mg/kg) by Kablan et al. (2019). However, the levels of histamine found in smoked fishes in Togo were also found in smoked fishes sold in New Zeeland market (346.4 and 681.8 mg/kg) by Fletcher et al. (1998).

Histamine is natural component of human cell (Durak-Dados et al., 2020; Podeur, 2014). During food intake process in human gut, low amounts of histamine are

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metabolized to a physiologically less active degradation product (Pawul-Gruba et al., 2014). It is difficult to determine the threshold dose body detoxification systems can eliminate. The threshold dose depends on multiple human factors. In fish consumption, epidemiological data show that histamine levels below 50 mg/kg do not cause any toxic effect. From 50 to 200 mg/kg, there is a possible risk of toxicity. From 200 to1000 mg/kg, there is a probable risk of toxicity. Above 1000 mg/kg, consumption of the food is considered to present a definite risk of toxicity (Bartholomew et al., 1987; Dalgaard et al., 2008). The mean value of histamine found in smoked fishes shows a real problem of public health.

The fresh fishes are highly contaminated by bacteria from Enterobacteriaceae family as TAFs, CTTs and *E. coli* at Lome fishery port. On smoking site, the rate of bacteria count was increased in addition of other germs as anaerobic sulphite reducing (ASR) and Staphylococcus aureus. In fact, in smoking site, the fishes come from fishery port 2-5 hours after harvest without a good refrigeration with inadequate sanitary fish storage conditions. Durak-Dados, and al. (2020) have shown that fundamental actions preventing bacterial spoilage and production of histamine were good refrigeration, good hygiene practices and appropriate processing procedures. It is important to respect the time and low temperature during unloading, transportation, storage, and processing of fish (Hungerford, 2010; McLauchlin et al., 2006).

V. CONCLUSION

This study showed that the sector of small-scale fishery must be organized and equipped to apply good practices hygiene to provide Togolese consumer a fresh fish less contaminated by microbial with a good indicator of freshness. Smoked fish samples collected from Togo markets contain very high amounts of histamine showing that Togolese population are exposed to high concentrations of histamine in smoked fish consumption.

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