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Determination of bacterial population and the presence of pesticide residues from some Cameroonian smoked and dried fish

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ABSTRACT

The objective of this study was to identify the dominant bacterial flora linked to the hygiene of dried and smoked fish in different geographical locations and to determine the presence of pesticide residues on the processed fish. Fish species were listed and collected. The bacterial ecology of dried and smoked fish from the northern zone of Cameroon was probed using a molecular technique employing variable regions of 16S rDNA profiles generated by PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis). The bacterial species were obtained by sequencing the PCR-DGGE bands and the pesticide residues by Chromatography coupled with mass spectrometry (GC-MS / MS), tested on 11 fish samples. Fifty three percent of the fish were smoked. Three types of pesticides have been identified on the dried fish samples. Among them, there were Cypermethrin (15 to 3600 µg/kg), Chlorpyrifos (19 to 8800 µg/kg) and Profenophos (62 to 92 µg/kg). The analysis of 16S rDNA profile and DNA sequencing revealed the presence of 32 bacterial species grouped into 22 genera. Dried fish were the most contaminated. Species such as *Vagococcus carniphilus, Kurthia gibsonii, Lysinibacillus endophyticus, Macrococcus caseolyticus* and *Bacillus atrophaeus* were present in the samples from all the locations. On a general note, the fish samples are of poor microbiological and sanitary quality.

Introduction

Fish is one of the most traded foods in the world. It is a very valuable source of protein and contains many essential micronutrients [2,7]. It plays an important role in food security in developing countries in general and in Sub-Saharan Africa

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in particular [17,41]. FAO estimates that fish makes up 22% of protein intake in Sub-Saharan Africa [41]. However, in the poorest countries, this rate may exceed 50%, especially when other sources of animal protein are expensive. More than 200 million Africans eat fish regularly [7]. Fresh, but most often smoked, dried or even when it is only powdery [41]. Post-capture losses are a real problem in the Sub-Saharan Africa sub-region where about 25% of fish are lost due to a lack of effective means of conservation and processing. Traditional techniques (salting, drying and smoking) are used to conserve and improve the availability of fish.

In Cameroon, fish is widely consumed and the annual consumption per capita rates between 13.6 and 19 Kg, representing a contribution of about 42.3 to 44% of animal protein and covering 9.5% of total energy needs [9]. Fishing, which is important from both a socioeconomic and food point of view, is organized around four major sectors: industrial fishing, artisanal marine fishing, inland fisheries and aquaculture. Maritime artisanal and inland fisheries are exclusively the responsibility of rural populations [9]. The northern part of Cameroon has the largest inland fishery and accounts for more than 70% of the domestic production in inland waters. However, after fishing and because of the inadequacy of the cold chain, about 75% of the catches are smoked or dried [2]. The smallest fish, consumed locally by those who have low incomes, are therefore not recorded in capture statistics [17]. These people can buy dried fish with 200 CFA francs (€ 0.30), an amount that cannot purchase meat (beef or pork), which at least cost 1200 FCFA (1.82 €) for 500 g. Smoked or dried fish contains less than 10% of water and can be easily preserved in at room temperature for 60 days, even if it oxidizes effortlessly [30]. Processed fish, usually packaged in boxes or recovery bags are kept in rooms where the environment is not controlled. The products are usually infested by insects, rodents and even mold. To fight against these, some processors and traders use toxic pesticides like dieldrin, to extend the shelf life of the products [2]. Moreover, even after four weeks of exposure to atmospheric air, these pesticides were still present on the fish [3]. The use of pesticides represents a real danger to health, not only Cameroonians, but also other African neighbourhoods like Nigeria, Chad and Centre Africa Republic who also consume these products. Subsequently, despite the fact that fish is a vital source of animal protein and micronutrients, it can also cause serious damage to health, in as much as to bring negative political and economic consequences [38].

Water composition, temperature and weather conditions have an influence on the bacterial communities as far as foodstuffs are concerned. The rDNA profiles generated by PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis) have been used to detect variability in microbial community on many foods [11,27]. Aquatic microorganisms are known to be closely associated with the physiological status of fish [37]. PCR-DGGE has been used to unveil variability in microbial community (bacteria, yeast, and fungi) structures inherent to fish [11,18]. The predominant microbial floras were used to determine the capture area, production process or hygienic conditions during post-harvest operations and differentiate the geographical location [27,37]. Treatments (marinade, smoking and drying) were instead applied on fish samples; it was possible to recover the geographical origin by using the DNA of the bacterial community on fish [23].

Previous studies including that of Maïworé et al. [23] have been carried out on fillets of some species of fresh fish, but not on the whole fish. The samples considered in the previous studies were that of the big fish, but among the consumers' families with limited incomes, the small whole fish are consumed much more. In addition, the samples analyzed in the previous work were dried and smoked under laboratory conditions [23] and the bacterial PCR-DGGE fingerprints revealed were not excised for identification.

The aim of this study is to identify the dominant bacterial flora by sequencing the PCR-DGGE bands of dried and smoked fish collected in rainy season. The effect of treatments and fish species on the bacterial flora was assessed. The different species of dried and smoked fish that were found were listed and the pesticide residues were tested on some samples.

Materials and methods

Study area and sampling

The northern part of Cameroon is crossed by some rivers such as Logone and Chari in the Far North region, Benoue in the north region and Ndjerem in the Adamaoua region. For this study, some species of dried and smoked fish were collected separately in public markets, depending on their availability, the mode of treatment and the species in the three northern regions of Cameroon: the Far North (Girvidig, Blangoua, Pouss and Maga), the North (Garoua and Lagdo) and Adamawa region (Ngaoundere and Tibati).

Traditional drying involves exposing the eviscerated (for larger) and non-eviscerated (for the smallest) fish in the sun for 8 to 10 days [2]. Larger fish are sliced while small ones are dried fully. During smoking, fish are exposed to smoke produced by using wet or dry wood for three days. For each species, depending on the size of the individuals and the treatment, at least five dry samples were collected. The samples collected were taken covering the months of July to August, kept in polythene bags and sent to the laboratory for the various analyzes.

The fish were identified according to their general characteristics (size and shape) and detailed (relative to the different parts). The fish existing in these areas have been recorded [22,39]. Pictures of fresh fish were taken from Fishbase version 04/2019 [12] and compared with the dried or smoked samples.

Analysis

Bacteriological analysis

DNA extraction. The DNA extraction was done according to El Sheikha et al. [10] method. From the whole dried and smoked fish samples, ten (10) g were taken. 16 mL of sterile peptone water (pH 7.0, Dickinson, France) were then added and the mixture was homogenized by vortex (Vortex Genie 2 SI-A256, USA) at 2500 rpm for 30 min. The homogenates were added to 0.3 g of glass marbles contained in Eppendorf tubes of 2 mL. The mixture was vortexed for 10 min and put in a refrigerated (4 °C) centrifuge (Biofuge, Heraeus Instruments, Allemagne) at 12 000 g for 15 min. The supernatant was discarded and the cell pellets were suspended in 100 μL TE (Tris-Ethylenediamine Tetra acetic Acid) solution [10 mM Tris–HCl; 1 mM EDTA, pH 8.0 (Promega, France)]. After homogenization, the extracted bacteria cells underwent enzymatic hydrolysis by adding 100 μL of lysozyme solution (25 mg/mL, Eurobio, France). The tubes were then kept in an ambient temperature (25±2 °C) for 5 min; 100 μL of proteinase K solution (20 mg/mL, Eurobio, France) were added and the mixture was incubated at 42 °C for 20 min. 50 μL of 20% Sodium Dodecyl Sulfate (SDS) was added to each tube, and incubated at 42 °C for 10 min. Then 400 μL of a sulfactant Cetyltrimethyl ammonium bromide (CTAB; Sigma-Aldrich, USA) in 20% NaCl 3 M were added to each tube, and incubated at 65 °C for 10 min. The extraction was done based on 700 μL of phenol/chloroform/isoamyl alcohol (25/24/1; v/v/v; Carlo Erba, France). The mixture was homogenized manually for 1 min and centrifuged at 12 000 × g for 20 min. The aqueous layer was transferred to a new Eppendorf tube. This step of extraction was repeated. The residual phenol was removed by extraction with 600 μL chloroform/isoamyl alcohol (24/1; v/v), and centrifuged for 15 min at 12,000 × g. The aqueous phase was transferred into a new tube; the DNA was stabilized with 30 μL of sodium Acetate (3 M, pH 5) and well mixed. The samples were precipitated by adding a volume (500 μL) of ice-cold isopropanol (100%, −70 °C) and stored at −20 °C for 12 h (overnight). After centrifugation at 12 000 × g for 30 min, the supernatant was eliminated, 500 μL of 70% ethanol was added to wash the DNA pellets and the tubes were centrifuged at 12,000 × g for 5 min. After discarding the ethanol, the DNA pellets were air-dried in an ambient temperature and then suspended in 100 μL of ultrapure water and stored at 4 °C until analysis.

PCR amplification, DGGE analysis and identification. Two μL of the total DNA extracted were pipetted onto an optical measurement surface of the Thermo Scientific™ NanoDrop 2000, a full-spectrum, UV–Vis spectrophotometers used to quantify and assess the purity of DNA. After the verification of the presence, the quantity and the quality of the DNA extracted, the V3 variable region of bacterial 16S rDNA was amplified by using a couple of primers described by Muyzer et al. [28]: GC338f (5 ĆGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCGCA 3 ; Sigma, France) and 518r (5 ÷ATTACCGCGGCTGCTGG-3 ; Sigma, France) [20,21]. A GC clamp of 40 nucleotides was added to the forward primer 5 óf 338f in order to insure that DNA fragment will remain partially double stranded [1]. The 50 μL reaction mixtures were constituted by 5 μL of the extracted and controlled DNA; 5 μL of 10X Ataq buffer, 3 μL of $MgCl_2$; 1 μL of dNTP; 10μL of Primer 1 (518r); 10 μL of Primer 2 (338f) and 0.25 μL of Ataq polymerase. The reactions of PCR were carried out in a thermocycler (PTC-100 Peltier Therma Cycler, MJ Research In., and USA). For amplification, the cycle was carried out at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 15–30 secondes, annealing at 52 °C for 15–30 secondes and extension at 72 °C for 15–30 secondes and a single final extension at 72 °C for 5 min. The verification of the quality of PCR products was done first by analyzing Aliquots (5 μL) of PCR products, by an electrophoresis in 2% w/v agarose gel with Tris-Acetate-EDTA (TAE) 1X buffer (40 mM Tris–HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2EDTA), stained with Ethidium Bromide (Promega, France) 50 μg/mL in TAE 1X. Secondly the PCR products were quantified by using a standard (DNA mass ladder 100 bp; Promega). The PCR products of good quality were then analyzed by Denaturing Gradient Gel Electrophoresis, by using a Bio-Rad code universal mutation detection system (Bio-Rad, USA), using first the procedure described by Muyzer et al. [28] and improved by Leesing [21]. The Polyacrlamide gels (8% w/v, Acrylamide/Bisacrylamide 37.5/1 of 0.8 mm thickness) were prepared using 30–60% Urea-formamide denaturing gradients (Promega, France). For the preparation of the gel, 16 mL of each of the cold solutions (stored at 4 °C) of 30 and 60% (100% corresponded to 7 M urea and 40% v/v formamide; Promega, France). To this mixture, 40 mg of ammonium persulfate (Promega, France) and 40 µL Tétraméthylenediamine (TEMED) (Promega, France) were added. A volume of 9 µL of blue/orange coloring agent (Promega, France) was added to 35 µL of PCR products and poured in each well on the polyacrylamide gel.

The electrophoresis was managed as follows: at 20 V for 10 min and 80 V for 12 h. The gels were stained for 30 min with Ethidium Bromide, rinsed and snapped based on a transilluminator UV at 318 nm and the image obtained was generated using gel smart system 7.3 (Clara vision, Ulis, France). Imaging and statistical analysis of individual lanes of the gel images were straightened using Image Quant TL software V. 2003 (Amersham Biosciences, USA). Banding patterns were standardized with two reference patterns included in all gels, the rDNA amplification products from the pure bacterial strains of *Escherichia coli* and *Lactobacillus plantarum*. This software helped to identify the bands and compare their positions with standard patterns. The DGGE fingerprints were scored by the presence and absence of co-migrating bands, independent of intensity. Pairwise community similarities were quantified using the Dice similarity coefficient (SD) [14] as follows: $SD = 2Nc/Na + Nb$ (1) where Na represents the number of bands detected in sample A, Nb the number of bands in sample B, and Nc the number of bands common to both samples. The similarity index was expressed within a range of 0 (completely dissimilar) to 100 (perfect similarity). The data generated, obtained were exploited for the main component analysis (CPA) by Statistica software (Version 7.1), and for the hierarchical ascending classification CAH by XLStat (Version 2014). For the identification of bacterial community, the DNA bands on DGGE gels were then excised with a sterile scalpel, eluted in 50 μL sterile ultrapure water and stored overnight at 4 °C. These extracted DNA were amplified again with non GC-clamp primers. The PCR products were sent to GATC Biotech (Germany) for a second DNA purification and sequencing. Research for similar sequences of 16S rDNA were performed using the basic local alignment search tool (BLAST) in GenBank (http://www.ncbi.nlm.nih.gov/)

after editing and trimming the sequences by BioEdit sequence alignment editor, version 7.2 in order to determine the closest known relative species [5].

Analysis of pesticides

Among the dried and smoked fish collected, 11 entire samples were packed and send to PHYTOCONTROL, a Laboratory of Phytosanitary Analysis located in Nîmes in France (www.phytocontrol.com). The samples were collected respectively in Lagdo, Tibati, Blangoua, Garoua, Pouss and Maga, some key towns of the northern part of Cameroon. The solid/cold liquid extraction, one of the most commonly used sorbent techniques in analyzing pesticide residues was used. This extraction was followed by the dispersive SPE purification Gas chromatography-mass spectrometry (GCMS), to identify different substances within a test sample. The internal method used by this laboratory is MOC3/26.

Results

Species of dried and smoked fish identified

The species of dried and smoked fish samples were proven. Table 1 shows the different fish samples collected, their sampling site, the technological treatments applied to them. Among these samples, 25 species were identified over three regions (Far North, North and Adamaoua). These fish grouped into 15 families have undergone treatments such as smoking and solar drying.

Among these species collected, there are: *Alestes macrolepidotus; Bagrus bagrus, Brycinus macrolepidotus, Claria anguilaris, Citharinus gibbosus, Chrysichthys laticeps, Chrysichtys nigrodigitatus, Gymnarchus niloticus, Hydrocynus goliath, Heterotis niloticus, Labeo coubie, Lates niloticus, Mormyrops anguilloides, Micralestes elongatus, Micralestes occidentalis, Mormyrus rume, Marcusenius senegalensis, Oreochromis niloticus, Protopterus annectens brieni, Petrocephalus bovei bovei, Parailia pellucida, Rhabdalestes septentrionalis, Synodontis clarias, Sarotherodon galilaeus, Schilbe mystus* and *Tilapia dageti*.

These fish are grouped into 15 families among which there are: Alestiidae; Arapaimidae; Bagridae; Centropomidae; Characidae; Cichlidae; Citharinidae; Clariidae; Claroteidae; Cyprinidae; Gymnarchidae; Mochokidae; Mormyridae; Protopteridae; Schilbeidae.

These results reveal that fish from the Mormyridae, Cichlidae and Schilbeidea families are the most represented with at least 45, 35 and 30 samples respectively. These families are in addition to Claridae and Bagridea those that are met on at least four sampling sites. The least represented families belong respectively to the families of Centropomidae, Citharinidae, Gymnarchidae and Mochokidae, each encountered on one sampling site.

On one hand, the site of Maga is one of the place in which several species are found. At this location, there are 44% of the whole smoked and dried fish species collected during the rainy season in the market places of the northern zone of Cameroon. On the other hand, Tibati's site has provided fewer species, with only two species of smoked fish. On the account of conservation technique used and the sampling sites, the results revealed that for Adamawa region, all the samples of Tibati (100%) were smoked. In the Far North region, 64% of the fish samples are dried while, 36% of the fish were smoked. In the Northern Region, 70% of fish are smoked while 30% were dried under sun. In general, of all the samples collected, 53% of the fish were smoked while 47% were dried.

PCR-DGGE profiles and identification of bacterial flora

Influence of the specie, origin and treatment on the profile of fish samples

The total DNA extracted from dried and smoked fish was amplified and the PCR products analyzed by DGGE. The DGGE profiles for the bacterial community of these different products were obtained. The DGGE fingerprints representing the influence of specie, origin and treatment on PCR-DGGE of smoked and dried fish are presented on Fig. 1. The analysis of this figure reveals the presence of 38 bands on the whole gel. These bands were comprised between 6 (ON5: *Orechromis niloticus* 5) and 20 (lane 11).

The samples from the same region, from the same fish species and that have undergone the same technological treatment have a higher similarity. That is the case of MS1 and MS2, two dried samples of the same species collected at Guirvidig in the far north region, with the highest similarity of 88.3%. The lowest similarity was obtained between ON5 of Adamaoua and B1 of Northern Region. On the other hand, some samples of the same species like CA1-CA4 and that have undergone the same treatment (smoked) have different bacterial profiles because their geographical origins are different. For a given geographical zone or region, the bacterial profiles are nearer. So whatever the specie or the technological treatment of fish, the geographical origin influences their bacterial profile (group in blue on the Fig. 1c). This is the case of the following samples: CA1, SG, MR, MA, MS1, MS2 collected in the far north region.

Amid the six samples that have at least 18 bands, four were amassed in the Far North region (MS1, MA, PBB1, SG, CA4 and CA1) and two only in the Northern Region. The calculation of the similarity indices revealed that, whatever the species of the fish, the highest similarity was obtained between the samples from the same sampling site. This is the case, for example, of CA3 and ON5 both coming from Adamaoua with a similarity equal to 71.43%. The samples SG and MA have a similarity of 66.67%, even if they are respectively smoked and dried. The dendrogram obtained (Fig. 1b) shows that in 45% of similarity, there are three main clusters: (1) the first cluster included the samples of fish of the North and Far North region.

Table 1
Smoked and dried fish from the three northern regions of Cameroon.

Fish species	Code	Local names	Families	Location	R	Traitments
Alestes macrolepidotus	AM1	Pelpélédji	Characidae	Blangoua	FN	Dried
Alestes macrolepidotus	AM2	Ketchoperadi	Characidae	pouss	FN	Dried
Bagrus bagrus	B1	kouroungou	Bagridae	Garoua	N	Dried
Bagrus bagrus	B2	kouroungou	Bagridae	Garoua	N	Smoked
Bagrus bagrus	B3	kouroungou	Bagridae	Blangoua	FN	Smoked
Brycinus macrolepidotus	BM	Azamaza	Alestiidae	Blangoua	FN	Dried
Claria anguilaris	CA1	Mobaladji	Clariidae	Maga	FN	Smoked
Claria anguilaris	CA2	Silure	Clariidae	Tibati	A	Smoked
Claria anguilaris	CA3	Silure	Clariidae	Ngaoundere	A	Smoked
Clarias anguillaris	CA4	Silure	Clariidae	Garoua	N	Smoked
Citharinus gibbosus	CG	Falia	Citharinidae	Garoua	N	Smoked
Citharinus gibbosus	CG1	Falia	Citharinidae	Maga	FN	Smoked
Chrysichthys laticeps	CL	kouroungou	Claroteidae	Lagdo	N	Dried
Chrysichtys nigrodigitatus	CN	kouroungou	Claroteidae	Lagdo	N	Smoked
Gymnarchus niloticus	GN1	Dansarki	Gymnarchidae	Maga	FN	Dried
Gymnarchus niloticus	GN2	Dansarki	Gymnarchidae	Maga	FN	Smoked
Hydrocynus goliath	HG	Ngniédji fendu	Alestidae	Blangoua	FN	Dried
Heterotis niloticus	HN	Kanga	Arapaimidae	Pouss	FN	Smoked
Heterotis niloticus	HN1	Kanga	Arapaimidae	Maga	FN	Smoked
Labeo coubie	LC	Sardine	Cyprinidae	Garoua	N	Smoked
Labeo coubie	LC	Sardine	Cyprinidae	Lagdo	N	Smoked
Labeo coubie	LC	Bidé	Cyprinidae	Maga	FN	Smoked
Lates niloticus	LN	capitaine	Centropomidae	Lagdo	N	Smoked
Mormyrops anguilloides	MA	Ndola 1	Mormyridae	Maga	FN	Dried
Mormyrops anguilloides	MA	Ndola 4	Mormyridae	Maga	FN	Smoked
Micralestes elongatus	ME	Alidangaye	Alestiidae	Blangoua	FN	Dried
Micralestes occidentalis	MO	Kadji	Alestiidae	Maga	FN	Dried
Mormyrus rume	MR	Ndola 2	Mormyridae	Maga	FN	Dried
Mormyrus rume	MR	Ndola 3	Mormyridae	Guirvidig	FN	Dried
Marcusenius senegalensis	MS	soudodjé	Mormyridae	Blangoua	FN	Dried
Marcusenius senegalensis	MS1	Ndola 5	Mormyridae	Guirvidig	FN	Dried
Marcusenius senegalensis	MS2	Ndola 6	Mormyridae	Guirvidig	FN	Dried
Marcusenius senegalensis	MS	Ndola 7	Mormyridae	Guirvidig	FN	Dried
Oreochromis niloticus	ON1	tilapia	Cichlidae	Garoua	N	Smoked
Oreochromis niloticus	ON2	tilapia fendu	Cichlidae	Garoua	N	Smoked
Oreochromis niloticus	ON3	Tilapia	Cichlidae	Garoua	N	Dried
Oreochromis niloticus	ON4	parawé	Cichlidae	Blangoua	FN	Dried
Oreochromis niloticus	ON5	Tilapia	Cichlidae	Tibati	A	Smoked
Protopterus annectens brieni	PAB	Boynadji	Protopteridae	Maga	FN	Smoked
Protopterus annectens brieni	PAB	Boynadji	Protopteridae	pouss	FN	Dried
Petrocephalus bovei bovei	PBB1	souda mouka	Mormyridae	Garoua	N	Smoked
Petrocephalus bovei bovei	PBB	Souda mouka	Mormyridae	Blangoua	FN	Dried
Petrocephalus bovei bovei	PBB	Souda fendu	Mormyridae	pouss	FN	Dried
Parailia pellucida	PP	Ngamré	Schilbeidae	Lagdo	N	Smoked
Rhabdalestes septentrionalis	RS	Ngamré	Schilbeidae	Garoua	N	Smoked
Rhabdalestes septentrionalis	RS	Ngamré	Schilbeidae	Lagdo	N	Dried
Synodontis clarias	SC	kouroungou	Mochokidae	Lagdo	N	Smoked
Sarotherodon galilaeus	SG	parawé	Cichlidae	Maga	FN	Smoked
Schilbe mystus	SM	solé	Schilbeidae	Lagdo	N	Smoked
Schilbe mystus	SM	solé	Schilbeidae	Lagdo	N	Dried
Schilbe mystus	SM	Malalaïdji	Schilbeidae	Maga	FN	Dried
Tilapia dageti	TD	parawé	Cichlidae	Blangoua	FN	Dried

FN: Far North; N: North; A: Adamaoua; R: Region.
Amongst these bands, only 15 main bands were selected and identified. Among all the samples, CA1 (Claria anguilaris) and MA (Mormyrops anguilloides), samples of Maga in the far north region have more bands with respectively 11 of the 15 identified samples and 13 over the 15 identified. MA thus has more species of bacteria than the other samples. On the other hand, ON5 has only 6 bands with 4 identified. Among these bands, the DGGE patterns also revealed the presence of some common bands on almost all the samples sites (bands 2, 3, 4, 6, 7, 8 and 9).

These fish do not have scales and grow in the same environment and in the same conditions. The second cluster is made of four eviscerated dry fish collected in the far north region (MS1, MS2, MA and MR) and four samples of entire smoked fish (PBB1, SG, CA4 and CA1).

PCR-DGGE profiles of dried fish

The total DNA extracted from dried fish was amplified and the PCR products analyzed by DGGE. The DGGE fingerprints representing the influence of the family on the PCR-DGGE of dried fish are presented in Fig. 2. The analysis of this figure reveals the presence of 47 bands on the gel. The number of bands was comprised between 13 (RS2) and 38 (ME). Among

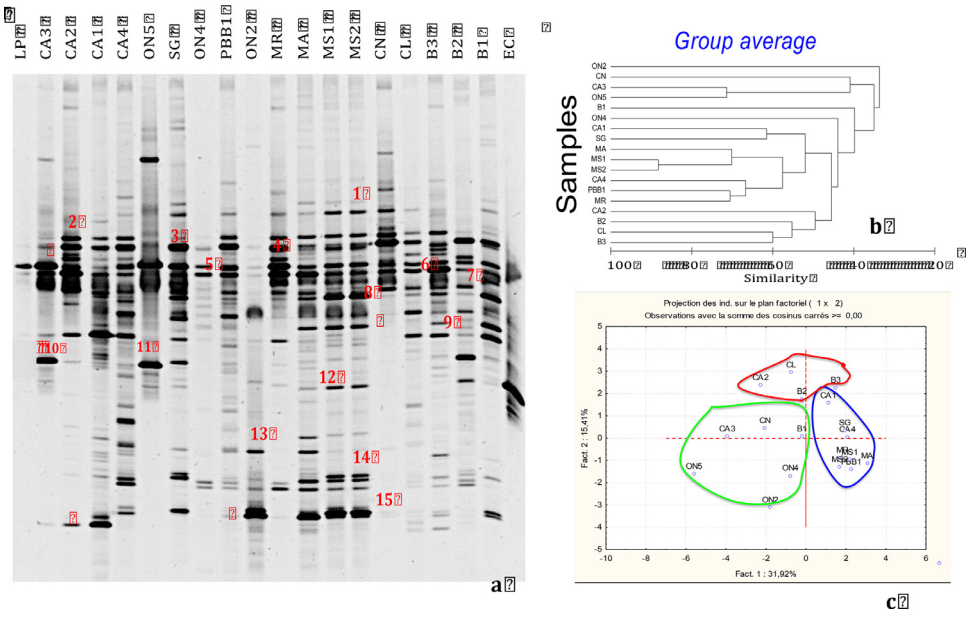

Fig. 1. Influence of the specie and origin on the PCR-DGGE profile of smoked and dried fish.

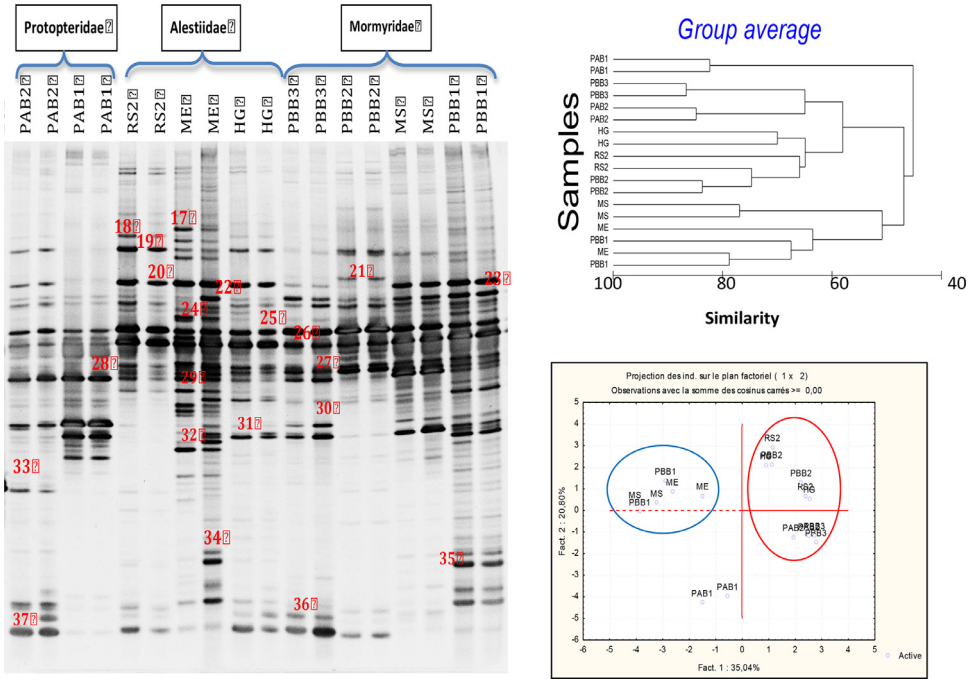

Fig. 2. Influence of the fish family on the PCR-DGGE profile of dried fish.

all these bands, 21 main bands were selected and identified. The sample ME, a dried sample of fish, collected in the far north region has a greater number of bands than the other samples, (38) with 17 bands identified. On the other hand, RS2 (*Rhabdalestes septentrionalis*) has only 13 bands with 10 identified. These DGGE patterns reveal also the presence of nine common bands to all the analyzed samples (19, 20, 23, 25, 26, 27, 28, 36 and 37). The dendrogram obtained (Fig. 2b) shows that in 56% of similarity, there are two main clusters: (1) the first cluster included the samples MS, ME and PBB1 which are the entire fish that were not eviscerated before drying. The second cluster is made up of 6 six samples of eviscerated dry fish (PBB3, RS2, HG, PAB2, PBB3 and PAB1).

Table 2
Bacteria identified on dried and smoked fish.

Bands	Bacteria identified	Percentage (%)	Accession
1PB	*Vagococcus carniphilus*	97.52	MG592277.1
2PB	*Kurthia gibsonii*	95.74	MN314360.1
3PB	*Lysinibacillus endophyticus*	97.44	MG712634.1
4PB	*Macrococcus caseolyticus*	98.14	MK791586.1
5PB	*Bacillus atrophaeus*	90.64	JQ389784.1
6PB	*Planococcaceae bacterium*	96.73	LK934680.1
7PB	*Lactobacillus delbrueckii subsp. bulgaricus*	99.38	HE858542.1
8PB	*Uncultured bacterium*	100.00	LN849501.1
9PB	*Clostridium amylolyticum*	94.96	KC331185.1
10PB	*Peptostreptococcus anaerobius*	95.97	LT675610.1
11PB	*Peptostreptococcus russellii*	97.10	MK424031.1
12PB	*Lysinibacillus endophyticus*	96.60	MG712634.1
13PB	*Staphylococcus xylosus*	98.65	MG819270.1
14PB	*Macrococcus caseolyticus*	99.01	MN314411.1
15PB	*Macrococcus brunensis*	98.36	KC864768.1
16PB	*Savagea faecisuis*	98.73	MG920791.1
18PB	*Macrococcus equipercicus*	98.12	MK093026.1
19PB	*Myroides marinus*	94.41	MH396758.1
20PB	*Enterococcus faecium*	94.38	AB627844.1
21PB	*Streptococcus gordonii*	99.27	GU418152.1
23PB	*Acetobacter pasteurianus*	98.76	KJ556521.1
24PB	*Staphylococcus xylosus*	97.12	MN294563.1
25PB	*Staphylococcus saprophyticus*	100.00	MN314503.1
26PB	*Lysinibacillus sphaericus*	97.37	KR919605.1
27PB	*Macrococcus caseolyticus*	98.64	KJ652696.1
28PB	*Acinetobacter calcoaceticus*	95.33	MK719803.1
29PB	*Tissierella praeacuta*	95.45	JX267093.1
30PB	*Clostridium saccharobutylicum*	97.78	MK253227.1
31PB	*Gemmatimonas aurantiaca*	91.38	NR_074708.2
32PB	*Clostridium carboxidivorans*	99.20	KU316945.1
33PB	*Clostridium scatologenes*	100.00	CP020953.1
34PB	*Clostridium vincentii*	97.10	KR857406.1
35BP	*Uncultured Clostridium sp.*	99.21	AB736204.1
37BP	*Clostridium acetobutylicum*	92.25	MH538942.1
40BP	*Vibrio alginolyticus*	98.98	MF319606.1
41BP	*Paraclostridium bifermentans*	97.44	MK932065.1
42BP	*Clostridium sp.*	98.53	MH209623.1

Identification of bacteria

DNA bands were excised from denaturing gels, amplified and sequenced. The following species (32) of bacteria identified and congregated into 20 genera (Table 2): *Vagococcus, Kurthia, Bacillus, Planococcaceae, Lactobacillus, Peptostreptococcus, Macrococcus, Savagea, Myroides, Enterococcus, Streptococcus, Acetobacter, Staphylococcus, Lysinibacillus, Acinetobacter, Tissierella, Gemmatimonas, Vibrio, Paraclostridium, Clostridium.* The diversity of bacteria species in dried fish was higher than in smoked fish.

An uncultured *Clostridium* and uncultured bacterium were also found among the identified bacteria. Among all these species identified, we noticed the presence of some common bands, both to dry and smoked fish independently of their specie and origin (Fig. 1): *Kurthia gibsonii, Lysinibacillus endophyticus, Macrococcus caseolyticus, Planococcaceae bacterium, Planococcaceae bacterium, Lactobacillus delbrueckii subsp. Bulgaricus*, Uncultured bacterium and *Clostridium amylolyticum*. The DGGE pattern reveals also the presence of eight common identified bacteria in all the dried fish samples analyzed: *Myroides marinus, Enterococcus faecium, Acetobacter pasteurianus, Staphylococcus saprophyticus, Lysinibacillus sphaericus, Macrococcus caseolyticus, Acinetobacter calcoaceticu* and *Clostridium acetobutylicum*.

The bacterial specie *Savagea faecisuis* was found only on smoked fish while 19 (or 59.4%) bacterial species among which: *Lysinibacillus sphaericus, Macrococcus equipercicus, Myroides marinus, Enterococcus faecium, Streptococcus gordonii, Acetobacter pasteurianus, Staphylococcus saprophyticus, Acinetobacter calcoaceticus, Tissierella praeacuta, Clostridium saccharobutylicum, Gemmatimonas aurantiaca, Clostridium carboxidivorans, Clostridium scatologenes, Clostridium vincentii, Uncultured Clostridium* sp., *Clostridium acetobutylicum, Vibrio alginolyticus, Paraclostridium bifermentans* and *Clostridium sp*. were found only on dried fish. The genus *Clostridium* was more represented by a percentage of 47%.

Among these isolated bacteria, about 74% of the bacteria isolated on the analysed fish are present in water, soils and the environment. The others 26% are present on the skin of animals, milk, fermented products and normal human flora. In the analyzed samples, there are some lactic acid bacteria like *Lactobacillus* and *Streptococcus*.

Pesticides residues in fish samples

The Table 3 reveals the different pesticides found on dried and smoked fish. Amongst the 11 fish samples analyzed, pesticides were not detected in 4 samples. On the seven others, active substances were detected. Among them, there were: Cypermethrin ($\alpha + \beta + \theta + \zeta$), with a concentration ranging from 15±5 (GN1) to 3600 ± 1000 µg/kg (ME); Chlorpyrifos with a concentration ranging from 19 ± 11 (ME) to 8800 ± 2000 µg/kg (PBB2) and Profenophos with a concentration ranging from 62 ± 30 (TD) to 92 ± 42 µg/kg (MS2). These results reveal also that pesticides are more used on dried fish from the far north region of Cameroon.

The contents of pesticide residues on the fish samples, were, however far above the standard. Thus, for Chlorpyriphos and Cypermethrine, the maximum residue limit (MRL) corresponds to 50 µg/kg of body weight [8,35], while for Profenofos this limit is 10 µg/kg or body weight [8]. The results obtained reveal that only 36.4% of fish analyzed comply with the standard. The analysis of the results abtained also reveals that 63.6% of the samples analyzed contain pesticide residues. About 57% of the contaminated samples contain 2 pesticide residues at the same time, 26% contain only one and 14.3% contain three residues.

We observed that the use of pesticides was not linked to a specific species of dried fish because the same pesticide residues can be found on fish of different species as well as from different locations. For example, it was the case of the samples collected in Blangoua: *Petrocephalus bovei bovei* on which the residues of Chlorpyrifos and Cypermethrine were found and on the other hand, *Tilapia dageti* treated with pesticides contains the residues of Cypermethrine and profenophos.

Discussion

Dried and smoked fish identified

This study was carried out on dried and smoked fish collected in the three northern regions of Cameroon: the Far North (Girvidig, Blangoua, Pouss and Maga), the North (Garoua and Lagdo) and Adamawa region (Ngaoundere and Tibati). The 15 families of fish identified, are those found in this geographical area. The families like Mormyridae, Alestiidae, Citharinidae, Centropomidae, Cyprinidae and Mochokidae are endemic in Africa, but the others are nevertheless, present on the location sites (Levêque and Paugy, 2006). According to these authors, the northern part of Cameroon belongs to the Nilo-soudanian province and the majority of the identified fish are endemic to this zone (12 families over 15 identified). The fact that the Mormyridae and Cichlidae and Schilbeidea families are the most represented amongst the samples can be explained by the fact that the majority of dried fish are of small size. The larger fish are generally sold fresh or smoked while the unsold ones and the smaller are dried. These small fish are dried with their head, bones and internal organ. These fish are concentrated source of several essential nutrients, compared to large fish that are generally not consumed entirely [7,17]. In addition, these small sun-dried fish are sold cheaper and thus allow the poorest people to have good source of protein. The fact that sampling was done during the rainy season explain that smoked fish were more numerous on the market than dried ones. During this period, ambient humidity does not promote fast solar drying of fish and fishermen have to use smoking method for that purpose, even for small fish such as *Parailia pellucida*.

Tibati location had a few processed fish due to the fact that the majority of the fish are sold fresh. Generally, in the northern area of Cameroon, fish are sold dried or smoked in order to increase their shelf life and to permit easy transportation, over long distances to other cities in the country and to some neighboring countries [40]. The north and far north regions of Cameroon, due to their location in the Sudano-Sahelian zone, are characterized by a hot and dry climate favoring the solar drying of fish. The small fish are automatically dried; some large fish are also dried when the catch is too small to justify a trip to the big cities or when the fish threaten to get rotten. Significant tonnages of Alestes, Brycinus and small Mormyridae are almost completely sold dried. Smoking is applied on large fish, previously eviscerated, scaled and on entire fish of reduced size [36].

PCR-DGGE profiles and identification of bacterial flora

The PCR-DGGE was a good molecular tool that allowed the study of complexity and behavior of fish microbial ecology [11]. The analysis of bacterial flora showing the influence of species, origin and treatment on the profile of fish samples revealed that the samples CA1 and MA, samples of Maga in the far north region had more bands and were therefore more contaminated by bacteria. This high rate of contamination first finds its origin on fresh fish that was directly linked to its environment [31]. The microorganisms were more concentrated in Maga's lake. This was due to the fact that fishermen mixed clay with cereal bran to trap fish [24]. This study also revealed a higher similarity between some fish of the same species, collected at the same location and that has undergone the same treatment. Therefore, the quality of smoked or dried fish is conditioned by the living environment of the fish [6].

Considered separately the CA1 sample, although smoked was not very dry and was entire, which could explain the high bacterial concentration. MA sample, on the other hand, was dry and gutted, but started putrefaction, which explains its high contamination.

This study revealed the presence of common bands or bacterial species representing those present throughout this geographical area as revealed by the works of Tatsadjieu et al. [37]. PCR-DGGE pattern is strongly linked to the microbial

Table 3
Pesticides residues detected on dried and smoked fish.

Samples	Species	T Traitment	Location	Région	Pesticides residue	Content (µg/kg)
ON5	*Oreochromis niloticus*	Smoked	Tibati	Adamawa		ND
ON3	*Oreochromis niloticus*	Dried	Garoua	North		ND
CG	*Citharinus gibbosus*	Smoked	Garoua	North		ND
CL	*Chrysichthys laticeps*	Dried	Lagdo	North	Cypermethrine($\alpha+\beta+\theta+\zeta$)	520± 180
PBB2	*Petrocephalus bovei bovei*	Dried	Blangoua	Far North	Chlorpyrifos Cypermethrine($\alpha+\beta+\theta+\zeta$)	8800 ± 20,002,300 ± 600
PAB2	*Protopterus annectens brieni*	Dried	Pouss	Far North		ND
ME	*Micralestes elongatus*	Dried	Blangoua	Far North	Chlorpyrifos Cypermethrine($\alpha+\beta+\theta+\zeta$)	190 ± 10 3600 ± 1 000
PBB3	*Petrocephalus bovei bovei*	Dried	Pouss	Far North	Chlorpyrifos Cypermethrine($\alpha+\beta+\theta+\zeta$)	290 ± 11 120 ± 50
TD	*Tilapia dageti*	Dried	Blangoua	Far North	Cypermethrine($\alpha+\beta+\theta+\zeta$) Profenophos	15 000± 5000 62 ± 3
GN1	*Gymnarchus niloticus*	Dried	Maga	Far North	Cypermethrine($\alpha+\beta+\theta+\zeta$)	15 ± 5
MS2	*Marcusenius senegalensis*	Dried	Guirvidig	Far North	Chlorpyrifos Cypermethrine($\alpha+\beta+\theta+\zeta$) Profenophos	35 ± 9 660 ± 23 92 ± 4.2

ND: not detected; ON5: Oreochromis niloticus 5; ON3: Oreochromis niloticus 3; CG: Citharinus gibbosus; CL: Chrysichthys laticeps; PBB2: Petrocephalus bovei bovei 2; PAB2: Protopterus annectens brieni 2; ME: Micralestes elongatus; PBB3: Petrocephalus bovei bovei; TD: Tilapia dageti; GN1: Gymnarchus niloticus 1; MS2: Marcusenius senegalensis 2.

environment of the fish because the skin is directly in contact with water. It was also shown that technological treatment had not affected traceability markers [23].

Among the dried samples fish, there was a cluster made of eviscerated ones. This was due to the fact that the viscera contain a lot of bacteria and their removal would considerably reduce their concentration on the final product.

It happens that 26% of identified bacteria are naturally present on the skin, feces, mouth and digestive tract of Human and animals. It easily reflects the fact that these fish products are constantly handled improperly. As soon as the fish are removed from the water, they are handled without protection in the boats, during processing; they are eviscerated, dried or smoked, spread out and even packaged in retrieving bags all this by bare hands without any protection. In addition, the presence of bacteria coming from animals is linked to the fact that, the processing and packaging of fishery products are carried out by persons living on the fish's processing place. In this environment, there are domestic animals like dogs, cats, goats, sheeps and even cows. All these parameters greatly influence the quality of dried or smoked fish, packaged in this environment (Ndiaye and Diei-Ouadi [29].

In addition, proper storage will permit to maintain the marketable quality and safety of the product [29]. The greatest proportion (74%) of the identified bacteria was linked to the environment in which the fish was first caught, processed, packaged and then sold showed also by other authors [42].

Some of the bacteria identified are part of the alteration flora of fish. This flora is associated with the degradation of fish and the production of undesirable odors and tastes. Among them, there are bacteria such as Vibrionaceae, Enterobacteria; Gram positive bacteria like: *Micrococcus, Bacillus, Staphylococcus* and some germs incriminated in healthiness, like Coliforms, *Proteus, Clostridium* [18]. The presence and the activity of these microorganisms in fish change with storage temperatures. Between 20–30 °C, the alteration flora is composed of Gram negative bacteria like Vibrionaceae and Enterobacteriaceae, capable of mesophilic fermentation of fish caught in polluted waters [18,19]. This goes in agreement with the results obtained in this study insofar as all these microorganisms were found.

Lactic acid bacteria like Streptococcus and Lactobacillus were present in our samples. Streptococcus sp. and Lactobacillus spp are usually found in abundance in some tropical, marine or freshwater fish where they are implicated in fish degradation [16].

The presence of a higher number of bacteria species in dried and smoked fish can be justified by the fact that, cold drying (40 °C) certainly have an inhibiting effect on growth and toxinogenesis, but with no real destructive effect on the microbial flora [18]. However, before the fish is completely dry and the bacterial growth stopped, there is an increasing in the bacterial concentration and consequently the expected DNA concentration. Traditional smoking, on the other hand, is most often done under higher temperature (around 100 °C). It is more effective than cold smoking (temperature $\leq$ 25 °C) insofar as it is more effective in destroying microbial flora, and smoking is significant depending on its duration [34]. In addition, wood smoke compounds such as phenol and acetic acid that are known for their antiseptic and bacteriostatic properties. This results in a lower number of bacteria in the smoked fish samples.

The presence of faecal *Streptococcus, Staphylococcus* and *Clostridium* reveal a fecal contamination and skin infections. In fact, these germs are typical of animal or telluric excrement of aquatic products (Jay, 2000). The presence of these strains is not surprising, according to the unsanitary conditions of the sampling sites, the producers, the production and drying equipment. *Staphylococcus* has a predominant effect on the characteristic flavor of fermented foods [33]. However, the presence of *Clostridium*, Enterobacteria, *Streptococcus* and *Staphylococcus* in these smoked and dried products indicates the need to take hygienic measures likely to reduce the risks associated with these types of microorganisms [4,18].

Pesticides content in fish

Both smoked and dried fish samples were analyzed. Pesticide residues were found only on the dried fish samples. The pesticide residues found in the samples in this study are part of the fat-soluble insecticides, generally used on food and vegetable crops, fruit trees, tomatoes, and cotton. These entire pesticides act by contact along with class II for chlorpyrifos and profenofos including class I for cypermetrine. These substances known under various trade names are products approved since 2014 in Cameroon [26]. In this study, we discovered that the presence of pesticide residues was not linked to a specific species of dried fish. This can be explained by the fact that fishermen and traders are especially concerned with the shelf life of their products than the consumer's health.

The pesticides are usually used before drying and during storage. In general, after fishing, the fish are maintained in the canoes for a long time and are thus exposed to flies, mishandled in an unprotected way and the packaging used is generally unhygienic. Furthermore, sun drying fish process is basically done between 3 and 10 days [13]. In this tropical area, flies are very often attracted by the smells of decomposition or fermentation of fish. To keep fish safe from these intruders, many people use unorthodox methods such as the use of certain unauthorized pesticides [2]. The fish are washed and then sprayed with a solution of insecticide locally called '*Pia-pia*'. This liquid or solid insecticide is imported from Nigeria (neighboring country). The fish, thus treated is ready to be dried away from flies and other insects. For a given fishing season, fishermen can produce between 500 kg and 1.8 tons of dried fish per season [25]. The dried fish are then stored in boxes previously treated with the same solution by hand spraying for 2 to 3 months for safety. Wholesalers admit that they use pesticides by injecting into cargo and bags and spraying all the storage spaces for smoked and dried fish. According to these persons, products are protected from parasites (insects of Dermestidaes' family) for more than a year [3]. It should also be noted that storage on the ground constitutes a factor of contamination and loss of products [29]. Authors like Ndrianaivo et al.

[30] have shown that the losses of dried or smoked fish are caused mainly by insect infestation varying between 10 and 60%. Storage on ground constitutes also a factor of contamination and loss of products [29]. The use of these chemicals can affect the quality of dried fish (flesh texture and taste) and could be toxic or harmful to the consumers (Abdoulllahi, 2018). These results revealed that certain contaminated samples contained between two to three pesticide residues at the same time. The presence of several pesticide residues in some samples could be the result of the fact that fishermen mix one or more pesticides for more effectiveness on dried or fish on the ones to be dried. These results also reveal that the traders use high concentrations of pesticides, with values going to very high rates reaching up to three hundred times the recommended prescription. However, the maximum concentration of Cypermethrin and Profenophos found in this study were lower than those uncovered in smoked fish from Mali, respectively 18 084 μg/kg and 182 μg/kg [15]. Despite the low share in the global pesticide trade (4%), Africa remains one of the regions where pesticides cause most problems, in as much to pinpoint accidental poisonings ushering thus more than 75% of fatal cases [32].

Conclusion

The aim of this study was to ascertain different species of smoked and dried fish sold on the markets of the northern part of Cameroon, to investigate the diversity of dominant bacteria on dried and smoked fish and the type and concentration of pesticides used to preserve the fish products. The results revealed the presence of 25 fish species grouped into 15 families of dried and smoked ones, with the Mormyridae as the most representative. Pesticides analysis disclosed the presence of higher cocentration of Cypermethrin ($\alpha + \beta + \theta + \zeta$), Chlorpyrifos and Profenophos residues on 64% of the analysed samples, with 100% of the dried samples contaminated. The diversity of identified bacteria exposed the presence of the genera *Vagococcus, Kurthia, Bacillus, Planococcaceae, Lactobacillus, Peptostreptococcus, Macrococcus, Savagea, Myroides, Enterococcus, Streptococcus, Acetobacter, Staphylococcus, Lysinibacillus, Acinetobacter, Tissierella, Gemmatimonas, Vibrio, Paraclostridium, Clostridium.* The quality of raw material, poor hygienic practices, and inappropriate handling practices during processing and selling are majors factors that contribute to the unsatisfactory microbiological quality of these products. Processors and sellers should be trained on the matter good hygiene and the handling practices in order to produce safe and sound fish product for consumption. To complete this study into it fulness, it will be necessary to follow the bacterial concentration at each transforming stage of the fish.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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