Journal Of Applied Microbiology
February 2018, Volume 124 Issue 2 Pages 559-571
http://dx.doi.org/10.1111/jam.13663
http://archimer.ifremer.fr/doc/00414/52557/
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Characterization of the spoilage potential of pure and mixed cultures of bacterial species isolated from tropical yellowfin tuna (*Thunnus albacares*)

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Abstract :

Aim

The spoilage potential of 28 bacterial strains isolated from spoiled raw yellowfin tuna was evaluated.

Methods and Results

Bacterial species were inoculated in irradiated tuna matrix. Chemical changes, bacterial growth and sensory quality were monitored during aerobic storage at 8°C. *Pseudomonas* spp., *Enterobacter* spp. and *Escherichia hermanii* had no spoiling effect. *Brochothrix thermosphacta* and *Carnobacterium divergens/maltaromaticum* developed moderate unpleasant odors. *Hafnia paralvei* and *Serratia* spp. released strong off-odors (pyrrolidine, sulfur/cabbage). No bacterial group (except *H. paralvei)* combined with *Pseudomonas* spp. deteriorated the sensory quality of tuna. When *C. divergens*/*maltaromaticum* was associated with *H. paralvei* or *B. thermosphacta*, the odor is close to the naturally contaminated tuna stored on the same conditions. The pH, Total Volatile Basic Nitrogen (TVBN) and Trimethylamine (TMA) were not correlated with the spoilage.

Conclusions

The bacterial species had a different impact on the sensory quality of the fish. The bacterial interactions leading to an enhancement or an inhibition of the spoilage potential and the bacterial growth.

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Significance and Impact of Study

The Specific Spoilage Organism (SSO) appears to be an association of Lactic Acid Bacteria (LAB) with *Enterobacteriaceae* or *B. thermosphacta. Pseudomonas*, often dominant at the sensory rejection time, is not a good quality indicator.

Keywords : Tuna, Spoilage potential, Bacterial species, Sensory quality, Bacterial interaction, *Pseudomonas*, Fish, Tropical

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Introduction

World catches of tuna and tuna-like species was multiplied by 10 since 1950 and represent more than 7.5 millions of tons in 2014. The yellowfin tuna (*Thunnus albacares*), predominant in tropical and subtropical waters, corresponds to 20% of this production value (FAO, 2016). The flesh is increasingly consumed raw such as sashimi and sushi. Fresh fish meat is an ideal substrate for the bacterial growth which is mainly responsible for the sensory deterioration (Gram and Huss, 1996, 2000; Leroi and Joffraud, 2011). Most of the studies of the microbiota of raw yellowfin tuna focuses on the histamine-producing bacteria potentially responsible for the human histamine-fish-poisoning illness (Du *et al.*, 2002; Emborg *et al.*, 2005; Guizani *et al.*, 2005; Ferrario *et al.*, 2012; Sika *et al.*, 2014). More recently, Silbande *et al.* (2016) monitored the quantitative and qualitative composition of the microbial ecosystem present in raw tuna steaks under different conditions of storage. At the sensory rejection time, *Brochothrix thermosphacta* and *Pseudomonas* spp. dominated the microbiota of products stored in air at 0°C while *B. thermosphacta* alone or a mix of *B. thermosphacta*, *Enterobacteriaceae* and lactic acid bacteria

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(LAB) prevailed in modified atmosphere-packed (MAP) and vacuum-packed (VP) products**,** respectively. Gram-negative bacteria such as *Pseudomonas* are well-known as predominant microorganism during chilled storage of fish in presence of O_2 (Ghaly *et al.*, 2010) while the anaero-aerotolerant LAB are more frequently isolated from VP and MAP fish, as well as lightly preserved seafood (Franzetti *et al.*, 2003; Leroi, 2010). *B. thermosphacta* and enteric bacteria have also been reported in CO_2/O_2 or CO_2/N_2 atmosphere and VP products (González-Rodríguez *et al.*, 2002; Emborg *et al.*, 2005; Hovda *et al.*, 2006; Olofsson *et al.*, 2007; Chaillou *et al.*, 2015; Parlapani *et al.*, 2015b). However, many of the bacterial species present at the sensory rejection time do not contribute to the sensory degradation of products and it is therefore important to identify the specific spoilage organisms (SSO) (Gram and Dalgaard, 2002). Depending on the SSO, it is generally admitted that the count should reach 8-9 Log CFU g^{-1} to observe the deterioration of fresh or processed cold-water fish (Leroi *et al.*, 2001). However, Du *et al.* (2001) and Silbande *et al.* (2016) reported that the organoleptic quality of raw yellowfin tuna steaks stored in air, VP and MAP was unacceptable at 6–7 Log CFU g^{-1}. The spoiling effects were characterized by slime formation, putrefactive odor and loss of the typical red color whose development is faster when storage temperature increases. The growth of spoiling bacteria and their sensory impact depend on the food matrix and the storage conditions but also on the interaction with other bacterial species (Joffraud *et al.*, 2006; Laursen *et al.*, 2006; Alfaro *et al.*, 2013). Identifying the SSO and understanding the mechanism involved in the spoilage of raw yellowfin tuna is important to predict shelf-life of products and develop preservative techniques.

The present study investigates the spoilage potential of several bacterial species previously isolated from tropical yellowfin tuna steaks (Silbande *et al.*, 2016). The

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ability of each isolate to produce unpleasant odors or color was assessed in irradiated yellowfin tuna flesh, both in pure and mixed culture.

Materials and Methods

Isolation, purification and identification of bacterial strains

Twenty-eight strains of *Brochothrix, Pseudomonas, Carnobacterium, Hafnia, Serratia, Enterobacter* and *Escherichia* were previously isolated from yellowfin tuna steaks stored in air at 0°C or VP and MAP (70% CO_2 - 30% O_2) at 4/8°C, at the sensory rejection time (13 days) (Silbande *et al.*, 2016). Isolates were purified twice on Brain Heart Infusion agar (BHI, Biokar Diagnostic, Beauvais, France) with 2% NaCl and maintained as frozen stocks at -80°C in a cryoprotector medium (BHI with 2% NaCl and 20% of glycerol). Strains have been identified by total 16S rDNA sequences analysis (1500 bp) as described by Macé *et al.* (2012). They are presented in Table 1.

Preparation of the pauci-microbial tuna matrix

Whole and gutted tunas, freshly caught off the coast of Martinique, were transported directly to the PARM laboratory. All the flesh (6 kg) was cut into approximately 2 cm^3 cubes with the greatest hygienic precautions (12°C-room, disinfection of material and equipment). These pieces were vacuum-packed in 500 g plastic bags, frozen at -80°C and irradiated between 5.9 to 6.6 KGy in a plant equipped with cobalt 60 source electron beam facilities (Ionisos, Pouzauges, France). The matrix was kept at -80°C. Before beginning the experiment, diced tuna was thawed in ambient water bath for 30 to 45 min and divided in several 30, 100 and 150 g portions in

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polyamide/polyethylene bags (oxygen transmission rate of 40-50 $cm^3/m^2/24$ h/atm at 23°C and 75% RH and CO_2 transmission rate of 146 $cm^3/m^2/24$ h/atm).

Challenge test: strain culture, sample inoculation and packaging

In the first trial of experiments, each species (mix of strains from the same species) or each genus (mix of strains from the same genus) were tested separately. In total, 9 groups of 2 to 4 strains were tested (Table 1). Each bacterial strain was pre-cultured individually in 10 ml of BHI with 2% NaCl at 20°C. After 24 to 48 h, 100 µl of the pre-culture was transferred into 10 ml of saline BHI and placed at 20°C until an absorbance-estimated concentration level of approximately 9 Log CFU ml^{-1}. The different strains of each group were mixed in equal proportions (final volume of 10 ml). Two successive dilutions (2-fold and 100-fold) of each group were carried out in tryptone-salt broth (Biokar Diagnostic, Beauvais, France). Volume of 0.6, 2 and 3 ml was directly incorporated in each of the 30, 100 and 150 g tuna portion, respectively, to achieve a final concentration in the flesh of approximately 5 Log CFU g^{-1} with an inoculation rate of 2% (v/w). These samples were mixed manually during 2 minutes. Similar portions of non-inoculated flesh (control) were also prepared. All the samples were stored aerobically at 8°C in vertical position. Sensory, microbiological and chemical analyses were carried out immediately after inoculation (day 0) and at day 3, 6, 9, 13 and 17. For sensory analysis, samples were placed at -80°C until the test. Similarly, a second trial consisted to combine by pair some of the nine groups. A combination of 4 groups was also prepared. In total, 11 combinations and an un-inoculated control, presented in Table 1, were prepared as previously described and analyzed at day 0, 6, 13 and 17.

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Sensory analysis

Before starting the experiment, a discussion was organized with 17 trained panellists of Ifremer to choose the relevant sensory descriptors, on the basis of the evaluation of non-irradiated and irradiated tuna after 0 and 9 days of storage at 8°C. For the single-group characterization, 12 panellists from the preliminary evaluation carried out the sensory analysis to determine the influence of the bacterial species on tuna spoilage. At each sampling date, session was performed in individual testing booths according to the procedure NF V 09-105 (AFNOR, 1995), equipped with a computerized system (Fizz, Biosystèmes, Couternon, France). One packet per batch (150 g) was thawed, divided in individual portions (20-25 g), placed in plastic bowls with lids and maintained in an oven at 18°C during the session. All products were coded with random 3-digit numbers and served to the panellists in a predefined order to avoid a bias due to the effect of the first group tested. The set of 10 samples (9 inoculated and 1 control) was scored by 2 different panellists with a minimum of 20 min interval. This allow minimizing the total quantity of tuna flesh for sensory analysis. A control (irradiated tuna at day 0), thawed morning of the test, was proposed at each session as a reference. At each session, panellists had to score an overall spoilage level based on odor on a continuous scale from 0 to 10 (6 was determined as the limit of acceptability) and then the following appropriate odor descriptors: marine/fish, vegetal, grilled, floor cloth, butter/caramel, acid, sour/fermented, pyrrolidine, feet/cheese, amine, sulfur/cabbage. Moreover, panellists had to evaluate the color intensity on a 10-point scale ranging from red/pink (0) to beige (10). In the second trial, the same procedure was repeated for the characterization of the different bacterial combinations, with minor differences: the panel was composed of 14 judges from the preliminary discussion, the set presented

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to panellists consisted of 12 samples (11 inoculated and 1 control) and 2 descriptors (rancid and fecal), often cited by panellists during the first test, were added to the list. Data of each sensory spoilage level and descriptor were submitted to two-way analysis of variance (ANOVA) with products and panellists as independent factors to identify significant product effects. Principal component analysis (PCA) and ascending hierarchical clustering (AHC) with standardization were realized for the odor profile of groups and combinations, respectively. The statistical treatments were carried out with Fizz 2.50 b 37 software (Biosystèmes), except the AHC which was performed with R software (3.2.1. version).

Enumeration of the bacterial groups

At each sampling date, one packet (30 g) of each group or combination was used for microbial analysis. The fish flesh was aseptically added to 120 ml of sterile tryptone-salt broth (Biokar Diagnostic) and stomached for 2 min with a stomacher 400 (Seward Ltd., London, UK) to obtain the mother solution. Depending on the potential development of the bacterial groups at each sampling date, one to seven 10-fold dilutions were carried out in tryptone-salt solution. For the 9 individual bacterial groups and controls, the total viable count (TVC) was enumerated by spread plating onto Long and Hammer with 1% NaCl plates (Van Spreekens, 1974) incubated 5 days at 20°C. For the 11 bacterial combinations, selective culture media were used to distinguish the different genus of bacteria. *Brochothrix* sp. (Bx), *Pseudomonas* sp. (Ps.) and *Carnobacterium* sp. (Ca) were enumerated by spreading 100 µl of diluted mother solution onto respective plates of Streptomycin Thallium Acetate Agar (Oxoid, Basingstoke, Hampshire, England) (2 days at 20°C), supplemented Cetrimide Fucidin Cephalosporin agar (Biokar Diagnostic) (2 days at

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20°C) and Nitrite Actidione Polymixin agar as prepared by Davidson and Cronin (1973) (3 days at 20°C, anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany)). *Hafnia* sp. (Ha) was counted in double layer pour plates of Violet Red Bile Glucose agar (Biokar Diagnostic) after 2 days at 30°C.

Chemical analysis

The pH value was measured with a pH-meter (Mettler Delta 320, AES, Combourg, France) in the mother solution prepared as described above for microbial analysis. Total volatile basic nitrogen (TVBN), trimethylamine (TMA) and trimethylamine-oxide (TMA-O) were determined in 100 g of flesh using the Conway micro-diffusion method (Conway and Byrne, 1933).

Results

1. Individual groups of bacteria

Chemical changes

The chemical results at day 0 and 17 are presented in Table 2. The data at day 3, 6, 9 and 13 are not shown as there is no major changes during storage. The control tuna flesh had a stable acidic pH all over the storage (average of 5.80 ± 0.06). The inoculated samples showed similar values of pH at the end of storage, except the groups Pp, Pg and Ha that caused an alkalinisation of around 1.7, 0.8 and 1.4 units, respectively. At the beginning of storage, value of TVBN was equal to 20.3 mg-N 100 g^{-1} and null for TMA. No significant productions of TVBN and TMA were noticed in the tuna flesh during 17 days of storage, both in control and in inoculated samples.

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Enumeration of the bacterial strains

The bacterial population changes in inoculated tuna samples stored for 17 days at 8°C are shown in Figure 1. The control count (non-inoculated flesh) remained below the detection threshold (1.7 Log CFU g^{-1}) during all the storage period (data not shown). Initially, the TVC of the inoculated samples ranged between 4.5–5.3 Log CFU g^{-1}, except for Pp group with a level of 3.7 Log CFU g^{-1}. At day 3, the counts of 3 groups (Pf, En and Es) ranged from 5.3 to 6.3 Log CFU g^{-1} and the others (Se, Pp, Ha, Bx, Ca and Pg) from 7.2 to 8.2 Log CFU g^{-1}. Thereafter, half of groups, including Pf, Bx, Se, Ca and Es, reached their maximum count of approximately 8–9 Log CFU g^{-1} from 6 days of storage while the others continued to grow, especially Pg, Pp and Ha with a level superior to 9–10 Log CFU g^{-1} at the end of storage.

Sensory quality

Figure 2 shows the sensory spoilage score of the control and the inoculated samples during the aerobic storage at 8°C. Figure 3 represents the standardized PCA performed with the mean scores of each odor descriptors. The descriptors and samples are simultaneously projected on the 1–2 plane (61.5% of the inertia). The first axis (40.6%) discriminates unspoiled samples with marine/fish, vegetal and grilled characteristics (right side of PCA) from spoiled samples with off-odors as sulfur/cabbage, amine, sour/fermented, feet/cheese, etc. (left side). The second axis (20.9%) discriminates samples with pyrrolidine odor (upper part) from butter/caramel (lower part). The sensory quality of the control remained stable until 9 days (<2), with marine/fish odor, and then presented slight sour/fermented and feet/cheese odors until the end of storage. The spoilage potential of the bacteria varied and three scenarios were observed. Scenario 1, no significant spoilage: groups Pg, Pf, Es and En did not spoil the tuna flesh. The score remained inferior to control (2–3) all over

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the storage with marine/fish and vegetal odors, except En after 17 days (score 5.0) for which some sulfur/cabbage and floor cloth off-odors were detected. Scenario 2, weak spoilage: groups Bx and Ca, and in a different way Pp, were considered as moderate spoilers. By the third day, Ca released butter/caramel (total spoilage score 3.4) and Bx sour/fermented, butter/caramel and acid fragrances (score 4.7). The sensory spoilage score then ranged between 4 to 5 all over the storage period and samples were represented on the lower part of the second PCA axis, essentially characterized by butter/caramel and acid odors. Ca group also released grilled odor at days 9 and 17. These samples were well represented on axis 3 of the PCA (inertia 10.6%, data not shown). Pp group was considered as a moderate spoiler due to the result at day 9 when a degree of spoilage superior to the limit of acceptability (6.0) and sour/fermented and sulfur/cabbage odors were detected by the panellists. However, all the other days, even after day 9, the samples were not considered as spoiled when comparing to the control. Scenario 3, strong spoilage: Ha and Se were the strongest spoiling bacteria with a spoilage score exceeding the limit of acceptability (6) from day 6 and day 9 until the end of storage for Ha and Se respectively. These samples were located on the left upper part of PCA and characterized by unpleasant odors such as pyrrolidine, floor cloth, amine and sulfur/cabbage. The red/pink color of the tuna flesh disappeared during the aerobic storage of all samples, except for the most spoiled samples (Ha and Se) and for the group Ca (data not shown).

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Combination of bacterial groups

Chemical changes

The initial pH of the control was similar to the first trial and no significant variations were observed for the control and inoculated samples (data not shown). The TVBN concentrations were around 20 mg-N 100g^{-1} and the TMA remained negligible all along the storage. The fresh yellowfin tuna flesh did not contain TMA-O (data not shown).

Enumeration of the bacterial strains

The spoilage potential of five groups of bacteria (Bx, Ca, Ha, Pp and Pfg) were tested in pair combination (Table 1). Pfg was a mix of Pf and Pg that did not spoiled the tuna flesh in the first trial whereas Pp lightly spoiled it. Those groups were selected because of their prevalence in spoiled tuna flesh (Silbande *et al.*, 2016). The groups were inoculated by pair (10 mixes in total) and in a more complex association of Bx, Ha, Pp and Pfg. Figure 4 presents the growth curves of bacterial groups inoculated in tuna, alone or in combination. The control flesh remained below the detection threshold (1.7 Log CFU g^{-1}) during all the storage period (data not shown). Overall, the bacterial growth was weaker when the groups were co-inoculated than alone and the differential at the end of storage was between 1 to 3 Log CFU g^{-1} depending on the combinations. As an example, Bx increased more rapidly alone and reached a rate of 9.2 Log CFU g^{-1} at day 17 while in association with Ca, Ha, Pp, Pfg and HaPpPfg, Bx never overpassed 6–7 Log CFU g^{-1}. The greatest inhibition was observed for *Pseudomonas*. Indeed, Pp and Pfg counts were 4-5 Log CFU g^{-1} lower in mix culture with Bx, Ca and Ha than in pure culture. In some case, the initial inoculum of *Pseudomonas* spp. was below the detection threshold (BxPp, HaPp and CaPfg) and this may explain the results. However, even

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with an inoculum of 5.2 Log CFU g^{-1} (HaPfg, CaPp, BxPfg,) this genus was inhibited and a total prevention of growth was observed with Ha and Ca. For the combination PpPfg, it was not possible to conclude as no selective medium was available to differentiate *Pseudomonas* species.

Sensory quality

Figure 5 shows the sensory spoilage score of the control and the inoculated samples obtained during the aerobic storage at 8°C. The classification of samples according to their sensory characteristics is presented in the AHC based on the first five principal components of the standardized PCA that is obtained with the mean scores of profiling odors (Figure 6). The control was characterized by marine/fish, vegetal and rancid odor and the sensory spoilage score ranged between 3 to 4. At day 6, none of the bacterial mixes significantly spoiled the products (Figure 5) and just weak butter/caramel, floor cloth and sour/fermented odors were noticed. After 13 or 17 days, only 3 combinations allowed the sensory rejection: BxCa, HaPfg and CaHa. The samples were characterized by strong fecal and sulfur/cabbage odors and their spoilage score were approximately 7–8. When *Carnobacterium* spp. was present, a sour/fermented odor was also significantly detected. All these samples were grouped at the left side of the AHC (Figure 6). All bacterial groups, even some considered as strong spoilers, combined with *Pseudomonas* spp. (Pp or Pfg) did not deteriorate the sensory quality of tuna, except HaPfg. The samples grouped together on the AHC with grilled and marine/fish odors, and sometimes butter/caramel, feet/cheese and sour/fermented odors. Surprisingly, the combination of the two *Pseudomonas* groups (PpPfg) led to feet/cheese odors that significantly increased the spoilage score to 5.6 at the end of storage. The last combination (BxHaPpPfg) was closed to the sensory acceptability limit at day 13 with a score of 5.5 and characterized by sour/fermented,

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floor cloth and pyrrolidine odors. Only pyrrolidine odor persisted at the end of storage and the spoilage score dropped to the same level as the control (4.1). At day 6, all the tuna samples, inoculated or not, presented a beige color with a score of approximately 5–7, except CaPfg that had a red/pink aspect (score 1.8). The control samples reached the maximum beige level of 7.7 at day 17 and presented a green discoloration of the flesh. Only the CaPfg combination had a similar trend. Surprisingly, the others led to a better red/pink color without green defects, particularly PpPfg, CaPp, HaPfg and CaHa (data not shown).

Discussion

The spoilage potential of bacterial species depends on the food matrix and storage conditions (Stohr *et al.*, 2001; Macé *et al.*, 2013, 2014; Pothakos *et al.*, 2014; Saraoui *et al.*, 2016). The physicochemical properties of the tropical yellowfin tuna flesh, studied by Dromer *et al.*, 2015, should favor the growth and spoilage activity of *Pseudomonas* spp. and various members of the family of *Enterobacteriaceae* and, depending on the storage conditions, of *Carnobacterium* spp. and *B. thermosphacta* because they grow well and able to metabolize the flesh constituents at pH<6. Tropical fish (especially those without O-TMA) conduct to the bacterial formation of acetoin/diacetyl (butter/caramel or cheesy off-odors) and esters (fruity off-odors) (Gram *et al.*, 2002; Dalgaard, 2005). In the previous research (Silbande *et al.*, 2016), tuna microbiota was studied using the Illumina sequencing method and revealed a dominance of these bacteria in the spoiled yellowfin tuna responsible of strong amine off-odors.

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Except some cases of *Pseudomonas*, inoculations were performed at approximately 5 Log CFU g^{-1}. This level was chosen to favor a fast colonization of the flesh and to rapidly observe the spoilage effect that usually occurs at the beginning of the stationary phase of bacterial growth (Gram and Huss, 1996). All cultures tested grew well on tuna cubes but the unpleasant odors appeared only for some groups of strains, which is in accordance with the SSO concept (Gram and Dalgaard, 2002). For each group, a mix of 2 to 4 strains per species was used to avoid the strain effect described by Stohr *et al.* (2001) and Pothakos *et al.* (2014).

From the 3rd day, 4 bacterial groups released significant off-odors. Bx and Ca emitted butter and sour odors while Se and Ha were characterized by pyrrolidine-like odors. Except for the Ca group, all the off-odors persisted in the samples and even led to the rejection of the product by the sensory panel for Se and Ha. Mesophilic enteric bacteria, especially the species *S. liquefaciens* and *H. alvei*, are identified as strong spoilers of meat, fish and seafood and are often associated with the production of malodorous diamines such as putrescine and cadaverine (Joffraud *et al.*, 2001; Stohr *et al.*, 2001; Ercolini *et al.*, 2009; Doulgeraki *et al.*, 2011; Jaffrès *et al.*, 2011; Macé *et al.*, 2013). The growth of these two organisms in red meat may cause a green discoloration probably due to the interaction between bacterial metabolites and myoglobin (Stanbridge and Davies, 1998). This has not been observed in this study where diced tuna inoculated with Se and Ha kept their red color, whereas the control became grey and green. Under controlled storage conditions (vacuum, modified atmosphere), members of *Enterobacteriaceae* are able to degrade TMA-O to TMA creating ammonia-like off-odors (Jørgensen *et al.*, 2001; Gram and Dalgaard, 2002). However, no production of TMA or TVBN was observed in the samples inoculated with Se, Ha, En and Es. The spoilage role of *C. divergens*

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and *C. maltaromaticum* is variable in meat and fish products, despite their ability to metabolize amino-acids and released unpleasant odors (Laursen *et al.*, 2005). In cold-smoked salmon, these organisms have a limited spoilage activity (Joffraud *et al.*, 2001; Stohr *et al.*, 2001; Brillet *et al.*, 2005) such as in tuna. Brillet *et al.* (2005) and Laursen *et al.* (2005, 2006) even showed that *C. maltaromaticum* may be applicable as protective cultures. Conversely, *B. thermosphacta* is often listed as a spoiling bacteria in meat and fish products (Vermeiren *et al.*, 2005; Papadopoulou *et al.*, 2012, 2013), capable to produce ketones, acids and alcohols (Joffraud *et al.*, 2001). This bacteria released strong butter, sour and nauseous off-odors in chilled and MAP cooked peeled shrimps (Laursen *et al.*, 2006; Jaffrès *et al.*, 2011). The inoculation in sterile cold-smoked salmon revealed that *B. thermosphacta* and *C. maltaromaticum* were characterized by 2-heptanone (blue-cheese odor) and 2,3-butanedione (butter smell), respectively (Joffraud *et al.*, 2001). *Pseudomonas* spp. are commonly classified as major spoiling bacteria of chilled fish stored in air (García-López *et al.*, 2004; Hovda *et al.*, 2007; Parlapani *et al.*, 2013, 2014, 2015a, 2015b; Parlapani and Boziaris, 2016). In tuna, Pg and Pf groups exhibited weak influence on the sensory quality despite a rapid growth up to 9–10 Log CFU g^{-1}. The Pp group developed a peak of strong off-odors at the middle storage period and then a moderate butter/caramel flavor. This could be due to a loss of some volatile compounds in the air. For this group of *Pseudomonas*, panellists noted “vanilla” and “lemon” odors that are close to the sweet and fruity odors described during the inoculation of *Ps. fragi* in sterile fish matrices (Gennari *et al.*, 1999; Ólafsdóttir *et al.*, 2006). The sensory differences observed between the *Pseudomonas* groups are in accordance with the fact that the spoilage potential of this genus is species-dependent (Dogan and Boor, 2003; Morales *et al.*, 2005; Arslan *et al.*, 2011).

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Joffraud *et al.* (2006) and Macé *et al.* (2013) have evidenced the effect of bacterial interactions on spoilage of seafood. Table 3 summarized the maximum spoilage score and significant odors developed in tuna inoculated with individual groups of bacteria and combinations of groups during the aerobic storage at 8°C. Ha was the greatest spoiling group. In association with Bx or Pp, which are individually considered as mildly spoilers, very weak off-odors were released. This may be attributed to the inhibition of Ha growth, which reached around 10 Log CFU g^{-1} in pure culture and 8 in co-culture. The odors of Ha alone were similar to Ha with Ca or Pfg. Contrary to the study of Macé *et al.* (2013) in salmon flesh, Ca did not inhibit the growth of Ha. However, Pfg did inhibit Ha, in the same proportion as Bx and Pp. Some metabiosis phenomena may explain this result and still need to be explored. All the associations with Bx exhibit the same sensory characteristics as Bx alone, except with Ca. Surprisingly, Bx and Ca which are weak spoilers when alone, strongly deteriorated the quality of flesh and the odors were comparable to naturally contaminated flesh at the end of storage, observed by Silbande *et al.* (2016). The spoiling effect obtained with the association of *B. thermosphacta* and *Carnobacterium* spp. has already been mentioned by Mejlholm *et al.* (2005) in cooked and peeled MAP shrimps. However, this result was not reproduced during the similar study of Laursen *et al.* (2006). In most cases, the presence of some *Pseudomonas* spp. (Pp or Pfg group) lowered the spoiling intensity of the other groups and this is particularly true for the HaPp mix. The combination of all the *Pseudomonas* groups (PpPfg) had the same maximum spoilage score as Pp but emitted different odors than the single spoilage groups (Pp, Pf and Pg) probably due to the mixture of all the metabolites individually produced.

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The main output of this study is that most of the *Pseudomonas* species tested do not spoil tuna, although this genus is often incriminated, as it dominates the microbiota at the sensory rejection time. Moreover, *Pseudomonas* spp. tends to attenuate the spoilage of other bacteria, which has not been reported in literature. *B. thermosphacta* and *Carnobacterium* spp. are moderate spoilers, with butter/caramel odors. *H. paralvei* and *Serratia* spp. are strong spoilers with fecal or sulfur odors. Therefore, the count of enterobacteria seems a better indicator of spoilage than the *Pseudomonas* one. The co-inoculations highlight the bacterial interactions conducting to an enhancement or an inhibition of the spoilage potential in the tuna flesh. When *Carnobacterium* spp. are combined with *H. paralvei* or *B. thermosphacta*, the odor profile is close to the naturally contaminated tuna stored on the same conditions and these associations could correspond to the SSO. A microbial ecosystem similar to that found by Silbande *et al.* (2016) (Bx, Ca, Ha, Pp, Pfg) did not reproduce the sensory quality of the tuna with its endogenous microbiota. No classical physico-chemical parameters such as pH, TVBN and TMA correlated with the spoilage process. More knowledge on the bacterial species metabolism is necessary to predict spoilage and manage the preservative techniques.

Acknowledgements

This research was financially supported by the territorial community of Martinique and the European Fisheries Fund (joint convention number: 013/DM/0335/3.1.1.a/38836). The authors are grateful to T. Guerrier for its valued contribution.

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Conflict of interest

All the authors approved the manuscript and its submission to the Journal of Applied Microbiology and declare having no competing interest in this work.

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Table 1: Bacterial species inoculated in tropical yellowfin tuna for the two trials of experiments

Trials	Bacterial species (strains SYP*)	Labels
Groups (mix of strains)	Control	C
	Brochothrix thermosphacta (SYP3105, SYP3111, SYP3123, SYP3149)	Bx
	Pseudomonas psychrophila/fragi (SYP3102, SYP3114, SYP3124, SYP3130)	Pp
	Pseudomonas fluorescens, fulva, monteilii/plecoglossicida (SYP3139, SYP3140, SYP3161, SYP3162)	Pf
	Pseudomonas gessardii/libanensis/synxantha, cedrina (SYP3116, SYP3117, SYP3118, SYP3138)	Pg
	Carnobacterium divergens, maltaromaticum (SYP3141B, SYP3142)	Ca
	Hafnia paralvei (SYP3128, SYP3136, SYP3137, SYP3143)	Ha
	Serratia grimesii/liquefaciens/proteamaculans (SYP3122, SYP3135)	Se
	Enterobacter asburiae/cancerogenus (SYP3157, SYP3158)	En
	Escherichia hermanii (SYP3159, SYP3160)	Es
Combinations (mix of groups, a group contains all the strains listed above)	Control	C
	B. thermosphacta + *Ps. psychrophila/fragi*	BxPp
	B. thermosphacta + *Ps. fluorescens, fulva, monteilii/plecoglossicida* + *Ps. gessardii/libanensis/synxantha, cedrina*	BxPfg
	B. thermosphacta + *H. paralvei*	BxHa
	B. thermosphacta + *C. divergens, maltaromaticum*	BxCa
	Ps. psychrophila/fragi + *Ps. fluorescens, fulva, monteilii/plecoglossicida* + *Ps. gessardii/libanensis/synxantha, cedrina*	PpPfg
	H. paralvei + *Ps. psychrophila/fragi*	HaPp
	C. divergens, maltaromaticum + *Ps. psychrophila/fragi*	CaPp
	H. paralvei + *Ps. fluorescens, fulva, monteilii/plecoglossicida* + *Ps. gessardii/libanensis/synxantha, cedrina*	HaPfg
	C. divergens, maltaromaticum + *Ps. fluorescens, fulva, monteilii/plecoglossicida* + *Ps. gessardii/libanensis/synxantha, cedrina*	CaPfg
	C. divergens, maltaromaticum + *H. paralvei*	CaHa
	B. thermosphacta + *H. paralvei* + *Ps. psychrophila/fragi* + *Ps. fluorescens, fulva, monteilii/plecoglossicida* + *Ps. gessardii/libanensis/synxantha, cedrina*	BxHaPpPfg

*SYP: strain code in the bacterial collection of Ifremer.

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Table 2: Total volatile basic nitrogen (TVBN), trimethylamine (TMA) (mg-N 100 g^{-1}) and pH measurements after 17 days of aerobic storage at 8°C in the samples inoculated with 9 groups of bacteria

Groups	Control (day 0)	Control (day 17)	Bx	Pp	Pf	Pg	Ca	Ha	Se	En	Es
TVBN	20.34	18.93	19.40	18.20	22.66	19.59	19.73	19.76	20.21	16.58	20.17
TMA	0.00	0.66	1.08	1.10	1.39	1.35	1.47	2.71	3.51	1.07	1.83
pH	5.76	5.84	5.92	7.53	5.69	6.55	5.57	7.20	5.73	5.68	5.74

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Table 3: Maximum spoilage score (scale 0-10) and significant odors developed in tuna inoculated with individual groups of bacteria (gray cells) and combinations of groups, during the aerobic storage at 8°C

	Bx	Pp	Pfg	Ha	Ca
Bx	5 butter/caramel				
Pp	3 sour/fermented butter/caramel	**7** **sour/fermented** **sulfur/cabbage** **"fruity, acid"**			
Pfg	4 butter/caramel	**6** **sour/fermented**	2		
Ha	3 butter/caramel	4 sulfur/cabbage	**8** **fecal** **sulfur/cabbage**	**9** **sulfur/cabbage** **pyrrolidine** **"fecal"**	
Ca	**7** **fecal** **sour/fermented** **sulfur/cabbage**	4 butter/caramel	3 sour/fermented	**8** **fecal** **sulfur/cabbage** **sour/fermented**	5 butter/caramel sour/fermented grilled

*Odors in brackets were not comprised in the list of descriptors but added by the panellists to describe the sample.
**Bold text indicate the sample with a spoilage score superior to the limit of acceptability of 6/10.

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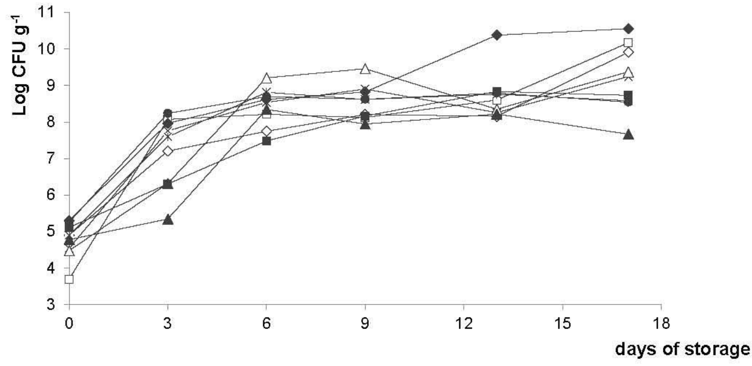
Figure 1



Legend:
(x): Bx
(□): Pp
(Δ): Pf
(◇): Pg
(✳): Ca
(◆): Ha
(●): Se
(■): En
(▲): Es

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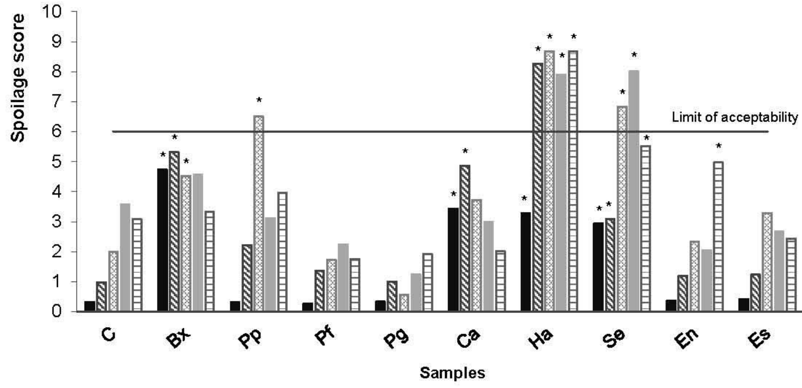
Figure 2



Legend:
(■): day 3
(▨): day 6
(▩): day 9
(■): day 13
(▤): day 17

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Figure 3

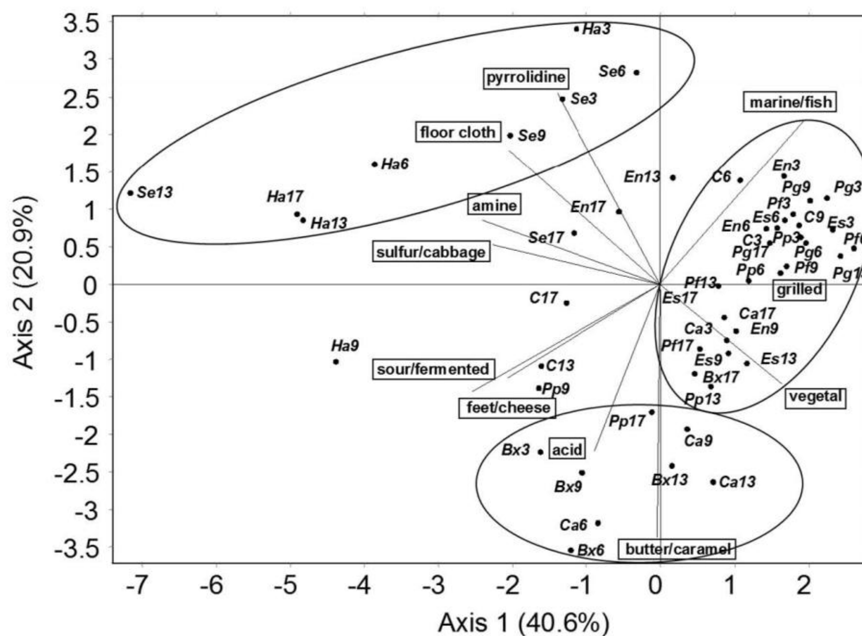

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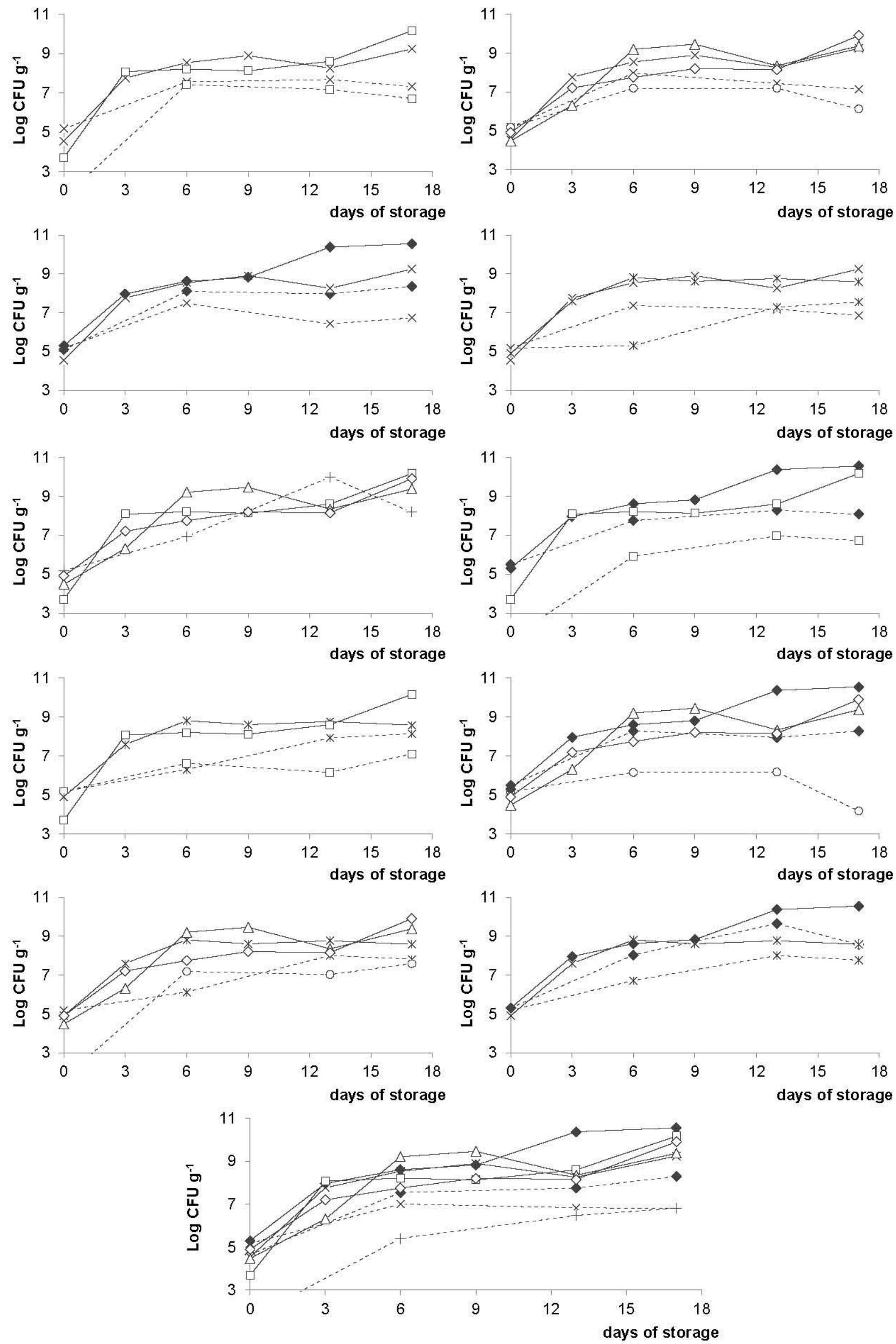
Figure 4



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Legend:
(x): Bx
(□): Pp
(Δ): Pf
(◇): Pg
(○): Pfg (mix of Pf and Pg)
(+): PpPfg (mix of Pp, Pf and Pg)
(✳): Ca
(◆): Ha
(●): Se
(■): En
(▲): Es

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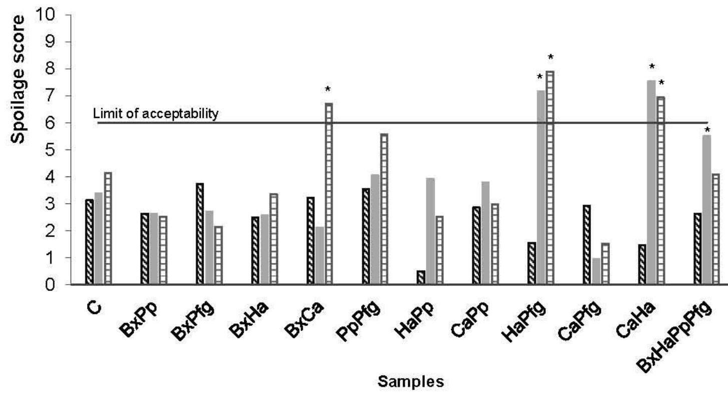
Figure 5



Legend:
(▧): day 6
(■): day 13
(▤): day 17

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Figure 6

HaPfg17 HaPfg13 CaHa17 CaHa13 BxCa17
fecal
sulfur/cabbage

C13 C6 C17
rancid
vegetal
marine/fish

CaPfg6 BxCa13 HaPp17 BxHa17 CaPfg17 PpPfg6 HaPp6 CaPfg13 HaPfg6 CaHa6 HaPp13 BxPfg17 BxPp17 CaPp6 BxHa13 BxPp13 BxHa6 BxPp6 BxPfg13
grilled
marine/fish

BxPfg6 BxHaPpPfg6 CaPp13 BxCa6 BxHaPpPfg17 BxHaPpPfg13 PpPfg17 CaPp17 PpPfg13
butter/caramel
feet/cheese
sour/fermented

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