Sanitary Investigation on Microbial and Heavy Metals of grilled fish sold in the night street market of Daloa (Ivory Coast).

Abstract

The quality of food sold in the street is a major public health concern. This research was conducted to study the sanitary quality of grilled fish sold in the streets of three districts of Daloa.Seasoned and unseasoned grilled fish were collected in 3 districts of Daloa, namely Commerce, Soleil and Tazibouo. A total of 150 unseasoned and 150 seasoned grilled fish were collected and analyzed for the detection and enumeration of *Enterobacteriaceae, Staphylococcus aureus* and fungal flora. We also tested for the presence of heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg) and zinc (Zn). The antibiotic resistance of *S. aureus* was also checked. The results showed that *Enterobacteriaceae* represented the highest level of microbial load in the samples of seasoned grilled fish from the 3 districts. Total coliform levels ranged from (7.05 ± 2.1) x 10^3 colony-forming units (CFU)/g to (4.1 ± 1.9) x 10^5 CFU/g and from 2.8 ± 3.3 x10^2 to 3.4 ± 2.1x10^3 CFU/g for thermotolerant coliforms. The presence of heavy metals showed higher values in seasoned grilled fish than in unseasoned grilled fish. The highest values of heavy metals are Al (1.98±0.120 mg/kg), Pb (1.46±0.23 mg/kg), Cu (0.7±0.1 mg/kg), Hg **(**0.40±0.201 mg/kg) and Hg (0.9±0.201 mg/kg). The microbiological quality of both types of fish samples was unsatisfactory. The presence of *S. aureus* resistance was noticed with 80% resistance to vancomycin, lincomycin, gentamycin and erythromycin but all strains tested were sensitive to imipenem.

Keywords: Fish seasoned, Microbial, Heavy Metals, Resistance, Daloa.

Introduction

Food is the primary consumer good used to satisfy the primary needs of all humans. The quality of food is crucial for the health of consumers [1]. Food is the primary means of transmission of micro-organisms when it is not consumed in a healthy manner. As a result, several million people suffer from foodborne illnesses each year [1-2]. Most of this occurs outside the home. In recent decades, the street food sector has grown in urban areas of low and middle-income, both by providing access to a variety of low-cost foods to low-income households and by providing employment opportunities for many urban residents. The street food sector also contributes to the economy of an urban and peri-urban agricultural sector [3]. Microbial contamination is an important health risk associated with street food. Outbreaks of foodborne disease have been linked to poor hygiene in restaurants and the consumption of food from street [4]. Diarrheal diseases due to contaminated and unhygienic food are among the leading causes of illness and death in low-income countries. Several epidemics have been attributed to the consumption of street food [5]. Foodborne diseases are mainly caused by pathogenic bacteria [6-7] and cause an estimated 48 million illnesses and 3,000 deaths per year [8-9]. The most commonly identified diseases are botulism, shigellosis campylobacteriosis, salmonellosis, listeriosis, cholera, and *Escherichia coli* and *Staphylococcus aureus* infections, are also widespread and pose a major threat to human health [10]. The main factors causing foodborne illnesses are food from unsafe sources, inadequate cooking, inappropriate storage temperatures, contaminated equipment and cross-contamination, and poor personal hygiene. Microbial contamination differs according to the nature and origin of the food [7].

In Côte d'Ivoire, fish is the most consumed source of protein [11]. Fish, like meat, is an important source of animal protein, which is generally richer in essential amino acids than vegetable protein and is more digestible. For non-meat eaters, fish is therefore a source of protein, with average contents ranging from 15 to more than 25 g of protein/100 g [12]. In terms of the distribution of processed products, the main commodity is smoked fish, which accounts for just 60%, followed by fermented-dried fish, which accounts for 24%, salted-dried fish with 17% and grilled fish with less than 1% [13]. Grilled fish is one of the modes of consumption of fishery products most appreciated by Ivorians. They provide nearly 70% of the animal protein consumed by Ivorians. These fishery resources have the advantage of being cheaper than red meat [14]. In the Upper Sassandra region, the fish sold on the markets come from the Guéssabo River [15]. The river is so polluted that there is a clear interaction between fish species and heavy metal levels [16]. Grilled fish is usually cooked and dressed in fresh vegetable. Vegetables are important components of a healthy and balanced diet. They are an extraordinary food source of nutrients, micronutrients, vitamins, and fiber for humans and are therefore essential for health and well-being [17]. The objective of this work is to assess the microbiological and heavy metal quality of grilled fish sold in restaurants in the city of Daloa. For this study, we looked for the germs responsible for toxicity such as total coliforms, thermo-tolerant coliforms, *Salmonella, S. aureus* and fungal flora as well as heavy metal toxicity.

Material and Methods

Experimental design

The study was carried out in the city of Daloa; it's in the Centre West of Côte d'Ivoire. Geographically, Daloa is located at 6° 53 north latitude and 6° 27 west longitudes [18]. Daloa is an urban center with a population of 705,378 [19]. The first step was to conduct an observational survey to select the sampling sites but also to see how the fish are grilled. The fish used in this study is *Cyprinus carpio*. The preparation of grilled fish is as follows: fresh fish is scaled, gutted from the inside and cleaned with drinking water. Then, an incision is made on both sides of the fish before it is marinated in a spicy sauce for a few minutes. It is then cooked over charcoal coals on an iron grill. Once the fish is cooked, it is dressed with a salad of finely chopped ready-to-use vegetables consisting of tomatoes (*Solanum lycopersicon* L), onions (*Allium cepa*), cucumbers (*Cucumis sativus*) and peppers (*Capsicum annuum*). Due to their height frequentation and selling, samples were collected from three sites (Soleil, Commerce and Tazibouo). The collection of grilled fish samples was done by purchase. In total, 300 grilled carp (*Oreochromis niloticus*) were purchased, 100 fish per site. From each site, 2 batches of fish were formed, namely one batch of 50 seasoned grilled fish and one batch of 50 unseasoned grilled fish. Each sample was placed in a sterile bag and transported to the laboratory at temperatures between 4°C and 6°C.

Isolation and enumeration of bacteria

The microbiological analysis consisted in looking for the following germs: Total and thermotolerant coliforms (NF ISO 4831), *Staphylococcus aureus* (NF EN ISO 6888-1), *Salmonella sp* (NF V 08 - 052), yeasts and molds (ISO 7954), [20]. For this, 25 g of each sample weighed aseptically were immersed in 225 ml of sterile distilled water for 15 min, vigorously agitated and subsequently left to stand for 30 min at room temperature, to allow detachment and revivification of the microorganisms. From this suspension, a series of decimal dilutions were then carried out. The 10^{-1} to 10^{-4} dilutions were seeded. Two types of inoculation were carried out during this manipulation. These are mass seeding which concerned Sabouraud media with chloramphenicol (Alpha Biosciences, USA), Violet Red Bile Lactose (VRBL, Diagnostici-Liofilchem, Italy**)** and Violet Red Bile Glucose (VRBG, Diagnostici-Liofilchem, Italy**)**, and surface seeding by spreading using the Baird Parker media (Alpha Biosciences), surface seeding by streaking using the Hecktoen medium (Biorad). The isolation and identification of *Salmonella* was carried out following the Adane *et al.* [21] protocol. After incubation, suitable Petri plates from different dilutions were selected and distinct bacterial colonies were counted with a colony counter. Total viable bacteria count (TVC) was subsequently deduced and expressed as colony-forming units per gram (CFU per g) of the sample.

The results were interpreted according to a two-class plan with reference to the microbiological criteria for fresh animal products (French legislative and regulatory guide, No. 8155 of 12 December 2000), setting the tolerance threshold at M $=10^3$ CFU/g or ml for total flora; 10 CFU/g or ml for fecal coliforms and thermotolerant coliforms including *S. aureus*; and the absence of *Salmonella* in 25 g of the product analyzed.

***Staphylococcus* strains identification**

A total of 30 suspect strains of *S. aureus* were isolated from the 2 types of fish. Standard microbiological methods for identification of microorganisms were used [22]. Then, the suspected *Staphylococcus* colony was subculture on Mueller-Hinton agar (bioMérieux, Marcy l'Etoile, France) and identified by Gram staining, catalase and Slidex Staph Plus test (bioMérieux, Marcy l'Etoile, France) and coagulase test with rabbit plasma [23]. Finally, the strains were analyzed by API Staph (bioMérieux, Marcy l'Etoile, France).

Antibiotic resistance of suspect *S. aureus* strains isolated from both types of fish

Antimicrobial susceptibility was determined by the disc diffusion method of Kirby-Bauer on agar Mueller-Hinton (bioMérieux, Marcy l'Etoile, France) as recommended by the Antibiogram Committee of the French Microbiology Society [24]. After 24 h at 37°C, inhibition zone was measured. For susceptibility to oxacillin, inoculum of 10^7 CFU/ml was prepared, and the plate was incubated at 37°C for 24 h on Mueller-Hinton agar plus 2% NaCl. The tested antibiotics (Bio-Rad, Marne la Coquette, France) were: pristinamycin; erythromycin; lincomycin, oxacillin, amoxicillin, ceftriaxone, gentamicin, tobramycin, sisomicin; oxytetracycline, tetracycline, trimethoprim / sulfamides, cefotaxime, ofloxacin, pefloxacin, vancomycin, rifampicin, and imipenem.

Determination of Heavy Metals (Pb, Zn, Cd, Cu and Mg)

The method of Ibrahim *et al*. [25] was used with a slight modification. Muscle and skin organs were dried using an electric oven at 150 °C overnight. One gram of each dry tissue was mixed with 10 ml of concentrated HNO_3 for one hour. Heating and evaporation until red vapour appeared, then cooling and adding 5 ml of 30% H_2O_2 and reheating until digestion was complete. Cooling of the sample and addition of deionized distilled water to a volume of 25 ml. The concentration of heavy metals (Pb, Zn, Cd, Cu) was determined by flame atomic absorption. For the determination of mercury, mix 1ml of the digested sample with 1ml of the separated solution (dithozone+ CCl_4) in a separate funnel. Repeat the separation three times. The organic layers were washed with weakly alkaline NH_4OH three times until the organic layer changed from green to orange and the last wash with acetic acid (2 M). Mercury concentrations were determined using a UV-visible spectrophotometer (485 λ).

Statistical analysis

The results were statistically analyzed by the variance method (ANOVA) using the STATISTICA 7.1 software (Statsoft, France). The comparison of means is carried out by the test of the smallest significant difference Tukey's test. The differences are significant when $p < 0.05$. When the probability is greater than 0.05 ($p > 0.05$) the statistical differences are not significant. In the event of a significant difference ($p < 0.05$) between the means, Tukey's test was performed to determine the different classes of homogeneity.

RESULTS

Bacterial load of samples of seasoned grilled fish

The average load of the various germs sought is shown in Table 1. The grilled fish taken from the Soleil district site showed no contamination with *Salmonella sp.*, unlike the samples from the 2 other sampling sites. Enterobacteria represent the highest bacterial load in samples from the 3 sites. The values range from $7.05 \times 10^3 \pm 2.1$ CFU/g to $4.1 \times 10^5 \pm 1.9$ for total coliforms and $2.8 \times 10^2 \pm 3.3$ to $3.4 \times 10^3 \pm 2.1$ CFU/g for thermotolerant coliforms. Fungal flora represents the less contaminant load in all samples compared to other investigated germs. According to the standards set, all fish from the different sites are unsatisfactory.

Table 1: Bacteria and fungi flora load of collected seasoned grilled fish.

Sampling site (District)	**Germs loads (CFU/g)**				
	Total coliforms	Thermotolerant coliforms	Fungi flora	*S. aureus*	*Salmonella sp.*
Commerce	$4.1 \times 10^5 \pm 1.9^a$	$1.3 \times 10^3 \pm 2.1^a$	$1.10^2 \pm 2.3^a$	$1.8\times10^3 \pm 3.2^a$	+
Tazibouo	$7.05 \times10^3 \pm 2.1^a$	$2.8 \times 10^{2\,\pm}3.3^a$	$2.3 \times 10^2 \pm 1.3$	$5.5\times10^3 \pm 5.1^a$	+
Soleil	$1.6 \times 10^4 \pm 1.2^a$	$3.4 \times 10^3 \pm 2.1^a$	$1.7\times10^2 \pm 3.2^a$	$7.1\times10^2 \pm 3.5^a$	-
Criteria [26]	10	10	10	10	Absence

+ : presence of *Salmonella sp*, - : absence of *Salmonella* sp ; Values assigned the same letter are statistically identical, at the 5% threshold.

Bacterial load of samples of unseasoned grilled fish

Microbiological analysis of unseasoned grilled fish showed a considerable decrease in the microbial load of all samples, and no *Salmonella* contamination was observed (Table 2). The microbial load for thermotolerant coliforms, fungal flora and *S. aureus* were all above the set standard (10 CFU/g). Total coliforms are the most abundant germs in the different samples. The highest value is 23×10^2 CFU/g for the samples taken at the soleil site**.**

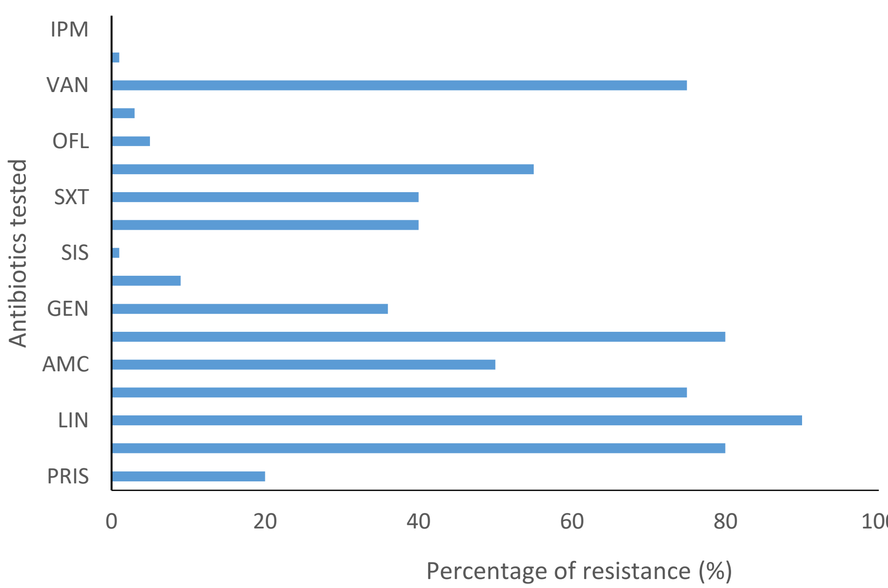
Table 2: Bacterial and fungal flora loads of unseasoned grilled fish.

Sampling site (District)	Microorganisms loads (CFU/g)				
	Total coliforms	Thermotolerant coliforms	Fungal flora	*S. aureus*	*Salmonella sp*
Commerce	$1.1 \times 10^{2} \pm 1.9^{a}$	$1.32 \times 10^{1} \pm 2.1^{a}$	$4.1 \pm 2,3^{a}$	$1.43 \times 10^{1} \pm 3.2^{a}$	-
Tazibouo	$2.1 \times 10^{2} \pm 2.1^{a}$	$2.73 \times 10^{1} \pm 3.3^{a}$	$8.1 \pm 1,3^{a}$	$5.45 \times 10^{1} \pm 5.1^{a}$	-
Soleil	$2.3 \times 10^{3} \pm 1.2^{a}$	$3.41 \times 10^{1} \pm 2.1^{a}$	9.1 ± 3.2^{a}	$1.76 \times 10^{1} \pm 3.5^{a}$	-
Criteria [26]	10	10	10	10	Absence

+ : presence of *Salmonella sp*, - : absence of *Salmonella* sp ; Values assigned the same letter are statistically identical, at the 5% threshold.

Antibiotic resistance of strains of *S. aureus*

The Figure 1 shows the proportion of *S. aureus* strains resistant to the different antibiotics tested. We notice that almost 80% of the strains are resistant to vancomycin, lincomycin, Gentamycin and erythromycin. On the other hand, all the strains tested are sensitive to imipenem.



PRIS : Pristinamycin; ERY: Erythromycin; LIN: Lincomycin; OXA: Oxacillin; AMC: Amoxicillin; CRO: Ceftriaxone; GEN: Gentamicin; TOB: Tobramycin; SIS: sisomicin; OXT: Oxytetracycline; TET: Tetracycline; SXT: Trimethoprim/sulfamides; CTX: Cefotaxime; OFL: Ofloxacine; PEF: Pefloxacin; VAN: Vancomycin; RIF: Rifampicin; IPM: Imipenem.

Figure 1: Antibiotics resistance profiles of suspects strains of *S. aureus*.

Concentration of heavy metals in seasoned and unseasoned grilled fish

Table 2 below shows the concentration of heavy metals in the two types of fish. The concentration of each metal is significantly higher in the seasoned grilled fish than in the unseasoned grilled fish. Zinc remains the metal with the highest concentration at 36.2 mg/Kg, although it is still within the standard of 75 mg/Kg. The other metals are above the standard for both types of fish.

Table 3: Mean concentration (mg/kg) of Pb, Zn, Cu, Cd and Hg in the tissues of the different type of fish

	Heavy metals concentration (mg/kg)					
Types of fish	Pb,	Zn	Cu	Cd	Hg	Al
Unseasoned grilled fish	0.9±0.14	17.5±0.70	0.5±0.02	0.5 ±0.2	0.48±0.16	0.823±0.50
Seasoned grilled fish	1.46±0.23	36.2±0.50	0.7±0.1	0.8±0.06	0.40±0.201	1.98±0.120
Norm [27]	[0.3-0.5]	75	0.2	0.2	[0.1-0.3]	1

Discussion

Fish and fish products play an important role in the diet of Africa's populations [28]. This study assessed the quality of seasoned and unseasoned grilled fish sold in three main districts of the city of Daloa. These are the Commerce, Tazibouo and Soleil districts. Street food vending is a thriving business, especially in the developing countries, and it is one of the most common business practices globally, as it generates income in many of the low-income households [29]. Street food vendors are estimated to feed more than 50% of the urban population in developing countries. However, street vended foods may be a source of many foodborne pathogens and illnesses if not regulated or properly handled [15]. The sanitary evaluation of grilled fish sold revealed the presence of germs, such as coliforms, thermotolerant coliforms, fungal flora, and *Salmonella* sp. which were found in both types of samples, namely seasoned and unseasoned grilled fish. These same types of germs were found in the studies of Israa *et al*. [25] and Attien *et al*. [15]. The source of contamination is diverse either from the places of fishing and the places of transformation. According to Kaffine *et al*. [30], this is due to the contamination of the products by various improper handling. Indeed, the technology used in artisanal processing is essentially empirical and rudimentary with several flaws in the production chain (non-compliance with hygiene rules, non-cleaning of equipment, raw material, hands of working staff).

Nevertheless, the load of total and thermotolerant coliforms remains very high in the samples of grilled fish. Among seasoned grilled fish, total coliforms ($4.1 \times 10^5 \pm 1.9$ CFU/g) and thermotolerant coliforms ($3.4 \times 10^3 \pm 2.1$ CFU/g) were higher than those recorded with unseasoned grilled fish samples for total coliforms ($23 \times 10^2 \pm 1.2$) and thermotolerant coliforms (3.41×10^1 CFU/g). The presence of *Enterobacteriaceae* would be linked to the fecal contamination of water sources by warm-blooded animals [31]. Poor handling and processing would also favor secondary contamination [32]. The high bacterial load observed in the seasoned grilled fish is thought to be due to the condiments (salad vegetables, tomatoes, onions, cucumbers, finely chopped) added for seasoning. This could be justified by post-contamination after cooking the fish by the vegetables [33]. This is corroborated by the microbiological quality results, with more than 50% of the seasoned grilled fish being of unsatisfactory microbiological quality and 10% being acceptable from the microbiological quality point of view. According to the work of Toe *et al.* [34] on vegetable salads used as seasoning, the vegetables are not disinfected and are simply soaked in water, which does not allow effective decontamination. In addition, the water used for soaking the vegetables is rarely changed, which can encourage the accumulation of bacteria, which are then transferred to other batches of uncontaminated products in subsequent washings, through cross-contamination. In some cases, vegetables were served with bare hands and the same utensils were used to cut animal food (chicken, fish, meat) and vegetables.

Enterobacteriaceae were the main bacteria contaminating the vegetables. The prevalence was 100%, 77.8% and 2.6% for *Enterobacteriaceae*, *Escherichia coli* and *Salmonella sp.*, respectively [35]. The presence of *Enterobacteriaceae* in raw vegetable salads can be explained by the fact that they are widespread and widely distributed in the environment via soil, water, animal and human intestines, and vegetables. Some enterobacteria are naturally present in the flora associated with vegetable cultivation [36]. Despite the benefits of eating fruit and vegetables, many studies have shown that that these foods, when consumed fresh, provide an ideal substrate for microbial contamination [37-38]. From a food safety perspective, these are known to be foods at risk of transmitting pathogenic microorganisms [34, 39],. The presence of *S. aureus* in both types of poisons is not as suppressive as it is a commensal bacterium of humans. Indeed, according to Todd *et al.* [41], *S. aureus* colonizes the skin and mucous membranes of food handlers. These food handlers can therefore constitute a source of contamination of food during its handling and processing. But this bacterium easily colonizes fish. Work by Baumgartner *et al*. [42] showed high prevalence in fish in Japan (87%) and northwestern Spain (43.5%); but a low prevalence rate (19.8%) was reported in Korea. The different and high prevalence rates of *S. aureus* found in this, and other studies could be attributed to differences in the treatment and hygiene practices of symptomatic and/or asymptomatic fish handlers. The isolated strains show a very high resistance to conventional antibiotics. In fact, nearly 80% of the strains were resistant to vancomycin, lincomycin, gentamycin and erythromycin. Our results are corroborated by the work of Attien *et al.* [43] and Obaidat *et al.* [44] who showed that most of the *S. aureus* strains isolated from meat and fish respectively are resistant to at least one classical antibiotic. The emergence of resistant strains is therefore becoming a public health concern for governments.

As for the presence of heavy metals in poisons, this concern is as important as the presence of bacteria. Indeed, heavy metals are among the major environmental pollutants, both because of the ubiquitous nature of their presence in the biosphere and because of their toxicity and potential bioaccumulation in many aquatic species, leading to devastating effects [45]. In the aquatic environment, heavy metals can accumulate in the organisms living there and reach concentrations in certain tissues or organs that are sometimes higher than those measured in water: this phenomenon is called "bioaccumulation". The fact that fish are also an important part of the diet raises concerns about health risks [46]. Analysis of tissues from unseasoned and seasoned grilled fish reveals the presence of heavy metals. These metals are present in varying proportions in the two types of fish studied. In the case of seasoned grilled fish, the values of heavy metals are high, especially the zinc (36.2 mg/kg), which are above the accepted values (75 mg/kg). This implies that the vegetables added for seasoning are the source. Most of the cadmium accumulated by humans comes from agricultural activities (use of phosphate fertilizers rich in cadmium, spreading of waste sludge in market gardening areas) but also from industrial activities (zinc and lead metallurgy, etc.) [47]. Indeed, most of the vegetables used come from market gardening.

Conclusion

This study has shown that the quality of grilled fish sold in the main districts of Daloa are foods at risk for the health of consumers. The great majority of seasoned grilled fish are of unsatisfactory microbiological quality. Seasoned grilled fish are mostly contaminated with *Enterobacteriaceae*, *S. aureus* and fungal flora. The isolated *S. aureus* strains were resistant to most of tested antibiotics. the dangers associated with microorganisms can be aggravated by the noticeable increases in this antibiotic resistance. In addition, sampled grilled fish shows high values of heavy metals. It will be useful to minimize contamination at each level through the application of good hygiene, handling measures of these foods and effective decontamination.

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