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Quality assessment of ice-stored tropical yellowfin tuna (*Thunnus albacares*) and influence of vacuum and modified atmosphere packaging

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Abstract :

Metagenomic, microbial, chemical and sensory analyses of *Thunnus albacares* from Martinique and stored in ice (AIR – 0 °C), vacuum (VP – 4/8 °C) and modified atmosphere packaging (MAP – 4/8 °C) (70% CO_2 – 30% O_2) were carried out. The organoleptic rejection of AIR tuna was observed at day 13 when total bacterial counts equaled 10^6–10^7 CFU g^{-1}. No extension of shelf-life was provided by VP and MAP. According to 16S rRNA gene sequence analyzed by Illumina MiSeq and PCR-TTGE, *Rhodanobacter terrae* was the main species of the freshly caught tuna. At the sensory rejection time, *Brochothrix thermosphacta* and *Pseudomonas* dominated the AIR products while *B. thermosphacta* alone or a mix of *B. thermosphacta*, *Enterobacteriaceae* and lactic acid bacteria (LAB) dominated the microbiota of MAP and VP products, respectively. The pH value remained stable in all trials, ranging from 5.77 to 5.97. Total volatile basic nitrogen (TVBN) and trimethylamine (TMA-N) concentrations were weak and not significantly different between batches. Lipid oxidation increased in the samples containing O_2 (MAP > AIR). The initial concentration of histamine was high (75–78 mg kg^{-1}) and stable up to 8 days but then significantly decreased in all trials to reach 25–30 mg kg^{-1}, probably due to the presence of histamine-decomposing bacteria.

Keywords : Seafood, Fish, Microbiology, Sensory, NGS, 16S rRNA gene

1. Introduction

The spoilage of food refers to a loss of initial quality rendering it less palatable or toxic for human consumption (Ashie et al., 1996; Gram et al., 2002). Fish flesh is an extremely perishable product due to both microbial spoilage and biochemical reactions occurring during processing and storage. The high water activity, high *postmortem* pH (> 6) and large amounts of low molecular weight components make it an ideal substrate for bacterial growth, which is essentially responsible for the sensory deterioration (Dainty, 1996; Gram and Huss, 1996; 2000; Shewan, 1971). The use of ice is probably the simplest method for delaying fish spoilage and the benefits in the tropics are greater than in cold or temperate areas (Liston, 1980; Poulter et al., 1981; Sumner et al., 1984). Moreover, VP and MAP in combination with refrigeration are widely used to extend the shelf-life of fish products (Cakli et al., 2006; Dalgaard et al., 1993; Goulas and Kontominas, 2007; Reddy et al., 1992; Sivertsvik et al., 2002).

Fishery products are also responsible for outbreaks resulting from contamination by bacterial pathogens, biotoxins, histamine, viruses or parasites (Galaviz-Silva et al., 2009). Histamine fish poisoning (HFP) is the first cause of fish-related foodborne infection in France, which is due to the ingestion of a high concentration of histamine produced by the bacterial decarboxylation of free histidine (Frank et al., 1985; Hungerford, 2010; Lehane and Olley, 2000). Scombroid fish, such as tuna, generally possess a high content of free histidine in their muscle tissues and are most commonly associated with HFP (Lukton and Olcott, 1958; Taylor and Eitenmiller, 1986).

Worldwide, the yellowfin tuna (*Thunnus albacares*) predominates in tropical and subtropical waters and 1.3 MT were caught in 2012 (FAO, 2014). In Martinique, *T.*

albacares represents one of the main pelagics caught by long-line fleets around fish aggregating devices. Approximately 85 T, with a value of more than 800 000 US dollars, were captured in 2010 (Reynal et al., 2011). In this region, the marine catch is carried out manually in traditional non-mechanized vessels operated by one or two fishermen. Post-harvest treatments are not standardized but fish are generally gutted on board, iced in a cooler box and sold along the road, sometimes more than 48 hours after fishing. Two HFP outbreaks (137 people) due to the consumption of yellowfin tuna were observed in 2007-2008 in Martinique (Duflos, 2009). Although some studies are available on the quality of raw yellowfin tuna, most of them concern tuna from the Indian or Pacific Ocean (Cramer et al., 1981; Du et al., 2001; Du et al., 2002; Emborg et al., 2005; Ferrario et al., 2012; Gill et al., 1987; Guizani et al., 2005; Kanki et al., 2007; Lee et al., 2003; Saito et al., 1996; Sika et al., 2014; Staruszkiewicz et al., 2004; Susanto et al., 2011; Widiastuti et al., 2013). In addition to a lack of information in tropical areas, none of these works used culture-independent microbial techniques, such as temporal temperature gradient gel electrophoresis (TTGE) or next-generation sequencing (NGS), to characterize the microbiota in detail. Recently, these methods have been successfully employed for the evaluation of microbial ecosystems of various seafoods such as cod, raw and smoked salmon and shrimp (Chaillou et al., 2014; Giacomazzi et al., 2004; Jaffrès et al., 2009; Leroi et al., 2015; Macé et al., 2012; Rachman et al., 2004).

The aims of this work were to monitor the quantitative and qualitative evolution of the microbiota of raw yellowfin tuna flesh and to investigate the effect of packaging storage on quality. Microbiological (culture-dependent and culture-independent methods), chemical and sensory analyses were carried out on tuna stored under air, VP and MAP.

2. Materials and Methods

2.1. Tuna sampling

A yellowfin tuna caught off the Caribbean coast of Martinique was purchased from a fish landing point at Bellefontaine (14°40'22''N; 61°09'51''W) in May 2014.The fish was bled and gutted on board and stored in ice in a large cooler box. The fish weight without head and tail was 37.8 kg. The tuna was cut by the seller into slices (4-5 cm thick) which were put into plastic bags and transported in a clean plastic ice box to the PARM laboratory within 45 min of slicing.

2.2. Storage conditions

Upon arrival, slices were placed in a room at 12°C. Each slice was portioned into 4 quarters with skin (steaks) weighing 400 to 600 g. Steaks were divided into 3 batches. For the first batch (VP), steaks were vacuum-packed individually in 80-µm thick plastic bags (Garcia de Pou, Girona, Spain) made of polyamide/polypropylene with a gas-permeability of 2.78 cm^3/m^2/day for water vapor, 19.95 cm^3/m^2/day for O_2 and 164.87 cm^3/m^2/day for CO_2 using a packaging machine (Multivac, Lagny sur Marne, France). For the second batch (AIR), steaks were placed in the same type of plastic bags. These samples were iced in a cooler box by alternating a layer of fish with a layer of ice and kept in a cold room (4°C). To maintain AIR samples at 0°C, melting water was drained off and ice was replaced when necessary. Steaks of the third batch (MAP) were placed on a filmed plastic tray and packed under a modified atmosphere (70% CO_2, 30% O_2) using a Meca 500 machine (Mecapack, Pouzauges, France). The properties of the polyamide/polypropylene film used (Pechiney, Paris,

France) were a thickness of 90 µm and a gas-permeability (cm^3/m^2/day at 23℃, 50% RH) of 4, 30, 120 and 6 for water vapor, O_2, CO_2 and N_2, respectively. VP and MAP samples were stored at 4℃ for the first week and t hen at 8℃, according to the French standard shelf-life validation of perishable and refrigerated food (AFNOR, 2010). For each batch, 3 steaks were analyzed at five predetermined time intervals and tested for sensory, bacteriological and chemical qualities until they were organoleptically unacceptable. The sampling times were 0, 6, 8, 10 and 13 days for AIR and 0, 8 and 13 days for VP and MAP. The mean value of the triplicate was used as a representative value of the sample.

2.3. Chemical analysis

2.3.1. Total volatile basic nitrogen (TVBN) and trimethylamine (TMA-N)

TVBN and TMA-N were determined for 100 g of fish using the Conway micro-diffusion method (Conway and Byrne, 1933).

2.3.2. pH

Twenty grams of fish muscle was stomached with 80 ml of distilled water in a stomacher 400 (Seward Ltd., London, UK) and the pH was measured immediately on the homogenate using a pH-meter (Inolab, Germany).

2.3.3. Thiobarbituric acid (TBA) index

The extraction procedure and TBA reaction described by Vyncke, 1970 were used. After cooling in tap water, 4 ml of methanol was added and absorbance of the methanol-water phase was read at 532 nm with a spectrophotometer (Jenway, Stafford, UK). The amount of malonaldehyde (MDA) was calculated by dividing the absorbance by the slope of the standard curve prepared using

1,1,3,3-tetraethoxypropane and multiplying by a factor of 86.4. TBA (mg-MDA kg^{-1}) was analyzed at the first and last sampling point for the 3 batches.

2.3.4. Histamine

Histamine content was determined in 50 g of fish muscle by a colorimetric assay method with a commercial rapid histamine test kit (RIDA®QUICK Histamin from R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's recommendations.

2.4. Sensory analysis

Based on preliminary visual and olfactory acuity tests, 6 people from the permanent staff of PARM were selected to evaluate the sensory quality of *T. albacares*. Two training sessions were carried out. First, panelists were trained with concentrated aromatic molecules in an ethanol solution (Table 1). These molecules had been identified as related to odors of spoiled fish (Joffraud et al., 2001; Selli et al., 2006). Panelists met for another session using fresh and spoiled samples of yellowfin tuna in order to establish a list of descriptors and a fairly uniform degree of sensory evaluation. The following attributes were retained: marine, amine, sulfur and pungent for odor and blood red, slightly pink, bleached, grayish, browned and greenish for color. At each sampling date, 4 to 5 panelists had to score the odor, color and texture of the tuna samples on a 10-point scale, with 0 representing fresh fish and 10 rotten fish. Panelists received a fresh flesh sample (tuna flesh that had been frozen at -80℃ and thawed for each sensory analysis sessi on) as a reference. The sample consisted of 20 grams of each tuna steak of the triplicate pooled in a single opaque bowl coded with random 3-digit numbers. An average of the levels of odor, color and texture was calculated for each sample to give an overall spoilage score. When a

batch reached a spoilage score equal to or more than 6 points, it was considered rejected and the experiment was stopped. For odor and color aspects, panelists selected zero, one or more attributes defined by an intensity class (slight, moderate and strong). Each characteristic was associated with an index (I) defined as follows:

$$I = \frac{1 \text{ x } PP_{SLIGHT} + 2 \text{ x } PP_{MODERATE} + 3 \text{ x } PP_{STRONG}}{100}$$

where PP is the percentage of panelists who selected the corresponding intensity class.

This index varies from 0 (the characteristic was not selected) to 3 (all panelists chose strong for the characteristic).

2.5. Enumeration of bacterial groups

From each sample, a 30-g portion of fish flesh was added to 120 ml of sterile Tryptone-salt broth (Biokar Diagnostic, Beauvais, France) containing 0.2% of Tween 80 (Sigma-Aldrich®, St Louis, MO, USA) and stomached for 2 min with a stomacher 400 (Seward) to obtain the mother solution. Total Mesophilic Viable Counts (TMVC) were enumerated on a pour plate of Plate Count Agar (PCA) (AES Laboratory, Bruz, France) with 0.5% NaCl after 3 days at 30℃. Total Psychrotrophic Viable Counts (TPVC), lactic acid bacteria (LAB) and *B. thermosphacta* were enumerated on spread plates of Long and Hammer agar with 1% NaCl (LH) (Van Spreekens, 1974), Elliker agar (ELK) and Streptomycin Sulfate Thallous Acetate Agar (STAA), respectively, according to Leroi et al., 2015. *Pseudomonas* spp. were counted on spread plates of CHROMagar™ (CHROMagar, Paris, France) after 24-48 h at 20℃. *Enterobacteriaceae* were enumerated on pour plates of Caso agar (Merck, Darmstadt, Germany) overlaid by Violet Red Bile Glucose Agar (VRBGA, BioRad, Marnes-la-Coquette, France) called Caso/VRBG and incubated for 2 days at 20℃.

186

187 **2.6. Isolation, purification and identification of bacterial isolates**

188 At the sensory rejection time, 22 bacteria were isolated from each storage condition
189 (AIR, VP and MAP). Isolates were selected by picking colonies with various
190 morphologies from plates: 10 colonies from LH and 3 colonies from ELK, STAA,
191 Caso/VRBG and CHROMagar *Pseudomonas*. Sixty-six collected isolates were
192 purified twice on Brain Heart Infusion agar (BHI, Biokar). Each isolate was then
193 characterized for Gram reaction with KOH (Gregersen, 1978), catalase activity by the
194 3% H_2O_2 method and cytochrome oxidase production by Bactident Oxidase reagent
195 (Merck, Darmstadt, Germany). Extraction of DNA and PCR amplification and
196 identification of the 16S rDNA sequences from purified colonies were monitored as
197 described by Macé et al., 2012.

198

199 **2.7. Total bacterial DNA extraction from tuna flesh**

200 At each sampling date, the 3 independent mother solutions of the triplicate were
201 pooled in equal proportions. Bacterial DNA from 8 ml of this solution was extracted
202 and purified as described by Macé et al., 2012. The concentration and purity of DNA
203 were assessed by optical density using a NanoDrop ND-1000 spectrophotometer
204 (Isogen, De Meern, The Netherlands). DNA samples were stored at −20℃ until they
205 were used for Temporal Temperature Gradient Gel Electrophoresis (TTGE) and 16S
206 rDNA amplicon sequencing analyses.

207

208 **2.8. Temporal temperature gradient gel electrophoresis analysis**

209 At each sampling date, bacterial DNA was analyzed by PCR-TTGE. Primers V3P2
210 and V3P3-GC-Clamp were used to amplify the V3 region of the 16S-rDNA (194 bp)

in the conditions described by Jaffrès et al., 2009. Determination of the size of the PCR products and TTGE analysis were performed as described previously (Jaffrès et al., 2009). Standardization, analysis and comparison of TTGE fingerprints were monitored using BioNumerics Software, version 7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) as described by Macé et al., 2012.

2.9. Bacterial 16S rRNA gene amplification and barcoded sequencing

Bacterial DNA extracted from the tuna matrix at each sampling date was analyzed by Illumina sequencing.

2.9.1. PCR reaction

16S rDNA PCR libraries targeting the V1-V3 hypervariable region (minimum amplicon size of 460 bp) were generated. Primers E9-29 and E514-430 (Brosius et al., 1981), specific for bacteria, were selected for their theoretical ability to generate the lowest amplification bias relative to amplification capability/ability among the various bacterial phyla (Wang and Qian, 2009). The oligonucleotide design included the Illumina overhang adapter sequence (Illumina Inc, San Diego, USA). The amplification mix contained 5 U of FastStart high fidelity polymerase (Roche Diagnostics, Basel, Switzerland), 1X enzyme reaction buffer, 200 µM dNTPs (Eurogentec, Liège, Belgium), 0.2 µM of each primer and 2 µl of genomic DNA in a final volume of 25 µL. Thermocycling conditions consisted of a denaturation step of 3 min at 94℃, followed by 30 cycles of 30 s at 94℃, 30 s at 56℃, and 1 min at 72℃, and a final elongation step of 8 min at 72℃. These amplifications were performed on an Ep Master system gradient apparatus (Eppendorf, Hamburg, Germany). The PCR products were checked on 1% agarose gel electrophoresis and the DNA fragments were purified using AMPure XP beads (Beckman Coulter Inc, California, United

States). A second PCR (index PCR) was performed to attach dual indices and Illumina sequencing adapters using the Nextera XT index kit (Illumina Inc, San Diego, United States) following the manufacturer's recommendations. The quantity of the products was assessed by Picogreen dsDNA quantitation assay (Isogen Life Science). Equal amounts of each of the index PCR products were pooled. This library was quantified and normalized using KAPA SYBR FAST qPCR kit (KAPA BIOSYSTEMS, Boston, United States) following the manufacturer's recommendations.

2.9.2. Illumina sequencing

Sequencing was performed with the Illumina MiSeq Sequencer to generate paired sequences 2x300 bases (Illumina Inc). The MiSeq system provided an instrument secondary analysis using the MiSeq Reporter software V2.5. The metagenomics workflow option must be selected with the 16S protocols.

2.9.3. Bioinformatic analysis

Image and data processing for amplicon sequencing was performed using the MiSeq reporter software V2.5 (Illumina Inc). FLASH was used to merge paired overlapped reads of both ends of fragment libraries (Magoc and Salzberg, 2011). The 16S rDNA sequence reads were processed using the MOTHUR software package (Schloss et al., 2009). The quality of all the sequence reads was de-noised using the PyroNoise algorithm implemented in MOTHUR and filtered according to the following criteria: minimal length of 450 bp, an exact match to the barcode and 1 mismatch allowed to the proximal primer. Sequences from multiplexed samples were assigned based on the presence of the unique barcodes assigned to each sample. The sequences that passed the quality check were aligned to the SILVA alignment database (Pruesse et al., 2007). These sequences have been deposited at the European Nucleotide

Archive (ENA) under the project accession number PRJEB11823. Chimeric sequences were detected using the chimera-slayer command included in the MOTHUR package, and potential chimeras were removed (Haas et al., 2011). The sequences were pre-clustered to reduce false operational taxonomic units (OTUs) produced by erroneous sequences. A distance matrix was prepared (distance = 3%) and the sequences were clustered to OTUs using the average neighbor algorithm. OTUs representing less than 0.01% of the total number of reads were considered artifacts and discarded. In the second phase of the experiment, the representative sequences of each OTU were compared with the SILVA microbial database using the Basic Local Alignment Search Tool (BLASTN) (Altschul et al., 1990; Gertz et al., 2006). For each OTU, a standard detailed taxonomic identification was given based on the identity (less than 1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not). The genus assignations obtained using MOTHUR and BLAST were compared and noted as unclassified in the case of a mismatch. Each bacterial population identified by metagenetics was analyzed as a proportion of reads, in order to deduce the bacterial flora proportion. The sequences corresponding to mitochondria or chloroplasts were removed prior to analysis. The MOTHUR program was also used to determine the Shannon diversity index, Chao1 and ACE richness indices and coverage estimator at the 3% distance level.

2.10. Statistical analysis

Data were analyzed using the software R (version 2.14.0). Descriptive statistics of means, standard deviation, linear regression, one-way ANOVA and Tukey's HSD post-hoc test were applied. A significance level of 5% was used.

3. Results

3.1. Sensory characteristics of raw tuna

The results of the sensory evaluation of raw yellowfin tuna samples are presented in Table 2. Fresh tuna steak (D0) was mainly characterized by a firm texture with a typical red blood color and a marine odor. After 8 days (D8), all batches received a spoilage score ranging between approximately 2 and 3 and were not significantly different from the fresh sample ($p>0.05$). However, VP and MAP products presented a slight discoloration of the fish flesh and MAP started to release an unpleasant amine odor and have a slightly greenish color. AIR, VP and MAP steaks were all considered unacceptable after 13 days (spoilage score ≥ 6). Spoilage characteristics as described by the sensory panel were amine, sulfur and pungent off-odors and a bleached, grayish, browned or greenish appearance. The texture of MAP tuna was clearly less firm than AIR and VP samples. Based on these results, it was concluded that VP and MAP do not significantly increase the shelf-life of tuna steaks. This observation may be explained by the fact that these samples were stored at 4/8℃ instead of 0℃ for AIR products. Although no statis tical difference was observed between samples, MAP slightly accelerated the spoilage process of tuna.

3.2. Chemical analysis

The pH value of raw tuna was very stable during storage, ranging from 5.77 to 5.97, and no significant difference was observed between the trials ($p>0.05$) (data not shown).

Figure 1 shows the TVBN and TMA-N results. At day 0, TVBN and TMA-N values were 16.3 ± 0.6 mg-N 100 g^{-1} and 1.0 ± 0.1 mg-N 100 g^{-1}, respectively. The production of TVBN was very low whatever the storage condition (maximum value of 20.3 ± 2.0 mg-N 100 g^{-1}) and there was no significant difference between trials. The maximal TMA-N concentrations were observed at the end of storage for MAP and VP samples reaching 4.6 ± 1.8 mg-N 100 g^{-1} and 9.0 ± 1.0 mg-N 100 g^{-1}, respectively, contrary to AIR products where no increase was recorded.

The initial content of TBA in fresh tuna flesh was 0.5 ± 0.8 mg-MDA kg^{-1}. In spoiled products (day 13), the production reached 2.9 ± 0.2, 0.6 ± 0.1 and 6.0 ± 0.8 mg-MDA kg^{-1} for AIR, VP and MAP samples, respectively (data not shown).

At day 0, the concentration of histamine in tuna flesh was 75.4 ± 0.5 mg kg^{-1}. Histamine was fairly stable up to 8 days except for VP samples in which the concentration increased slightly to 83.9 ± 1.2 mg kg^{-1}. At the sensory rejection time (day 13), histamine concentrations had decreased significantly to reach 25.3 ± 1.5, 27.3 ± 1.4 and 30.7 ± 1.9 mg kg^{-1} in AIR, VP and MAP samples, respectively.

3.3. Enumeration of the different bacterial groups

According to the culture-dependent methods, all batches presented a relatively similar bacterial evolution (Figure 2). No difference was observed between TPVC and TMVC. Before tuna packaging (day 0), both counts reached around 3.5 Log CFU g^{-1}. A very small increase (not statistically different) was recorded in MAP samples after 8 days (+ 0.5 Log CFU g^{-1}) whereas a growth of 1.5 and 2.0 Log CFU g^{-1} units was observed in AIR and VP tuna, respectively. At day 13, TPVC and TMVC reached 6-7 Log CFU g^{-1} in all batches. The counts of *Enterobacteriaceae* and *Brochothrix* were similar to total counts. Although the initial concentration of LAB was around the

detection threshold (1.7 Log CFU g^{-1}), they grew rapidly and reached the same level as the other flora. The *Pseudomonas* count was below the detection threshold till day 10 and then increased to reach 4.6 ± 0.6, 4.8 ± 0.5 and 3.6 ± 0.3 Log CFU g^{-1} in AIR, VP and MAP spoiled tuna, respectively. When samples were considered unacceptable by the sensory panel (day 13), all the counts, except *Pseudomonas*, were around 6 to 7 Log CFU g^{-1} (no statistical differences between trials).

In the 3 batches of tuna, the majority of the bacterial isolates were identified as *B. thermosphacta* (55% of the isolates) and as *Pseudomonas* spp. (*cedrina*, *fluorescens*, *fulva, gessardii/libanensis/synxantha, plecoglossicida/monteilii*, *psychrophila/fragi*) (24%). The others were isolated exclusively from VP and MAP samples and belonged to the *Enterobacteriaceae* family (*Serratia grimesii/liquefaciens/proteamaculans*, *Hafnia paralvei*, *Enterobacter asburiae/cancerogenus*, *Escherichia hermanii*) (18%) and to the LAB group (*Carnobacterium divergens*, *C. maltaromaticum*) (3%).

3.4. TTGE analysis

TTGE analysis was used to monitor the evolution of the microbiota composition in yellowfin tuna (AIR, VP and MAP) (Figure 3). The migration level of DNA fragments was compared with those of 14 bacterial species chosen as references; twelve of them isolated from tuna (this study) and 2 others (*Lactococcus piscium* and *Psychrobacter aquaticus*) from salmon. As shown in Figure 3, eight different TTGE profiles were found among the *Pseudomonas* isolates. Fresh tuna (day 0) showed a very weak intensity fingerprint due to a low concentration of bacterial DNA. Among the detected bands, 1 corresponded to *B. thermosphacta* (k) and 3 (a, e and n) were not clearly defined. In addition to the presence of *B. thermosphacta* in all batches at

day 8, AIR and VP profiles revealed additional bands assigned to diverse *Pseudomonas* spp. and to LAB for VP products. At the rejection point (day 13), the 3 batches presented a common major band assigned to *B. thermosphacta*. Nevertheless, minor band profiles were different between batches. *Pseudomonas* spp. diversity was greater in AIR and VP samples than in MAP product. In VP, LAB (*L. piscium* and *C. divergens*) and *Enterobacteriaceae* (*H. paralvei*) were also present. However, the assignment of bands marked in line e was not clear. In fact, these bands could be assigned to *C. divergens* species, *Pseudomonas* spp. strain n°6 or *P. aquaticus*. The lines a, g, m and n on the figure showed bands that could not be assigned to reference strain profiles and were thus considered unknown.

3.5. Illumina sequencing analysis

Table 3 summarizes the number of OTUs as well as the estimation of coverage, richness and diversity of the different tuna samples. Following quality checks, sequencing of total DNA extracted from the 7 samples of tuna (days 0, 8 and 13) yielded a total of 48 980 bacterial 16S rRNA sequence-read counts (mean value of 5 442 ± 1 729) and 721 OTU counts. After 13 days, the number of OTUs decreased in AIR and MAP samples whereas it slightly increased in VP tuna. For each sample, the predicted number of OTUs (estimated by the Chao1 and ACE indices) was close to the number of observed OTUs and the coverage values exceeded 99%. Table 4 details the different bacterial genera/species found in tuna products and Figure 4 represents a synthetic view of the most abundant microbial genera (> 100 total reads). At the beginning of the experiment, *Rhodanobacter terrae* dominated the microbiota of tuna (76%) but other species such as *B. thermosphacta* (5%), *Pseudomonas* spp. (1%), *Methyloversatilis* sp. (0.9%), *Ralstonia* sp. (0.7%),

385 *Psychrobacter* spp. (0.5%) and many unclassified OTUs were detected at low levels
386 (Table 4). *R. terrae* disappeared almost completely during storage under the 3
387 conditions, except in MAP samples at day 8 where it still represented 61% of the
388 microbial ecosystem. From day 8 until the end of storage, a mix of *B. thermosphacta*
389 (~65%) and *Pseudomonas* spp. (~30%) constituted the dominant microbiota of AIR
390 tuna steaks. For MAP products, *B. thermosphacta* represented only 33% of the
391 microbiota after 8 days of storage but became the major organism at day 13 (99.9%).
392 In VP tuna, despite the fact that *B. thermosphacta* represented 95% at day 8, a large
393 diversity was observed after 13 days of packaging (Shannon index = 2.08). Thus,
394 several different species dominated the microbiota, which was composed of *B.*
395 *thermosphacta* (36%), *Enterobacteriaceae* (29%), LAB (21%), *Pseudomonas* (3%)
396 and many unclassified OTUs with a low occurrence. *Enterobacteriaceae* were mainly
397 composed of *H. paralvei* and LAB with the genus *Lactococcus* (including *L. piscium*)
398 and *Carnobacterium* (including *C. maltaromaticum*), but also with *Leuconostoc*
399 *gelidum* and *Lactobacillus crispatus* (Table 4). Among *Pseudomonas,* the species
400 *collierea*, *deceptionensis*, *psychrophila*, *syncyanea* and *teatrolens* were found but
401 this genus comprises more than 200 species and many OTUs could not be clearly
402 identified. Unclassified bacteria represented less than 5% of sample reads, except for
403 fresh tuna and VP samples at day 13 which were equal to 12% and 7%, respectively.
404

405 **4. Discussion**

406 In the present study, quality changes in tropical *T. albacares* were analyzed. The
407 choice of the gas composition for the MAP product was based on the study of
408 Yesudhason et al., 2010 in which an atmosphere of 70% CO_2 – 30% O_2 extended the
409 shelf-life of steaks of a tropical scombroid species (*Scomberomorus commerson*) by

10 days. Based on this result and previous researches (Emborg et al., 2005; Goulas and Kontominas, 2007; Lalitha et al., 2005; Özogul et al., 2004; Ravi Sankar et al., 2008; Ruiz-Capillas and Moral, 2001; Ruiz-Capillas and Moral, 2005), we anticipated an extension of the shelf-life of packed products by several days and the initial sampling plan was designed accordingly. Finally, all batches were rejected by panelists after 13 days of storage and just 3 data points were collected for VP and MAP products. Nonetheless, in our study, samples were not stored at the same temperature (AIR at 0℃, VP and MAP at 4/8℃) and t his difference may have an influence on shelf-life (Du et al., 2001; Du et al., 2002) and may reduce the preservative effect of packaging (Sivertsvik et al., 2002).

The fact that the initial TVBN concentration was higher than that usually found in fresh fish was regularly observed for pelagic fish (Edirisinghe et al., 2007; El Marrakchi et al., 1990; Malle et al., 1983; Pérez-Villarreal and Pozo, 1990; Surendran et al., 1989). Although some significant increases were noted at day 13, none of the batches exceeded the acceptability limit of 30-35 mg-N 100 g^{-1} for TVBN and 10-15 mg-N 100 g^{-1} for TMA-N, indicating that these indices are not reliable to characterize the spoilage of yellowfin tuna (Huss, 1996). The TBA index reflects a higher oxidation of tuna lipids in samples packed with oxygen (MAP and AIR) and at day 13, MAP products exceeded the limit reported in the literature, which is between 5 and 8 mg-MDA kg^{-1} (Beltran and Moral, 1990; Nunes et al., 1992). However, the sensory panel did not detect any rancid off-odor.

A high free histidine content is generally found on yellowfin tuna flesh (Emborg et al., 2005) and may rapidly lead to the synthesis of a large amount of histamine causing HFP. Freshly caught yellowfin tuna generally contain 10 to 30 mg kg^{-1} of histamine (Guizani et al., 2005; Sika et al., 2014; Widiastuti et al., 2013). In the present study,

435 the initial histamine amount was high (≈ 80 ppm). At day 0, none of the main bacterial
436 species producers (i.e. *Photobacterium phosphoreum*, *Morganella morganii*,
437 *Raoultella* spp., …) was detected by culture-independent techniques. However, the
438 production of histamine from *R. terrae*, the main bacterium found in fresh tuna, is
439 unknown. *R. terrae* is a gram-negative bacteria belonging to the *Xanthomonadaceae*
440 family, first isolated from soil from a ginseng field in South Korea and described by
441 Weon et al., 2007. To our knowledge, species from the *Rhodanobacter* genus have
442 never been found in marine products. No strains could be isolated from our samples
443 with the culture conditions used. Further studies are necessary to verify whether this
444 species is commonly found in *T. albacares* and to evaluate its role in spoilage and
445 histamine production. Thereafter, the histamine concentration dropped while the TVC
446 reached a level of 10^6 CFU g^{-1}, as observed by Guizani et al., 2005. Previous studies
447 suggest a proliferation of histamine-decomposing bacteria (Ababouch et al., 1996;
448 López-Sabater et al., 1996; Sato et al., 1994; Silva et al., 1998) but this has never
449 been experimentally confirmed in tuna. *Bacillus*, *Staphylococcus*, LAB and other
450 strains are able to oxidize biogenic amines such as histamine (Alvarez and Moreno-
451 Arribas, 2014).
452 Regarding culture-dependent results, TMVC and TPVC had a similar increasing
453 trend, as found by Mohan et al., 2010. After 13 days, total microbial counts reached
454 6-7 Log CFU g^{-1}, which is considered the limit of acceptability for freshwater and
455 marine fish (ICMSF, 1986). Initially, a majority (93%) of gram-negative bacteria was
456 detected. The top five genera of fresh tuna were *Rhodanobacter*, *Brochothrix*,
457 *Pseudomonas*, *Methyloversatilis* and *Ralstonia*. Chaillou et al., 2014 studied the
458 bacterial diversity of various meat and seafood products by pyrosequencing. At day
459 0, except for *Brochothrix*, the tuna ecosystem differs widely from cod and salmon,

460 possibly due to the composition of the flesh or the environmental parameters, and
461 particularly to the fact that cod and salmon originate in temperate water.
462 PCR-TTGE and Illumina sequencing methods revealed a selection of specific
463 bacteria during spoilage. Under AIR at 0℃, *B. thermosphacta* and *Pseudomonas*
464 spp. became dominant. *B. thermosphacta* is a spoiling bacteria often isolated in MAP
465 poultry, meat and seafood (Hovda et al., 2007b; Jaffrès et al., 2009; Kakouri and
466 Nychas, 1994; Koutsoumanis and Nychas, 1999; Mamlouk et al., 2012; Mejlholm et
467 al., 2005; Nychas et al., 2008) or in lightly preserved VP products (Olofsson et al.,
468 2007), but more rarely from fish stored under air (Noseda et al., 2012). On the
469 contrary, *Pseudomonas* spp., particularly *P. fragi*, is often reported to be responsible
470 for sweet and fruity off-odors in fish flesh stored under air (Gennari et al., 1999; Gram
471 and Huss, 1996; Hovda et al., 2007a; Hozbor et al., 2006; Koutsoumanis and
472 Nychas, 1999; Miller et al., 1973; Ola and Oladipo, 2004; Parlapani et al., 2015a;
473 Parlapani et al., 2015b; Parlapani and Boziaris, 2016; Ravi Sankar et al., 2008). In
474 agreement with previous studies (Lalitha et al., 2005; Lopez-Galvez et al., 1995;
475 Özogul et al., 2004; Ravi Sankar et al., 2008), CO_2 has a bacteriostatic effect which
476 delays the growth of various flora, and Pseudomonas are particularly sensitive to
477 CO_2 (Ehsani and Jasour, 2012; Koutsoumanis et al., 2000). This probably explains
478 why MAP products presented the lowest bacterial diversity, with *B. thermosphacta*
479 representing over 98% of the spoiled products. In VP tuna, *B. thermosphacta*
480 dominated at day 8 but after 13 days, *Enterobacteriaceae* and LAB represented a
481 high proportion of the microbiota. Vacuum is well known to prevent the growth of
482 strict aerobic bacteria, such as *Pseudomonas* and *Rhodanobacter*, and to favor LAB
483 (Leroi, 2010). Finally, despite the fact that some differences in the evolution of the
484 tuna microbiota were observed, the 3 batches had the same shelf-life.

In conclusion, the results of PCR-TTGE and Illumina sequencing analyses are similar and provide data on the bacterial spoilage of yellowfin tuna flesh. Knowledge about the microbial changes and quality deterioration is important for assessing shelf-life and could contribute to constructing predictive models. However, supplementary research is needed to characterize the spoilage potential of the bacteria isolated from tuna and develop rapid quality control methods for the local fishing sector.

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References

Ababouch, L.H., Souibri, L., Rhaliby, K., Ouahdi, O., Battal, M., Busta, F.F., 1996. Quality changes in sardines (*Sardina pilchardus*) stored in ice and at ambient temperature. Food Microbiology 13, 123-132.

AFNOR, 2010. NF V 01-003. Lignes directives pour la réalisation de tests de vieillissement microbiologique - Aliments périssables et très périssables réfrigérés. Hygiène des aliments

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

Alvarez, M.A., Moreno-Arribas, M.V., 2014. The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. Trends in Food Science & Technology 39, 146-155.

Ashie, I.N.A., Smith, J.P., Simpson, B.K., 1996. Spoilage and shelf□life extension of fresh fish and shellfish. Critical Reviews in Food Science and Nutrition 36 (1&2), 87-121.

515 Beltran, A., Moral, A., 1990. Gas chromatographic estimation of oxidative
516 deterioration in sardine during frozen storage. Lebens-Wissen Technology 23,
517 499-504.

518 Brosius, J., Dull, T.J., Sleeter, D.D., Noller, H.F., 1981. Gene organization and
519 primary structure of a ribosomal RNA operon from Escherichia coli. Journal of
520 Molecular Biology 148, 107-127.

521 Cakli, S., Kilinc, B., Dincer, T., Tolasa, S., 2006. Comparison of the shelf lifes of map
522 and vacuum packaged hot smoked rainbow trout (*Onchoryncus mykiss*).
523 European Food Research and Technology 224, 19-26.

524 Chaillou, S., Chaulot-Talmon, A., Caekebeke, H., Cardinal, M., Christieans, S.,
525 Denis, C., Desmonts, M.H., Dousset, X., Feurer, C., Hamon, E., Joffraud, J.J., La
526 Carbona, S., Leroi, F., Leroy, S., Lorre, S., Macé, S., Pilet, M.F., Prévost, H.,
527 Rivollier, M., Roux, D., Talon, R., Zagorec, M., Champomier-Vergès, M.C., 2014.
528 Origin and ecological selection of core and food-specific bacterial communities
529 associated with meat and seafood spoilage. International Society for Microbial
530 Ecology 1 (14), 1751-7362.

531 Conway, E.J., Byrne, A., 1933. An absorption apparatus for the micro-determination
532 of certain volatile substances: The micro-determination of ammonia. Biochemical
533 Journal 27, 419.

534 Cramer, J.L., Nakamura, R.M., Dizon, A.E., Ikehara, W.N., 1981. Burnt tuna:
535 conditions leading to rapid deterioration in the quality of raw tuna. Marine
536 Fisheries Review 43 (6), 12-16.

537 Dainty, R.H., 1996. Chemical/biochemical detection of spoilage. International journal
538 of food microbiology 33, 19-33.

539 Dalgaard, P., Gram, L., Huss, H.H., 1993. Spoilage and shelf-life of cod fillets packed
540 in vacuum or modified atmospheres. International journal of food microbiology
541 19, 283-294.

542 Du, W.X., Kim, J., Cornell, J.A., Huang, T.S., Marshall, M.R., Wei, C.I., 2001.
543 Microbiological, sensory, and electronic nose evaluation of yellowfin tuna under
544 various storage conditions. Journal of Food Protection 64 (12), 2027-2036.

545 Du, W.X., Lin, C.M., Phu, A.T., Cornell, J.A., Marshall, M.R., Wei, C.I., 2002.
546 Development of biogenic amines in yellowfin tuna (*Thunnus albacares*): Effect of
547 storage and correlation with decarboxylase-positive bacterial flora. Journal of
548 Food Science 67 (1), 292-301.

549 Duflos, G., 2009. Le risque histamine dans les produits de la pêche. Bulletin de
550 l'académie vétérinaire de France Tome 162 N°3, 241- 245.

551 Edirisinghe, R.K.B., Graham, A.J., Taylor, S.J., 2007. Characterisation of the volatiles
552 of yellowfin tuna (*Thunnus albacares*) during storage by solid phase

553 microextraction and GC-MS and their relationship to fish quality parameters.
554 International Journal of Food Science and Technology 42, 1139-1147.

555 Ehsani, A., Jasour, M.S., 2012. Microbiological properties and biogenic amines of
556 whole Pike-Perch (*Sander Lucioperca*, Linnaeus 1758): a perspective on fish
557 safety during postharvest handling practices and frozen storage. Journal of Food
558 Science 77 (12), M664-M668.

559 El Marrakchi, A.E., Bennour, M., Bouchriti, N., Hamama, A., Tagafatit, H., 1990.
560 Sensory, chemical, and microbiological assessments of Moroccan sardines
561 (*Sardina pilchardus*) stored in ice. Journal of Food Protection 53, 600-605.

562 Emborg, J., Laursen, B.G., Dalgaard, P., 2005. Significant histamine formation in
563 tuna (*Thunnus albacares*) at 2°C—effect of vacuum-and modified atmosphere-
564 packaging on psychrotolerant bacteria. International journal of food microbiology
565 101, 263-279.

566 FAO, 2014. The state of world fisheries and aquaculture 2014. Rome. 223 pp. URL:
567 http://www.fao.org/3/a-i3720e/index.html.

568 Ferrario, C., Pegollo, C., Ricci, G., Borgo, F., Fortina, M.G., 2012. PCR detection and
569 identification of histamine□forming bacteria in filleted tuna fish samples. Journal
570 of Food Science 77 (2), M115-M120.

571 Frank, H.A., Baranowski, J.D., Chongsiriwatana, M., Brust, P.A., Premaratne, R.J.,
572 1985. Identification and decarboxylase activities of bacteria isolated from
573 decomposed mahimahi (*Coryphaena hippurus*) after incubation at 0 and 32℃.
574 International journal of food microbiology 2 (6), 331-340.

575 Galaviz-Silva, L., Goméz-Anduro, G., Molina-Garza, Z.J., Ascencio-Valle, F., 2009.
576 Food safety issues and microbiology of fish and shellfish, in: Heredia N,
577 Wesley,I., Garcia S (Eds.), Microbiologically Safe Foods. John Wiley & Son,
578 Hoboken, pp. 227-254.

579 Gennari, M., Tomaselli, S., Cotrona, V., 1999. The microflora of fresh and spoiled
580 sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) Sea and stored
581 in ice. Food Microbiology 16, 15-28.

582 Gertz, E.M., Yu, Y.K., Agarwala, R., Schaffer, A.A., Altschul, S.F., 2006.
583 Composition-based statistics and translated nucleotide searches: improving the
584 TBLASTN module of BLAST. BMC biology 7, 4-41.

585 Giacomazzi, S., Leroi, F., L'Henaff, C., Joffraud, J.J., 2004. *rpoB*□PCR amplified
586 gene and temporal temperature gradient gel electrophoresis: a rapid tool to
587 analyse bacterial strains representative of cold□smoked salmon microflora.
588 Letters in applied microbiology 38, 130-134.

589 Gill, T.A., Thompson, J.W., Gould, S., Sherwood, D., 1987. Characterization of
590 quality deterioration in yellowfin tuna. Journal of Food Science 52 (3), 580-583.

591 Goulas, A.E., Kontominas, M.G., 2007. Effect of modified atmosphere packaging and
592 vacuum packaging on the shelf-life of refrigerated chub mackerel (*Scomber*
593 *japonicus*): biochemical and sensory attributes. European Food Research and
594 Technology 224, 545-553.

595 Gram, L., Huss, H.H., 2000. Fresh and processed fish and shellfish. The
596 microbiological safety and quality of food 1, 472-506.

597 Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products.
598 International journal of food microbiology 33, 121-137.

599 Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002.
600 Food spoilage—interactions between food spoilage bacteria. International journal
601 of food microbiology 78, 79-97.

602 Gregersen, T., 1978. Rapid method for distinction of Gram-negative from Gram-
603 positive bacteria. European Journal of Applied Microbiology and Biotechnology 5,
604 123-127.

605 Guizani, N., Al-Busaidy, M.A., Al-Belushi, I.M., Mothershaw, A., Rahman, M.S., 2005.
606 The effect of storage temperature on histamine production and the freshness of
607 yellowfin tuna (*Thunnus albacares*). Food Research International 38, 215-222.

608 Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannoukos, G.,
609 Ciulla, D., Tabbaa, D., Highlander, S.K., Sodergren, E., Methe, B., De Santis,
610 T.Z., Petrosino, J.F., Knight, R., Birren, B.W., 2011. Chimeric 16S rRNA
611 sequence formation and detection in Sanger and 454-pyrosequenced PCR
612 amplicons. Genome research 21, 494-504.

613 Hovda, M.B., Lunestad, B.T., Sivertsvik, M., Rosnes, J.T., 2007a. Characterisation of
614 the bacterial flora of modified atmosphere packaged farmed Atlantic cod (*Gadus*
615 *morhua*) by PCR-DGGE of conserved 16S rRNA gene regions. International
616 Journal of Food Microbiology 117, 68-75.

617 Hovda, M.B., Sivertsvik, M., Lunestad, B.T., Lorentzen, G., Rosnes, J.T., 2007b.
618 Characterisation of the dominant bacterial population in modified atmosphere
619 packaged farmed halibut (*Hippoglossus hippoglossus*) based on 16S rDNA-
620 DGGE. Food Microbiology 24, 362-371.

621 Hozbor, M.C., Saiz, A.I., Yeannes, M.I., Fritz, R., 2006. Microbiological changes and
622 its correlation with quality indices during aerobic iced storage of sea salmon
623 (*Pseudopercis semifasciata*). LWT 39, 99-104.

624 Hungerford, J.M., 2010. Scombroid poisoning: a review. Toxicon 56, 231-243.

625 Huss, H.H., 1996. Assurance de la qualité des produits de la mer. FAO 334, 13-17.

626 ICMSF, 1986. Microorganisms in Foods. 2. Sampling for microbiological analysis:
627 Principles and specific applications. 2nd ed. Toronto: University of Toronto press.

628 Jaffrès, E., Sohier, D., Leroi, F., Pilet, M.F., Prévost, H., Joffraud, J.J., Dousset, X.,
629 2009. Study of the bacterial ecosystem in tropical cooked and peeled shrimps
630 using a polyphasic approach. International journal of food microbiology 131, 20-
631 29.

632 Joffraud, J.J., Leroi, F., Roy, C., Berdague, J.L., 2001. Characterisation of volatile
633 compounds produced by bacteria isolated from the spoilage flora of cold-smoked
634 salmon. International journal of food microbiology 66, 175-184.

635 Kakouri, A., Nychas, G.J.E., 1994. Storage of poultry meat under modified
636 atmospheres or vacuum packs: possible role of microbial metabolites as
637 indicator of spoilage. Journal of Applied Bacteriology 76 (2), 163-172.

638 Kanki, M., Yoda, T., Tsukamoto, T., Baba, E., 2007. Histidine decarboxylases and
639 their role in accumulation of histamine in tuna and dried saury. Applied and
640 Environmental Microbiology 73 (5), 1467-1473.

641 Koutsoumanis, K., Nychas, G.J.E., 1999. Chemical and sensory changes associated
642 with microbial flora of Mediterranean boque (*Boops boops*) stored aerobically at
643 0, 3, 7, and 10℃. Applied and Environmental Microb iology 65 (2), 698-706.

644 Koutsoumanis, K.P., Taoukis, P.S., Drosinos, E.H., Nychas, G.J.E., 2000.
645 Applicability of an Arrhenius model for the combined effect of temperature and
646 CO_2 packaging on the spoilage microflora of fish. Applied and Environmental
647 Microbiology 66 (8), 3528-3534.

648 Lalitha, K.V., Sonaji, E.R., Manju, S., Jose, L., Gopal, T.K.S., Ravisankar, C.N.,
649 2005. Microbiological and biochemical changes in pearl spot (*Etroplus suratensis*
650 Bloch) stored under modified atmospheres. Journal of applied microbiology 99,
651 1222-1228.

652 Lee, S., Joo, S.T., Alderton, A.L., Hill, D.W., Faustman, C., 2003. Oxymyoglobin and
653 lipid oxidation in yellowfin tuna (*Thunnus albacares*) loins. Journal of Food
654 Science 68 (5), 1664-1668.

655 Lehane, L., Olley, J., 2000. Histamine fish poisoning revisited. International journal of
656 food microbiology 58, 1-37.

657 Leroi, F., 2010. Occurrence and role of lactic acid bacteria in seafood products. Food
658 Microbiology 27 (6), 698-709.

659 Leroi, F., Cornet, J., Chevalier, F., Cardinal, M., Coeuret, G., Chaillou, S., Joffraud,
660 J., 2015. Selection of bioprotective cultures for preventing cold-smoked salmon
661 spoilage. International journal of food microbiology 213, 79-87.

662 Liston, J., 1980. Microbiology in fishery science, in: Connell J J (Ed.), Advances in
663 fishery science and technology. Fishing News Books Ltd, Farnham, England, pp.
664 138-157.

Lopez-Galvez, D., De La Hoz, L., Ordonez, J.A., 1995. Effect of carbon dioxide and oxygen enriched atmospheres on microbiological and chemical changes in refrigerated tuna (*Thunnus alalunga*) steaks. Journal of Agricultural and Food Chemistry 43, 483-490.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernádez-Herrero, M., Roig-Sagués, A.X., Mora-Ventura, M.T., 1996. Sensory quality and histamine formation during controlled decomposition of tuna (Thunnus thynnus). Journal of Food Protection® 59, 167-174.

Lukton, A., Olcott, H.S., 1958. Content of free imidazole compounds in the muscle tissue of aquatic animals. Journal of Food Science 23 (6), 611-618.

Macé, S., Cornet, J., Chevalier, F., Cardinal, M., Pilet, M., Dousset, X., Joffraud, J., 2012. Characterisation of the spoilage microbiota in raw salmon (*Salmo salar*) steaks stored under vacuum or modified atmosphere packaging combining conventional methods and PCR–TTGE. Food Microbiology 30 (1), 164-172.

Magoc, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957-2963.

Malle, P., Nowogrocki, G., Tailliez, R., 1983. Correlation entre l'indice d'alteration et l'azote basique volatil total pour divers poissons marins. Sciences des Aliments 3, 311-322.

Mamlouk, K., Macé, S., Guilbaud, M., Jaffrès, E., Ferchichi, M., Prévost, H., Pilet, M.F., Dousset, X., 2012. Quantification of viable *Brochothrix thermosphacta* in cooked shrimp and salmon by real-time PCR. Food Microbiology 30, 173-179.

Mejlholm, O., Bøknæs, N., Dalgaard, P., 2005. Shelf life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging. Journal of applied microbiology 99, 66-76.

Miller, A., Scanlan, R.A., Lee, J.S., Libbey, L.M., 1973. Identification of the volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas fragi*. Applied Microbiology 25, 952-955.

Mohan, C.O., Ravishankar, C.N., Gopal, T.S., Lalitha, K.V., Kumar, K.A., 2010. Effect of reduced oxygen atmosphere and sodium acetate treatment on the microbial quality changes of seer fish (*Scomberomorus commerson*) steaks stored in ice. Food Microbiology 27, 526-534.

Noseda, B., Islam, M.T., Eriksson, M., Heyndrickx, M., De Reu, K., Van Langenhove, H., Devlieghere, F., 2012. Microbiological spoilage of vacuum and modified atmosphere packaged Vietnamese *Pangasius hypophthalmus* fillets. Food Microbiology 30 (2), 408-419.

Nunes, L.M., Batista, I., De Campos, M.R., 1992. Physical, chemical and sensory analysis of sardine (*Sardina pilchardus*) stored in ice. Journal of the science of food and agriculture 59, 37-43.

Nychas, G.J.E., Skandamis, P.N., Tassou, C.C., Koutsoumanis, K.P., 2008. Meat spoilage during distribution. Meat Science 78, 77-89.

Ola, J.B., Oladipo, A.E., 2004. Storage life of croaker (*Pseudotholitus senegalensis*) in ice and ambient temperature. African Journal of Biomedical Research 7, 13-17.

Olofsson, T.C., Ahrné, S., Molin, G., 2007. The bacterial flora of vacuum-packed cold-smoked salmon stored at 7℃, identified by direct 1 6S rRNA gene analysis and pure culture technique. Journal of applied microbiology 103, 109-119.

Özogul, F., Polat, A., Özogul, Y., 2004. The effects of modified atmosphere packaging and vacuum packaging on chemical, sensory and microbiological changes of sardines (*Sardina pilchardus*). Food Chemistry 85, 49-57.

Parlapani, F.F., Kormas, K.A., Boziaris, I.S., 2015a. Microbiological changes, shelf life and identification of initial and spoilage microbiota of sea bream fillets stored under various conditions using 16S rRNA gene analysis. Journal of the Science of Food and Agriculture 95 (12), 2386-2394.

Parlapani, F.F., Verdos, G.I., Haroutounian, S.A., Boziaris, I.S., 2015b. The dynamics of *Pseudomonas* and volatilome during the spoilage of gutted sea bream stored at 2° C. Food Control 55, 257-265.

Parlapani, F.F., Boziaris, I.S., 2016. Monitoring of spoilage and determination of microbial communities based on 16S rRNA gene sequence analysis of whole sea bream stored at various temperatures. LWT - Food Science and Technology 66, 553-559.

Pérez-Villarreal, B., Pozo, R., 1990. Chemical composition and ice spoilage of albacore (*Thunnus alalunga*). Journal of Food Science 55 (3), 678-682.

Poulter, R.G., Curran, C.A., Disney, J.G., 1981. Chill storage of tropical and temperate water fish - differences and similarities, Proceedings of the II-R-conference on advances in the refrigerated treatment of fish, 1981-4. Boston (USA), pp. 111-124.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glockner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic acids research 35, 7188-7196.

Rachman, C., Fourrier, A., Sy, A., De La Cochetiere, Marie France, Prevost, H., Dousset, X., 2004. Monitoring of bacterial evolution and molecular identification of lactic acid bacteria in smoked salmon during storage. Le Lait 84 (1-2), 145-154.

Ravi Sankar, C.N., Lalitha, K.V., Jose, L., Manju, S., Gopal, T.K.S., 2008. Effect of packaging atmosphere on the microbial attributes of pearlspot (*Etroplus suratensis* Bloch) stored at 0–2℃. Food Microbiology 25, 518- 528.

743 Reddy, N.R., Armstrong, D.J., Rhodehamel, E.J., Kautter, D.A., 1992. Shelf-life
744 extension and safety concerns about fresh fishery products packaged under
745 modified atmospheres: a review. Journal of Food Safety 12, 87-118.

746 Reynal,L., Demanèche, S., Guyader, O., Bertrand, J., Berthou, P., Dromer, C.,
747 Bruneau, M., Bellanger, M., Merrien, C., Guegan, F., Lespagnol, P., Pitel, M.,
748 Jézéquel, M., Leblond, E., Daurès, F., 2011. Projet pilote du Système
749 d'Informations Halieutiques (SIH) Martinique (2007-2010). Premières données
750 sur la pêche en Martinique (2009-2010). Ifremer-SIH-2013/06/30,
751 http://archimer.ifremer.fr/doc/00156/26762/, 176 p.

752 Ruiz-Capillas, C., Moral, A., 2001. Residual effect of CO_2 on hake (*Merluccius*
753 *merluccius* L.) stored in modified and controlled atmospheres. European Food
754 Research and Technology 212, 413-420.

755 Ruiz-Capillas, C., Moral, A., 2005. Sensory and biochemical aspects of quality of
756 whole bigeye tuna (*Thunnus obesus*) during bulk storage in controlled
757 atmospheres. Food Chemistry 89, 347-354.

758 Saito, H., Ishihara, K., Murase, T., 1996. Effect of prey fish lipids on the
759 docosahexaenoic acid content of total fatty acids in the lipid of *Thunnus*
760 *albacares* yellowfin tuna. Bioscience, biotechnology, and biochemistry 60 (6),
761 962-965.

762 Sato, T., Fujii, T., Masuda, T., Okuzumi, M., 1994. Changes in number of histamine-
763 metabolic bacteria and histamine content during storage of common mackerel.
764 Fisheries science 60 (3), 299-302.

765 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B.,
766 Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres,
767 B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur:
768 open-source, platform-independent, community-supported software for
769 describing and comparing microbial communities. Applied and Environmental
770 Microbiology 75, 7537-7541.

771 Selli, S., Rannou, C., Prost, C., Robin, J., Serot, T., 2006. Characterization of aroma-
772 active compounds in rainbow trout (*Oncorhynchus mykiss*) eliciting an off-odor.
773 Journal of Agricultural and Food Chemistry 54, 9496-9502.

774 Shewan, J.M., 1971. The microbiology of fish and fishery products—a progress
775 report. Journal of applied bacteriology 34 (2), 299-315.

776 Sika, A.E., Ake Assi, Y., Koffi-Nevry, R., Biego, H., 2014. Histamine contents in tuna
777 loins (*Thunnus* sp.) produced in côte d'ivoire and its relation with bacterial load.
778 International Journal of Science and Research 3 (6), 2347-2351.

779 Silva, C.C.G., Da Ponte, D.J.B., Enes Dapkevicius, M.L.N., 1998. Storage
780 temperature effect on histamine formation in big eye tuna and skipjack. Journal
781 of Food Science 63 (4), 644-647.

Sivertsvik, M., Jeksrud, W.K., Rosnes, J.T., 2002. A review of modified atmosphere packaging of fish and fishery products - significance of microbial growth, activities and safety. International Journal of Food Science and Technology 37, 107-127.

Staruszkiewicz, W.F., Barnett, J.D., Rogers, P.L., Benner, R.A.J., Wong, L.L., Cook, J., 2004. Effects of on-board and dockside handling on the formation of biogenic amines in mahimahi (*Coryphaena hippurus*), skipjack tuna (*Katsuwonus pelamis*), and yellowfin tuna (*Thunnus albacares*). Journal of Food Protection 67, 134-141.

Sumner, J.L., Gorczyca, E., Cohen, D., Brady, P., 1984. Do fish from tropical waters spoil less rapidly in ice than fish from temperate waters? Food Technology in Australia 36 (7), 328-334.

Surendran, P., Joseph, J., Shenoy, A., Perigreen, P., Mahadeva K, Gopakumar, K., 1989. Studies on spoilage of commercially important tropical fishes under iced storage. Fisheries research 7, 1-9.

Susanto, E., Agustini, T.W., Ritanto, E.P., Dewi, E.N., Swastawati, F., 2011. Changes in oxidation and reduction potential (Eh) and pH of tropical fish during storage. Journal of Coastal Development 14 (3), 223-234.

Taylor, S.L., Eitenmiller, R.R., 1986. Histamine food poisoning: toxicology and clinical aspects. CRC critical reviews in toxicology 17 (2), 91-128.

Van Spreekens, K.J.A., 1974. The suitability of a modification of Long and Hammer's medium for the enumeration of more fastidious bacteria from fresh fishery products. Archiv für Lebensmittelhygiene 25 (10), 213-219.

Vyncke, W., 1970. Direct determination of the thiobarbituric acid value in trichloracetic acid extracts of fish as a measure of oxidative rancidity. Fette-Seifen-Anstrichmit tel 72, 1084-1087.

Wang, Y., Qian, P.Y., 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PloS one 4, e7401.

Weon, H.Y., Kim, B.Y., Hong, S.B., Jeon, Y.A., Kwon, S.W., Go, S.J., Koo, B.S., 2007. *Rhodanobacter ginsengisoli* sp. nov. and *Rhodanobacter terrae* sp. nov., isolated from soil cultivated with Korean ginseng. International Journal of Systematic and Evolutionary Microbiology 57, 2810-2813.

Widiastuti, I., Putro, S., Fardiaz, D., Trilaksani, W., Inaoka, T., 2013. Changes in freshness of steak and loin tuna (*Thunnus albacares*) during 15 day-chilled storage. Journal of Fisheries and Aquatic Science 8 (2), 367-377.

Yesudhason, P., Gopal, T.K.S., Ravishankar, C.N., Lalitha, K.V., Kumar, A., 2010. Effect of potassium sorbate and modified atmosphere packaging on the shelf-life

820 extension of seer fish (*Scomberomorus commerson*) steaks during iced storage.
821 Journal of Food Biochemistry 34 (2), 399-424.

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List of figures

Figure 1: TVBN (light) and TMA-N (dark) production (mg-N $100g^{-1}$) in raw tuna flesh packed under air at 0℃ (AIR), vacuum packaging at 4/8℃ (VP) and modified atmosphere packaging at 4/8℃ (MAP) after 0, 8 and 13 days. Values with different superscript letters are significantly different ($p > 0.05$). Bars represent standard deviations.

Figure 2: Changes in spoiling bacteria (Log CFU g^{-1}) during air storage at 0℃ (□), 4/8℃ vacuum packaging (○) and modified atmosphere packaging (◊) of yellowfin tuna steaks. 1: total psychrotrophic viable counts (TPVC), total mesophilic viable counts (TMVC); 2: *Brochothrix* (Bx), *Pseudomonas* (Ps); 3: *Enterobacteriaceae* (Ent), lactic acid bacteria (LAB).

Figure 3: TTGE profiles of the 16S rDNA gene V3 regions obtained by PCR amplification from total bacterial DNA extraction of fresh tuna (D0) and tuna flesh packed under air at 0℃ (AIR), vacuum packaging at 4/8℃ (VP) and modified atmosphere packaging at 4/8℃ (MAP) at each date of sampling (D). Lanes *Brochothrix thermosphacta*; *Hafnia paralvei*; *Carnobacterium divergens*; *Carnobacterium maltaromaticum*; *Pseudomonas* spp. 1-8, *Lactococcus piscium*, *Psychrobacter aquaticus*: profiles of pure strain. Assignment of bacterial reference strain profiles to PCR-TTGE bands obtained from tuna matrix: b, *L. piscium*; c, *Pseudomonas* sp. 2; d, *Pseudomonas* sp. 5; e, *C. divergens* or *Pseudomonas* sp. 6 or *P. aquaticus*; f, *Pseudomonas* sp. 8; h, *Pseudomonas* sp. 3; i, *H. paralvei*; j, *Pseudomonas* sp. 3; k, *B. thermosphacta*; l, *B. thermosphacta*; a, g, m and n, unassigned bands with the bacterial reference strain profiles, considered unknown.

Figure 4: Relative abundance (%) at the genus level (> 100 reads) based on the classification of partial 16S rDNA sequences of bacteria from fresh tuna (D0) and tuna packed under air at 0℃ (AIR), vacuum at 4/8℃ (VP) and modified atmosphere at 4/8℃ (MAP) at each date of sampling (D).Others comprise bacterial genera with less than 100 reads. Unclassified represents genera/species not assigned OTUs.

856 Table 1: Aromatic molecules used for the odor training session of panelists.

Aromatic molecules	PubChem CID	Related odor
Diacetyl *butane-2,3-dione*	650	Rancid butter
Octenol *oct-1-en-3-ol*	18827	Mushroom
Pyrrolidine *pyrrolidine*	31268	Sperm, Bleach
Dimethyl disulfide *(methyldisulfanyl)methane*	12232	Gas, Sulfur
Hexenol *(Z)-hex-3-en-1-ol*	5281167	Cut grass
Trimethylamine *N,N-dimethylmethanamine*	1146	Fishy, Amine
Calone *7-methyl-1,5-benzodioxepin-3-one*	120101	Marine, Cucumber
Ethyl isobutyrate *ethyl 2-methylpropanoate*	7342	Fruity (strawberry, overripe kiwi)
Butyric acid *butanoic acid*	264	Cheese (parmesan)

857 Italics indicate IUPAC (International Union of Pure and Applied Chemistry) names

858 Table 2: Spoilage score and sensory characteristics of fresh tuna (D0) and each storage trial (AIR, VP and MAP) after 8 days (D8)
859 and 13 days (D13).

		D0	D8			D13		
		Fresh tuna	AIR	VP	MAP	AIR	VP	MAP
Spoilage score*		2.08 ± 1.08^{a}	1.92 ± 1.16^{a}	3.00 ± 1.41^{a}	3.07 ± 2.09^{a}	6.33 ± 1.78^{b}	6.75 ± 2.53^{b}	6.83 ± 1.99^{b}
Odor								
Level**		2.25 ± 0.96^{acd}	1.75 ± 0.96^{ac}	3.00 ± 0.82^{ab}	1.40 ± 0.55^{a}	6.25 ± 2.22^{bc}	7.50 ± 1.29^{b}	6.50 ± 3.00^{bd}
Characteristics (I)***	marine	2.00	2.00	1.25	1.80	1.00	0.75	1.00
	amine	0.00	0.00	0.00	0.20	1.50	1.75	1.50
	sulfur	0.00	0.00	0.00	0.00	0.00	1.50	0.50
	pungent	0.00	0.00	0.00	0.00	0.25	1.25	0.75
Color								
Level**		1.75 ± 0.96^{a}	2.75 ± 1.50^{ab}	3.75 ± 1.71^{abc}	5.00 ± 2.00^{bc}	7.00 ± 1.15^{cd}	8.50 ± 1.29^{d}	7.00 ± 0.82^{cd}
Characteristics (I)***	blood red	2.25	1.25	1.50	1.20	0.00	0.25	0.25
	slightly pink	1.25	1.00	1.50	2.20	0.75	0.25	1.25
	bleached	0.00	0.00	0.00	0.00	1.25	0.00	1.00
	grayish	0.00	0.00	0.00	0.00	1.00	0.50	1.50
	browned	0.00	0.00	0.00	0.00	1.25	2.50	1.25
	greenish	0.00	0.00	0.00	1.40	1.00	0.25	1.25
Texture								
Level**		2.25 ± 1.50^{ab}	1.25 ± 0.50^{a}	2.25 ± 1.50^{ab}	2.80 ± 1.64^{abc}	5.75 ± 2.06^{bc}	4.25 ± 2.63^{abc}	7.00 ± 2.16^{c}

860 AIR: Tuna stored under air at 0℃; VP: Tuna stored u nder vacuum packaging at 4℃ for 7 days followed by 8℃; MAP: Tuna stored under modified atmosphere pa ckaging (70%
861 CO_2, 30% O_2) at 4℃ for 7 days followed by 8℃.
862 $^{a, b, c, d}$ Tukey HSD test on ANOVA results ($p < 0.05$). Attribute scores with the same letter are noted significantly different ($p < 0.05$).
863 * Mean of the overall rating of odor, color and texture ± SD.
864 ** Panel mean score (scale from 0: non-spoiled to 10: very spoiled) ± SD.
865 *** I = (1 x percentage of panelists who selected slight + 2 x percentage of panelists who selected moderate + 3 x percentage of panelists who selected strong) / 100.

866 Table 3: Estimated sample coverage (ESC) and OTU diversity and richness of the
867 16S rRNA gene libraries from the Illumina sequencing analyses of fresh tuna (D0)
868 and tuna packed (AIR, VP and MAP) at each date of sampling (D).

Samples		OTUs	Reads	ESC (%)*	Shannon*	Chao1*	ACE*
Fresh tuna (D0)		104	4029	99.69	1.41 ± 0.08	82.54 ± 16.16	81.65 ± 13.81
AIR							
	D6	56	6530	99.84	1.14 ± 0.06	47.90 ± 11.20	46.69 ± 9.15
	D8	69	4565	99.62	1.31 ± 0.06	85.45 ± 47.69	68.81 ± 25.53
	D10	96	4550	99.60	1.42 ± 0.07	80.16 ± 20.91	83.03 ± 21.45
	D13	57	4198	99.73	1.20 ± 0.06	59.55 ± 31.03	56.74 ± 23.89
VP							
	D8	97	6685	99.58	0.76 ± 0.08	88.57 ± 32.60	80.76 ± 21.35
	D13	110	3148	99.75	2.08 ± 0.07	81.41 ± 18.24	76.75 ± 11.53
MAP							
	D8	94	6871	99.60	1.51 ± 0.06	82.70 ± 27.00	78.72 ± 20.11
	D13	38	8404	99.68	0.19 ± 0.04	82.92 ± 70.50	80.04 ± 47.62

869 AIR: Tuna stored under air at 0℃; VP: Tuna stored un der vacuum packaging at 4℃ for 7 days followed by 8℃;
870 MAP: Tuna stored under modified atmosphere packaging (70% CO_2, 30% O_2) at 4℃ for 7 days followed by 8℃;
871 OTUs: Operational taxonomic units; ESC: Estimated sample coverage.
872 * Calculated with MOTHUR at the 3% distance level (values ± SD).

873 Table 4: Sequence-read counts of bacterial genera/species identified by Illumina
874 Illumina sequencing for fresh tuna (D0) and for each storage trial (AIR, VP and MAP)
875 at each date of sampling (D). Unclassified represents genera/species not assigned
876 OTUs.

OTUs (genera/species level)	Fresh tuna	AIR				VP		MAP	
	D0	D6	D8	D10	D13	D8	D13	D8	D13
Brochothrix thermosphacta	211	5901	2849	2579	2796	6319	1132	2262	8310
Rhodanobacter terrae	3069	11	32	155	3	2	1	4194	1
Pseudomonas sp.	5	398	868	737	1217	27	104	6	13
Hafnia paralvei	0	8	0	0	0	42	877	0	0
Pseudomonas deceptionensis	0	34	310	492	0	11	0	0	0
Pseudomonas teatrolens	0	27	329	365	0	5	0	0	0
Lactococcus sp.	0	2	1	0	0	61	451	0	2
Brochothrix sp.	31	71	35	56	68	61	86	34	49
Lactococcus piscium	0	0	0	0	0	0	116	0	0
Carnobacterium sp.	2	1	0	4	0	15	72	10	3
Carnobacterium maltaromaticum	3	0	3	2	0	54	8	3	0
Methyloversatilis sp.	37	0	0	0	2	0	0	9	0
Pseudomonas collierea	39	0	0	0	3	0	0	0	0
Ralstonia sp.	27	0	0	0	0	0	0	8	0
Hafnia sp.	0	0	0	0	0	5	29	0	0
Lactobacillus crispatus	0	15	1	1	0	9	2	1	0
Bacteroides vulgatus	0	0	0	0	0	0	21	2	3
Streptococcus salivarius	0	2	0	2	0	10	0	4	5
Burkholderia sp.	20	0	0	0	0	0	0	0	0
Flavobacterium frigidarium	0	0	0	12	0	0	0	8	0
Pseudomonas psychrophila	0	6	11	2	0	0	0	0	0
Pseudomonas syncyanea	0	4	12	1	0	0	0	1	0
Rhodanobacter sp.	17	0	0	0	0	0	0	0	0
Lactobacillus salivarius	0	0	0	1	0	12	0	0	1
Hydrogenophilus sp.	13	0	0	0	0	0	0	0	0
Leuconostoc gelidum	0	1	0	0	0	0	8	3	0
Propionibacterium acnes	3	0	1	2	0	0	0	6	0
Psychrobacter glacincola	11	0	0	0	0	0	0	0	0
Shewanella arctica	0	0	0	11	0	0	0	0	0
Lactobacillus sp.	1	0	0	0	9	0	0	0	0
Prevotella sp.	0	3	2	0	0	4	0	0	0
Staphylococcus epidermis	0	9	0	0	0	0	0	0	0
Pseudarcicella sp.	0	1	0	1	0	0	6	0	0
Psychrobacter sp.	8	0	0	0	0	0	0	0	0
Psychrobacter arcticus	8	0	0	0	0	0	0	0	0
Photobacterium sp.	6	0	0	0	0	0	0	0	0
Staphylococcus sp.	6	0	0	0	0	0	0	0	0
Telluria sp.	0	0	4	2	0	0	0	0	0
Bacillus sp.	0	0	0	0	5	0	0	0	0
Granulicatella sp.	4	0	0	0	0	0	0	0	0
Propionibacterium sp.	0	0	0	0	0	0	1	3	0
Pelomas sp.	3	0	0	0	0	0	0	0	0
Achromobacter arsenitoxydans	2	0	0	0	0	0	0	0	0
Aeromonas rivuli	2	0	0	0	0	0	0	0	0
Alistipes sp.	0	0	0	0	2	0	0	0	0
Lactococcus lactis	2	0	0	0	0	0	0	0	0
Shigella sp.	2	0	0	0	0	0	0	0	0
Lactobacillus algidus	0	0	0	0	0	1	0	0	0
Unclassified	497	36	107	125	93	47	234	317	17
Total	4029	6530	4565	4550	4198	6685	3148	6871	8404

877

1 Figure 1

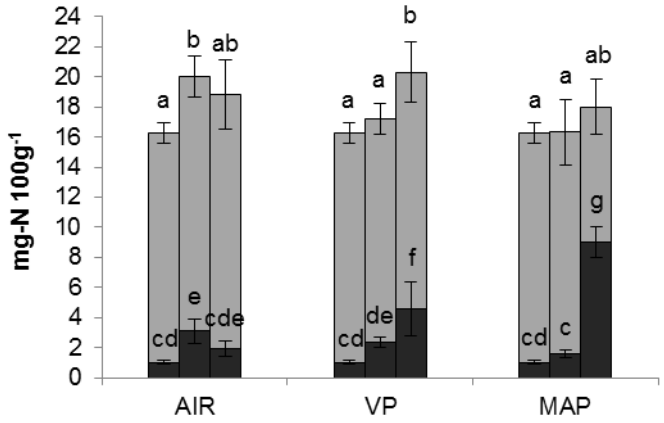

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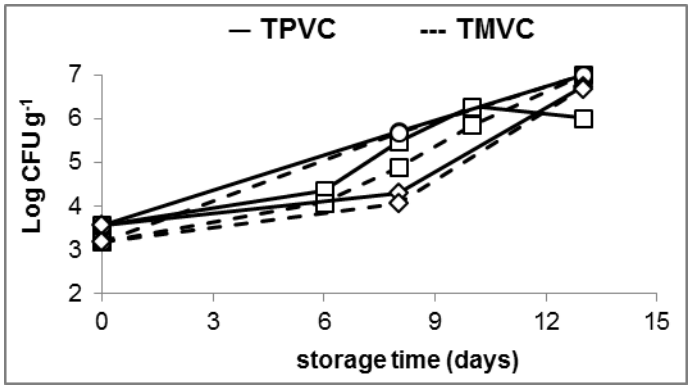
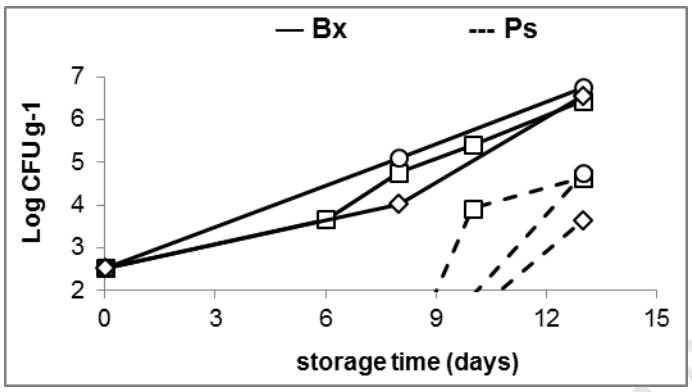
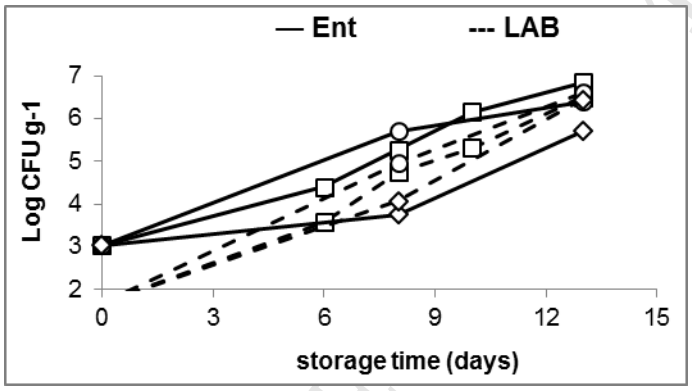
Figure 2







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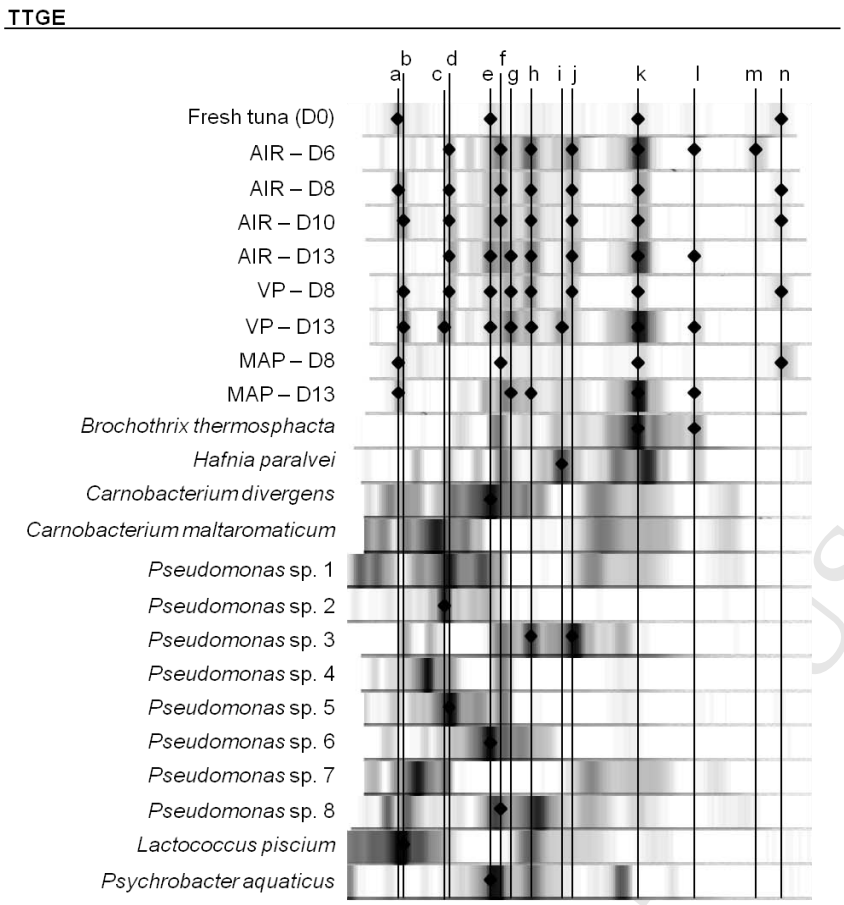

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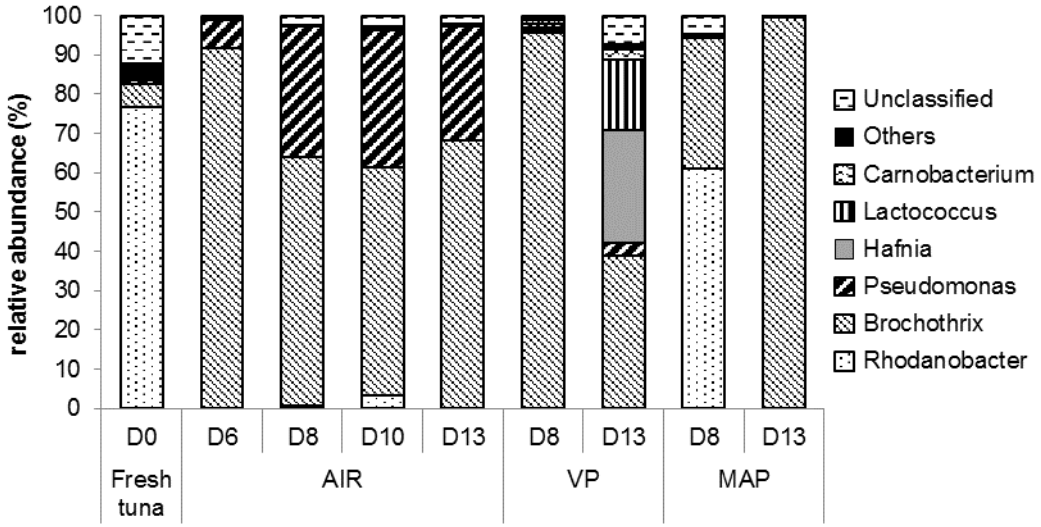
1 Figure 4



2

Highlights

-A polyphasic approach to characterize the microbial ecosystem of tuna is described

-*Rhodanobacter terrae*, never isolated from seafood, is dominant in fresh tuna

-Tuna placed under air, vacuum and modified atmosphere have the same shelf-life

-*Brochothrix thermosphacta* is the main bacterial species found in spoiled tuna

-A significant reduction in histamine is detected at the sensory rejection point