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Metabolomics and bacterial diversity of packaged yellowfin tuna (*Thunnus albacares*) and salmon (*Salmo salar*) show fish species-specific spoilage development during chilled storage

Elina Jääskeläinen[a*], Louise M.A. Jakobsen[b], Jenni Hultman[c], Nina Eggers[b], Hanne C. Bertram[b] and Johanna Björkroth[a]

[a]Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

[b]Department of Food Science, Aarhus University, Aarslev, Denmark

[c]Department of Microbiology, University of Helsinki, Helsinki, Finland

[*] corresponding author

Elina Jääskeläinen

Agnes Sjöbergin katu 2 (P.O. Box 66)

FI-00014 Helsinki University

Finland

Email: Elina.L.Jaaskelainen@helsinki.fi

Tel +358 29 4157 167

Abstract

Microbial (colony counts, 16S rRNA gene amplification), chemical (pH, ^{1}H-NMR spectroscopy) and sensory changes in raw Atlantic Salmon (*Salmo salar*) and tuna (*Thunnus albacares*) fillets stored under vacuum at 3°C were evaluated over a period of 12 days. Both species of fish are globally important and among the ten most consumed fishes in the world. Although the sensory analyses showed a decrease in the quality of both fish species, only the salmon fillets were considered spoiled at the end of the storage period. In salmon, trimethylamine was the main spoilage product and bacterial colony counts reached an average of 7.3 log10 cfu/g. The concentration of glucose decreased and the concentration of organic acids increased during storage revealing glucose fermentation. *Photobacterium* was the dominating genus in the salmon studied. In the tuna studied, the bacterial colony counts reached only an average of 4.6 log10 cfu/g. The dominating bacteria in tuna were *Pseudomonas* spp. Glucose levels did not decrease, suggesting that amino acids and lactate most likely acted as carbon sources for bacteria in tuna. In conclusion, the study revealed that salmon was clearly a more perishable fish than tuna.

Keywords: Fish quality; Photobacterium; Pseudomonas

Abbreviations:

AQ, acquisition time; FIDs, free induction decays; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; IMP, Inosine´5-monophosphate; LAB, lactic acid bacteria; MAP, modified atmosphere packaging/packed; OTU, Operational taxonomic units; SSO, specific spoilage organism; TMA, Trimethylamine, TMAO, Trimethylamine N-oxide; TSP, sodium trimethylsilyl [2,2,3,3-D4]-1-propionate; VP, Vacuum packaging/packed; WSE, water-soluble extracts

1. Introduction

Fish are among the healthiest foods and provide us with valuable unsaturated and omega-3 fatty acids. However, they are also one of the most perishable food items. Microbial activity is the major cause of raw fish spoilage (Mikš-Krajnik *et al.*, 2016), even though the first changes are caused by the endogenous enzymes of fish. It is estimated that 30% of fish landed/caught is lost through microbial activity alone (Amos, 2007). Microbial spoilage proceeds fast because of the presence of large amounts of low-molecular-weight compounds, high water activity and high post-mortem pH (>6) in fish muscles. Refrigeration is necessary to extend the shelf-life of fish and is often combined with vacuum packaging (VP) to restrict the growth of aerobic spoilers (Dalgaard *et al*, 1993; Goulas and Konominas, 2007; Silbande *et al.*, 2016).

Various species of fish have become popular with consumers. In this study, we compared two commercially important fish species, yellowfin tuna (*Thunnus albacares*) and Atlantic salmon (*Salmo salar*), stored under similar conditions. Tuna is predominant in tropical and subtropical waters and fresh tuna fillets exported globally represent a very valuable commodity. In Europe the value of retail sales of fresh tuna was €780 million in 2011 according to the Pacific Islands Forum Fisheries Agency (*https://www.ffa.int/node/567*) representing the main tuna fishing countries. Worldwide, the most farmed fish is Atlantic salmon. The annual export value of Atlantic salmon produced in Norway was about €5000 million in 2014 (Norwegian Seafood Council 2015).

The aim of the present study was to determine the bacterial diversity and metabolite changes as studied by metabolomics during the shelf-life of tuna and salmon. For this purpose, we used proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, which is a powerful technique for explorative food analyses (Trimigno *et al.*, 2015). Former studies exploiting the potential of NMR-based

metabolomics have shown a great potential of the methodology for monitoring of storage-induced metabolite changes in fish (Ciampa *et al.*, 2012; Shumilina *et al.*, 2015; Shumilina *et al.*, 2016) and seafood (Aru *et al.*, 2016). However, NMR spectroscopy has only rarely been used to monitor changes of complex microbial populations in food systems (Ercolini *et al.*, 2011). The link between the results of bacterial sequence data and metabolomics helped us to increase the understanding of bacterial growth and concomitant activity of the microbial pathways in salmon and tuna during cold storage.

2. Material and methods

2.1. Sample preparation

The fish used in this study were fresh farmed Atlantic salmon (*Salmo salar*) fillets from Norway and fresh yellowfin tuna fillets (*Thunnus albacares*) from the Maldives. Both were transported on ice (temperature 0 ± 1.5°C). The tuna were caught less than 48 hours before the start of the study. The salmon were gutted/eviscerated four days and filleted one day before delivery. Both species of fish were processed as fillets (tuna 2-4 kg; salmon 1800 ± 200 g). The tuna was vacuum packed in the Maldives whereas the salmon was transported under ambient atmosphere. According to the importer, the tuna had a shelf-life of 12 days and the salmon a shelf-life of 11 days. Three independent replicates from tuna and eight from salmon (two different farms) were used.

After arriving at the laboratory, the fish fillets were sliced into approximately 200-g slices and packaged individually (Multivac A 300/168; Sepp Haggenmuller KG, Wolfertschwenden, Germany) under vacuum using a high-barrier film 90 µm thick with an oxygen transmission rate of 1 $cm^3 m^{-2}$ 24 h^{-1} atm^{-1} at 23°C and 75% relative humidity (Finnvacum, Östersundom, Finland). In addition, two whole salmon fillets were left unpacked and stored at ambient atmosphere. The

packages were stored at 3°C in the dark. After 0, 2, 6, 9 and 13 days of storage all analyses were performed on each sample except the unpacked samples, which were only analysed on day 6.

2.2. Microbial enumeration

Colony counts of total psychrotrophic bacteria and sulphur reducing bacteria from each individual package at each time of sampling were performed according to the Nordic Committee on Food Analysis (NMKL) 184 methods (Anonymous, 2006), which is a routine method for the determination of the aerobic count and specific spoilage organisms in fish and fish products. Briefly, the determination of psychrotolerant bacteria was performed on Long and Hammer agar by the spread plating method and the plates was incubated aerobically at 15± 1 °C for 5-7 days. Luminous colonies on Long Hammer agar were counted in a dark room. Iron agar was used both to estimate total number of colonies and the hydrogen sulphide producing bacteria (colonies show black color due to precipitation of iron sulphide). The pour plating and incubation at 20-25°C for 72± 6 hours was used for iron agar.

2.3. Sensory evaluation

Sensory analyses were performed by a trained panel consisting of five individuals. The pool of panelists consisted of people experienced in sensory evaluation of fish. For these analyses, the fish samples were equilibrated at room temperature. A sample from the same fillet was stored fresh (day 0) in the freezer (−20°C) and used as a reference. A five-point evaluation scale was used for both odour and appearance (1 = severe defect, spoiled, 2 = clear defect, spoiled, 3 = mild/slight defect, satisfactory, 4 = good, 5 = excellent). The panelists evaluated both categories at the same time and described the observed deficiencies. The sample was considered spoiled when the median of the grades given was 2 or less.

2.4. Physical parameters

The pH of the fish was measured using a pH meter (Inolab 720, WTW, Weilheim, Germany) from Stomacher homogenates with 0.1% peptone saline at a ratio of 1:10 (w/w) after 1 min of homogenisation.

2.5. 16S rRNA amplicon sequencing

2.5.1. DNA extraction

DNA was extracted directly from the fish samples as in Hultman *et al.* (2015), by using 15 ml of the 1:10 homogenate. Briefly, the majority of the meat cells were removed by centrifugation (3 min, 200 rcf, Eppendorf 5810 R, Eppendorf AG, Hamburg, Germany) after which the bacterial cells from the supernatant were collected by a second round of centrifugation (3 min, 10,000 rcf). DNA was extracted from the cell pellets by bead beating a GES-phenol-chloroform extraction with FastPrep Lysing matrix E tubes (MP Biomedicals, Santa Ana, CA, USA). The lysing matrix tubes were bead beaten for 40 s at 5.5 m/s in a FastPrep-24 instrument (MP Biomedicals), after which the tubes were incubated on ice for 5 min and centrifuged for 10 min at 13,000 rcf (Eppendorf, Hamburg, Germany). A volume of 500 µl of chloroform was added to the upper layer and the tubes were centrifuged for 10 min after vortexing. The nucleic acids were precipitated with 1/10 volume of 3 M sodium acetate, 1 µl of glycoblue (Invitrogen, Carlsbad, CA, USA) and 3 × ethanol. The pellets were washed with 70% ethanol and eluted in 50 µl of sterile nuclease-free water.

2.5.2. PCR amplification and 16S rRNA sequencing

PCR amplification of the V3–V4 region of the 16S rRNA gene was performed in two steps. The first round of amplification was done with the primers 341F1-4 and 785R1-4, which contain partial

Illumina TruSeq adapter sequences in the 5′ ends, using Phusion polymerase with GC buffer and 2.5% DMSO (New England Biolab, MA, USA). The cycling conditions were as follows: initial denaturation at 98°C, followed by 15 cycles at 98°C for 10 seconds, 65°C for 30 seconds and 72°C for 10 seconds, and a final extension for 5 minutes at 72°C. The amount of template DNA used was approximately 25 ng. The PCR run included a PCR negative control without template DNA. The PCR products were purified with exonuclease I (Thermo Fisher Scientific, Waltham, MA, USA) and thermosensitive alkaline phosphatase (FastAP; Thermo Fisher Scientific, Waltham, MA, USA). A second PCR round was performed with full-length TruSeq P5 and Index containing P7 adapters. The cycling conditions were identical to the previous ones except that 18 instead of 15 cycles were run. The final PCR products were purified with Agencourt® AMPure® XP magnetic beads (Beckman Coulter, CA, USA), pooled and sequenced on the Illumina MiSeq platform at the Institute of Biotechnology, University of Helsinki, Finland. All 16S rRNA gene sequences have been deposited in the European Nucleotide Archive (accession no. PRJEB25372). For 16S rRNA reads were joined with PEAR (Zhang *et al.*, 2014) with default options and quality trimmed using USEARCH fastq filter command with fastq maxee 1 and fastq minlen 350 parameters. Unique sequences were identified with the UPARSE pipeline (Edgar, 2013) with derep full length command. Operational taxonomic units (OTUs) were clustered, chimeras removed and reads were mapped to reference sequences with the cluster otus command with minsize 2 parameter and usearch global command with id 0.97 parameter. Taxonomic classification of OTUs was done using the classify.seqs command in mothur (Schloss *et al.*, 2009) using the RDP naïve Bayesian Classifier (Wang *et al.*, 2007) against the Silva 128 database (Quast *et al.*, 2013) with classifier cutoff=60.

2.6 ^{1}H-NMR spectroscopic analysis

2.6.1. Metabolomics sample preparation

Lyophilised fish samples were extracted by a chloroform/methanol/water method (Yde *et al.*, 2014). The water-soluble extracts (WSE) were collected, dried and stored at − 80°C until ^{1}H-NMR analysis. On the day of ^{1}H-NMR measurements, the pellet was dissolved in 550 µl D_2O, 25 µl H_2O and 25 µl D_2O containing 0.05% (w/v) sodium trimethylsilyl [2,2,3,3-D4]-1-propionate (TSP) and transferred to a 5 mm NMR tube.

2.6.2. ^{1}H NMR spectroscopy

The NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer (Bruker BioSpin, Gmbh, Rheinstetten, Germany) operating at a frequency of 600.13 MHz for ^{1}H and equipped with a 5 mm ^{1}H TXI probe (Bruker BioSpin, Rheinstetten, Germany). Spectra were obtained using a 1D nuclear Overhauser enhancement spectroscopy (NOESY)-presat pulse sequence (noesypr1d), to suppress water molecule signals, at a target temperature of 298 K. The acquisition parameters used were: 64 scans (NS), spectral width (SW) = 7289 Hz (12.15 ppm), acquisition time (AQ) = 2.25 sec, 32768 data points (TD). The relaxation delay between successive scans were 5 sec. The free induction decays (FIDs) were multiplied by a line-broadening function of 0.3 Hz prior to Fourier transformation.

2.6.3. Processing of ^{1}H NMR data for analysis

The acquired ^{1}H NMR spectra were phase-corrected and baseline-corrected by polynomials using the TopspinTM 3.0 software (Bruker BioSpin, Gmbh, Rheinstetten, Germany). In Matlab (Version R2014, The MathWorks Inc., United States), data were referenced to TSP at 0.0 ppm. Selected

metabolites were quantified using Chenomx NMR Suite 8.1.2 (Chenomx Inc, Edmonton, AB, Canada).

3. Results

3.1. Microbiological analysis, pH and amplicon sequencing

On the day of arrival, total psychrotolerant bacterial counts were between log 3 and log 4, indicating good microbial quality of the fish (Fig. 1). The bacterial counts of VP tuna reached their maximum level on day 9, but only a 0.5 log increase was observed. In VP salmon, the bacterial growth was faster and the highest colony counts (log 7.5) were reached already on day 6. Cultivation results indicated that salmon was more susceptible to bacterial growth than tuna. Compared to salmon with pH 6.1-6.3 (Fig. S1), the lower pH 5.9 of tuna was probably one of the essential factors restricting bacterial growth. In salmon the psychrotopic counts on Long Hammer agar were higher than the total counts in Iron agar.

H_2S-producing bacteria were observed in salmon (Fig. 1) and to some extent (<5%) also in tuna. Bioluminescence was detected in salmon (Fig. 1). There was no difference between psychrotolerant bacterial colony counts in unpacked and in VP salmon. On iron agar, colony counts were 100 times higher in unpacked salmon (log 6) than in those packed under vacuum (log 4), but H_2S producers were at a similar level.

In 16S rRNA amplicon sequencing, a total of 275 different OTUs were obtained. OTUs assigned to *Pseudomonas* spp. and *Lactobacillus* spp. dominated in the communities of tuna (Fig. 2). However, a *Lactobacillus* species was identified in only one tuna sample (Fig. S2). A BLASTN search to

NCBI showed the best hit for those OTUs to be *Lactobacillus algidus* (100% match). In salmon > 97% of OTUs were assigned to *Photobacterium* spp. under VP on and after day 6 and on average 87% under air on day 6. The same OTUs dominated in salmon from different farmers. The main OTUs, which represent 90% of all *Photobacterium* OTUs assigned to unknown species, have been found earlier in meat and Arctic fishes (Chaillou *et al*., 2014; Ward *et al.*, 2009). The same *Pseudomonas* OTUs were found in salmon and tuna. The main OTUs, which represented 96% of all *Pseudomonas* OTUs, were assigned to *Pseudomonas fluorescens*. Results indicate that similar *Photobacterium* and *Pseudomonas* species occured both in tuna and in salmon.

3.2. Sensory analysis

According to the sensory panel, the quality of the tuna declined from good to satisfactory on day 9 and was satisfactory also on the last day analysed (Fig S3). The appearance was described as grey and the odour as marine, muddy and sweet. All salmon samples were considered satisfactory on day 9 and spoiled on day 13 (Fig S3). The appearance of spoiled salmon was described as pale and slimy, and the odour as nauseous, strong fishy and pungent. Unpacked salmon had good sensory-evaluated quality on day 6. Sensory evaluation indicated that the off-odours in tuna could be ascribed to aldehydes and in salmon to amines.

3.3. Metabolomics

A total of 45 WSE extracts from tuna and 90 extracts from salmon muscles were analysed by ^{1}H NMR spectroscopy. A representative spectrum is shown in Figure 3. The spectrum displays approximately 100 resonances and a total of 33 metabolites were assigned and identified (Table 1). The main metabolites (~ 1500 mg/100 g of fish) in both species of fish were creatine and anserine. Lactate concentrations were also high (1500 mg/100g) and this provides a good carbon source for

bacteria that can utilise lactate. In general fish typically contains only low carbohydrate concentrations; we measured average glucose concentrations of 518 and 89 mg/100g in tuna and salmon, respectively. The changes in glucose concentrations during storage (decrease in salmon and increase in tuna) indicate that in salmon glucose may have been used as a carbon source for bacteria.

Inosine´5-monophosphate (IMP) decreased throughout the storage period both in tuna and salmon (Table 1). Inosine content increased clearly (~100 mg/100g) during storage in tuna and similarly hypoxanthine concentrations increased in salmon. This indicates that the main ATP-related compound found at the end of shelf life was inosine in tuna and hypoxanthine in salmon.
Both fish contained high amounts of free amino acids and on-going proteolysis during storage increased the concentrations further (Table 1). Increasing concentrations of alanine, leucine and phenylalanine (> 5 mg/ 100g) were clearly seen. Methionine, tyrosine, valine and isoleucine displayed a modest increase during storage. Taurine concentrations increased (from 65 mg/100g to 105 mg/100g) in salmon and did not change (~ 35 mg/100g) in tuna during storage. Also histidine and threonine concentrations decreased (≥ 8 mg/100g) during storage, which is probably related to bacterial growth. Histidine was the most abundant free amino acid both in tuna and in salmon. Tuna contained ca. 75% more histidine than salmon did, 1770 mg/100g and 1015 mg/100g respectively, but in this study we did not observe decarboxylation to histamine. The levels of methionine and threonine concentrations were also about double in tuna compared to salmon. Valine, leucine, isoleucine, phenylalanine and tyrosine concentrations were basically at similar levels in both fish species. The concentrations of alanine and taurine were threefold higher in salmon compared to tuna.

The levels of the organic acids acetate and succinate were higher in salmon than in tuna. At the beginning of storage, both species of fish contained acetate corresponding to ca. 3 mg/100g. During storage, acetate concentrations remained constant in tuna and increased in salmon to 76 mg/100g. Initially succinate levels were 9 mg/100g and 7 mg/100g in salmon and tuna, respectively, and at the end of storage the concentrations had increased to 35 mg/100g and 13 mg/100g, respectively. Tuna contained noticably higher amounts of lactate than salmon. At the beginning of storage, the levels were 4175 mg/100g in tuna and 1502 mg/100g in salmon, and on the final day of storage the concentrations were 3859 mg/ 100g and 1714 mg/100g, respectively. In tuna, the decrease in lactate concentration may indicate microbial utilisation of lactate.

Trimethylamine N-oxide (TMAO) concentrations were 4 times lower in tuna than in salmon, 23 and 87 mg/100g, respectively, at the beginning of storage and at the same levels 15 and 12 mg/100g, respectively, at the end of the storage period. Both fish species contained very low amounts of trimethylamine (TMA) (≤ 1 mg/100g) at the beginning of storage. In tuna, TMA production was low (concentration at the end of storage 2 mg/100g), whereas TMA production was high in salmon (concentration at the end of storage 33 mg/100g). This finding showed a high rate of TMAO reduction to TMA, which was the most probable reason for off-odour and spoilage. TMA levels in unpacked salmon were two and a half times lower than under VP on day 6 (Table 1).

The formation of biogenic amines was low. The concentration of cadaverine increased from 15 to 23 mg/100g in tuna and 15 to 40 mg/100g in salmon. Tyramine levels did not increase significantly (< 1 mg/100g) and other biogenic amines were not detected.

4. Discussion

Fish and other types of seafood are an important source of protein, nutrients and omega-3 long-chain polyunsaturated fatty acids worldwide. People are recommended to consume about 225 g (8 oz) of seafood a week (*Dietary Guidelines for Americans, 2010).* The world's wild fish stocks are limited and would not be sustainable for this recommendation, especially because around 4-5 million tonnes of trawled shrimp and fish are lost every year due to spoilage (Unklesbay, 1992).

Tuna and salmon are among the ten most consumed species of fish in the world. The two fish species have different characteristics because tuna lives freely in tropical waters and salmon is farmed in colder waters. Still no notable differences were detected in the contents of amino acids except tuna´s higher histidine and salmon´s higher alanine concentrations. Some bacteria, e.g. *Photobacterium phosphoreum* (Gram and Huss, 2000), can decarboxylate free histidine to histamine, which can cause an illness called scombroid fish poisoning, also known as histamine poisoning (Taylor, 1986). In our study, in spite of high histidine concentration, histamine production in tuna was non-detectable, probably due to the domination of *Pseudomonas*, which is a weak histamine producer (Gram and Huss, 2000). Reported histidine concentrations in salmon have varied between 10 to 1000 mg/ 100g (wet wt) concentrations (Belghit *et al.*, 2018; Mente *et al.*, 2003) and it is evident that under certain conditions the development of histamine can also occur in salmon (FAO/WHO 2018). In addition to histamine, other important biogenic amines in fish are tyramine, tryptamine, putrescine and cadaverine (Visciano *et al.*, 2012). Biogenic amines are also related to fish spoilage, since they accumulate as a result of an on-going proteolysis and amino acid decarboxylase activity of microorganisms (Visciano *et al.*, 2012). Fish freshness has been evaluated using more than one single biogenic amine (Veciana-Nogués *et al.,* 1997). In our study,

the freshness evaluation using biogenic amines as indicators was not relevant because of the low accumulation rate detected (Table 1).

In contrast to biogenic amines, TMA levels clearly correlated with sensory evaluation quality in salmon. TMA is the main odourant characteristic of degrading seafood. Spoilage of fish by production of TMA is mainly caused by *Shewanella putrefaciens*, *P. phosphoreum*, *Vibrionaceae spp.*, *Aeromonas spp.* and *Enterobacteriaceae* (Gram and Huss, 2000). In our study, *Photobacterium* was the most abundant genus in salmon and most likely responsible for reducing TMAO to TMA. A TMA content of ≥ 29.5mg/100g is considered to be unacceptable in fish (Shumilina *et al.*, 2016). This level was reached on day 9 in salmon (Table 1); however, the sensory quality was still satisfactory according to our sensory panel (Figure S3). When the TMA levels reached 33 mg/100g on day 13, the panel evaluated the salmon to be spoiled. Similar levels of TMA in salmon have also been described earlier (Sokolová, 2017). In the present study, TMA levels in tuna were low (≤ 2 mg/100g) but higher values (≥ 25 mg/100g) have been reported (Jinadasa *et al.*, 2015). Our results indicate that TMA can be considered a quality indicator for salmon but not for tuna.

Hypoxanthine is formed by the autolytic decomposition of nucleotides and is also produced by bacterial activity. The rate of bacterial hypoxanthine formation is higher than autolysis (Gram and Huss, 1996). Several bacteria, ie. *P. phosphoreum* and *Pseudomonas spp.*, can form hypoxanthine from inosine or IMP. A correlation between hypoxanthine and TMA in many fish species has been reported in previous studies (Gram and Huss, 2000). Also, in our study the hypoxanthine concentration increased faster than TMA concentrations in salmon (Table 1). The increase in hypoxanthine was followed by the increase in TMA, which makes hypoxanthine a potential spoilage reporter of onset of spoilage.

Salmon is commonly sold as unpacked or under VP. We studied unpacked and VP fillets which were packed after 4 days of ambient atmosphere storage on ice when the initial contamination was at the level of < 4 log cfu/g. Regardless of the packaging type of salmon, log 7 cfu/g bacterial levels were reached after 6 days. On day 6 after delivery, TMA levels were higher in VP (15.8 mg/100g) than in unpacked salmon (5.6 mg/100g). The proportion of *Pseudomonas* was higher in unpacked salmon fillets than in vacuum packed ones, 9.3% and 0%, respectively, and *P. phosphoreum* was the dominating species (≥ 89%) under both atmospheres. The finding of higher counts on Long Hammer agar than in iron agar is most likely associated with the higher NaCl concentration, plating methods and lower incubation temperature being more favorable to *P. phosphoreum* (Abgrall and Cleret 1990; Dalgaard *et al.*, 1993; van Spreekens 1974; Dalgaard *et al.*, 1997) The increased CO_2 content under VP was probably an essential inhibitory factor of *Pseudomonas* growth. However, our results show that packaging did not affect spoilage in fish as much as has been described in meat (Ercolini *et al.*, 2011). In earlier studies, *Pseudomonas* spp. was found to be the major specific spoilage organism in aerobically stored salmon (Mikš-Krajnik *et al.*, 2016), while anaerobically *P. phosphoreum* has been the most commonly reported spoilage bacterium (Emborg *et al.*, 2002) causing rapid and strong spoilage (Macé *et al.*, 2013). In our study, the same OTU assigned to *P. fluorescens* dominated in VP tuna, but lost competition to *Photobacterium* in ambient and VP stored salmon. Although the same OTU may indicate the same species, it must be noted that the genus *Pseudomonas* spp. contains high heterogeneity and biodiversity within its species (Tryfinopoulou *et al.*, 2002), which could explain the different fitness properties and sensitivity to CO_2. Even if *P. fluorescens* has high spoilage activity (Liu *et al.*, 2018), in our study the growth in tuna was slow and the spoilage potential low. Similarly, Silbande *et al.* (2018) reported that *Pseudomonas* has no spoilage effect in tuna. However, they noticed that *Pseudomonas* combined with *Hafnia paralvei* deteriorated the sensory quality of tuna. In our study the proportion of

Enterobacteriaceae was relatively low, ≤ 6%, (Figure 2). Amplicon sequencing method is a well-established technique for studying bacterial diversities. But DNA extraction and PCR amplification may have an effect on the bacterial community profiles obtained since especially the proportitions of enterobacteria have been suggested to remain underestimated (Ercolini *et al.*, 2011).

Lactic acid bacteria (LAB) are frequently isolated from VP and MAP fish, as well as lightly preserved seafood (Franzetti *et al.* 2003; Silbande *et al.*, 2016). In tuna LAB together with *Enterobacteriaceae* and *Brochothrix thermosphacta* were detected dominating at the sensory rejection time (Silbande *et al.*, 2016). In our study *Lactobacillus* was the dominanting group (> 80%) with *Pseudomonas* (> 9%), *Enterobacteriaceae* (>5 %) and *Brochothrix* (> 1%) in one tuna fillet at the end of storage. *Pseudomonas* dominated in the other tuna fillets during storage. On the delivery day bacterial diversity was quite wide *Pseudomonas* and *Shewanella* forming the major groups. (Figure 2). Shewanella is an important fish SSO (Herbert *et al.*, 1971; Ge *et al.*, 2016; Gram *et al.*, 1987). In our study *Shewanella* strains were not competitive against *Pseudomonas* in tuna under VP.

The concentration of carbohydrates was higher in tuna than in salmon (Table 1). However, the concentrations did not decrease in tuna, thus indicating a limited consumption for bacterial growth. In fact, the preferred carbon sources for the dominating genus in tuna, *Pseudomonas*, are organic acids or amino acids, rather than glucose (Rojo, 2010). In salmon, the concentration of carbohydrates decreased (Table 1). The dominating genus, *Photobacterium*, produced several different organic acids during carbohydrate fermentation. The increase in organic acids in salmon was clearly seen in our study, whereas it was not evident in tuna (Table 1). The production of

organic acids decreased the pH of salmon fillets, but the higher initial lactate concentration in tuna retained the pH of tuna at a significantly lower level (Fig. S1).

In meat, glucose, lactic acid, and certain amino acids are the precursors for metabolites connected to spoilage, and the level and accessibility of the precursors affect the rate and extent of spoilage (Nychas *et al.*, 1998 and 2008). In our study, TMAO and glucose were the main precursors for salmon spoilage. Glucose concentration in salmon (Vargas-Chacoff *et al.*, 2016) is about half that of glucose concentrations in meat (Meinert *et al.*, 2008; Meinert *et al.*, 2009). No distinct metabolites causing quality deterioation for tuna were recognised, but lactate and free amino acids were most probably growth facilitators for bacteria. Bacterial counts and sensory evaluation showed that salmon was clearly a more perishable fish than tuna.

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Table 1. Metabolites quantitated by ^{1}H NMR spectroscopy under vacuum packaged (VP) or ambient (A) atmosphere stored (3 °C)salmon and tuna. The values (mg/ 100g) represent the means of the 3 tuna, 6 salmon under vacuum and 2 salmon in an ambient atmosphere. The maximum dispersion between replicates was 30%.

	Tuna (VP) day					Salmon (VP) day					Salmon (A) day
Metabolite	0	2	6	9	13	0	2	6	9	13	6
Amino acids											
Alanine	56,1	68,6	59,8	65,5	63,2	136,8	179,5	167,3	210,5	206,9	154,1
Anserine	3124,4	3263,9	3268,3	3177,3	3202,9	1866,5	1969,4	1814,6	2103,9	1867,0	1818,0
Creatine	2077,0	1956,7	1980,2	1986,6	1965,5	1370,3	1597,1	1431,8	1658,5	1515,8	1358,0
Glutamate	19,0	13,9	15,8	20,3	19,0	62,9	61,6	53,9	63,5	36,4	62,1
Glycine	11,7	10,7	11,6	13,2	13,1	96,3	116,5	106,2	118,2	111,3	90,1
Histidine	1742,3	1825,6	1785,9	1775,6	1734,4	1001,3	1049,3	956,1	1093,9	988,1	944,1
Isoleucine	19,6	20,4	17,2	21,2	20,3	15,2	18,0	16,0	18,4	18,7	15,2
Leucine	34,4	39,5	34,9	41,1	40,5	28,3	32,1	30,5	37,5	43,8	26,6
Methionine	15,4	16,2	15,1	18,0	18,9	7,4	8,0	7,9	9,5	10,4	8,3
Phenylalanine	9,1	13,0	12,5	13,0	14,0	6,7	7,9	9,1	13,8	19,2	8,0
Taurine	39,3	33,4	34,2	36,6	33,1	64,5	51,8	72,5	121,0	103,3	61,7
Threonine	36,0	33,3	34,4	35,5	33,9	15,5	17,3	19,6	20,4	18,8	15,9
Tyrosine	7,9	8,6	9,2	10,2	9,0	6,5	9,3	9,1	9,2	10,5	13,5
Valine	35,3	36,7	33,3	37,9	37,3	24,7	29,3	29,2	35,7	42,7	25,6
Organic acids											
3-Hydroxybutyrate	32,3	29,6	31,9	31,9	31,0	13,0	14,6	14,4	15,6	14,9	13,6
Acetate	3,3	3,4	3,5	3,5	3,6	3,4	4,1	14,8	44,3	76,8	14,2
Fumarate	4,5	6,0	4,8	5,3	5,1	0,4	0,6	0,8	0,6	0,3	0,4
Lactate	4175,0	3906,4	4011,7	3882,9	3858,8	1502,4	1746,9	1621,9	1809,9	1714,5	1506,0

Succinate	6,6	17,8	8,7	11,6	12,9	9,5	12,7	11,6	26,0	35,9	3,0
Carbohydrates											
Glucose	402,8	517,3	509,0	582,0	576,8	95,8	131,2	79,3	55,1	83,6	29,6
Glucose-6-phosphate	517,6	626,2	572,1	657,6	643,3	296,9	405,1	434,2	377,4	239,8	265,9
Nucleotides											
ATP	30,9	62,5	46,3	58,6	28,9	17,4	20,1	22,1	19,4	18,3	29,6
Hypoxanthine	89,9	67,0	68,6	86,9	87,9	15,3	21,9	40,1	82,6	117,3	40,4
IMP	582,7	506,3	431,1	366,0	327,5	377,6	449,7	256,1	160,9	72,1	258,5
Inosine	258,7	243,1	365,3	338,7	357,4	202,1	241,7	288,9	318,2	233,1	277,0
Amines											
Cadaverine	15,2	18	15,4	23,5	22,8	15,6	22,6	20,2	31,4	40,6	19,2
Dimethylamine	21,2	21,3	21,7	22,0	21,3	12,4	13,2	11,8	13,8	12,9	12,0
Methylamine	1,0	0,9	0,8	1,0	1,0	1,0	0,6	0,5	0,5	0,5	0,5
Trimethylamine	1,5	1,6	1,9	2,1	2,8	0,4	0,5	14,6	30,8	33,1	5,6
Trimethylamine N-oxide	23,7	17,5	13,3	17,2	15,9	87,0	72,7	42,2	21,3	12,7	57,2
Tyramine	11,0	11,2	8,8	10,2	11,3	3,5	3,7	3,7	3,7	4,1	4,2
Choline	12,3	13,1	13,0	13,2	12,8	15,4	15,5	15,9	18,2	18,6	16,2
Uracil	15,0	13,9	14,5	16,2	14,0	6,1	7,6	6,8	8,2	7,7	6,8

Figure captions

Figure 1. Bacterial counts and growth curves: psychrotrophic (red square), bioluminescent (black circle), total (blue triangle) and sulphur producing bacteria (green cross) in Atlantic salmon from two farms (A, B) and Yellowfin tuna (C) stored under vacuum at 3°C determined by plating. Data reported are mean values from three replicates, error bars represent standard deviation.

Figure 2. The distribution of bacterial OTUs in products from two farms of salmon (A, B) and tuna (C) stored at 3°C under vacuum, unless otherwise stated, based on 16S rRNA amplicon sequencing. Depending on the diversity of the 16S rRNA gene sequences, classification results are shown either at the genus or family level. Data are expressed as the mean of 2–3 replicates, except ambient atmosphere in day 6, which represents only one. Average percentages above 5% are plotted.

Figure 3. Representative 1 H NMR spectrum of a tuna (A) and salmon (B) sample (day 13) with numbers indicating assignments.

1: lactic acid

2: creatine

3: succinate

4: glucose

5: inosine

6: anserine

7: histidine-containing compound

8: hypoxanthine

9: formate

10: inosine 5' monophosphate (IMP)

11: niacinamide

12: guanine

13: phenylalanine

14: methionine

15: alanine

16: trimethylamine (TMA)

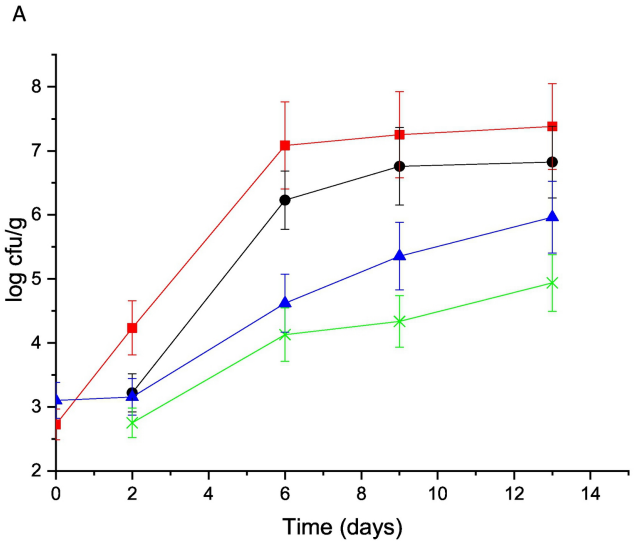
17: trimethylamine N-oxide (TMAO)

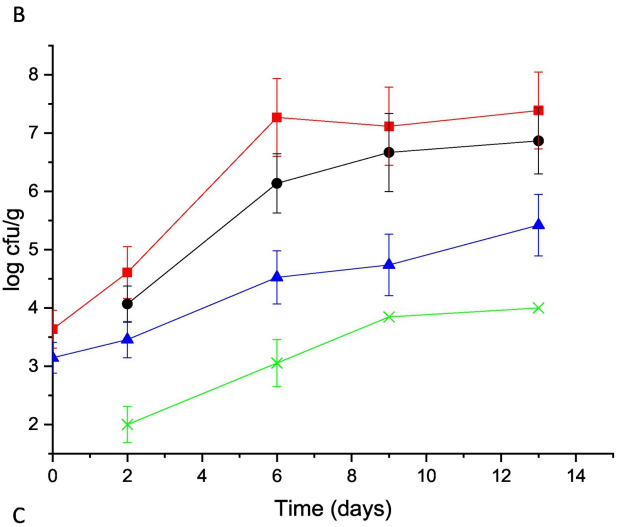
18: acetate

19: glycine

Highlights:

- *Pseudomonas* spp. was the dominating genus in tuna under vacuum.
- *Photobacterium* spp. was the dominating genus in salmon under vacuum.
- Lactate and amino acids were important carbon sources for bacteria in tuna.
- Glucose and TMAO were the precursors of spoilage metabolites in salmon.
- TMA correlated with sensory evaluation quality in salmon.





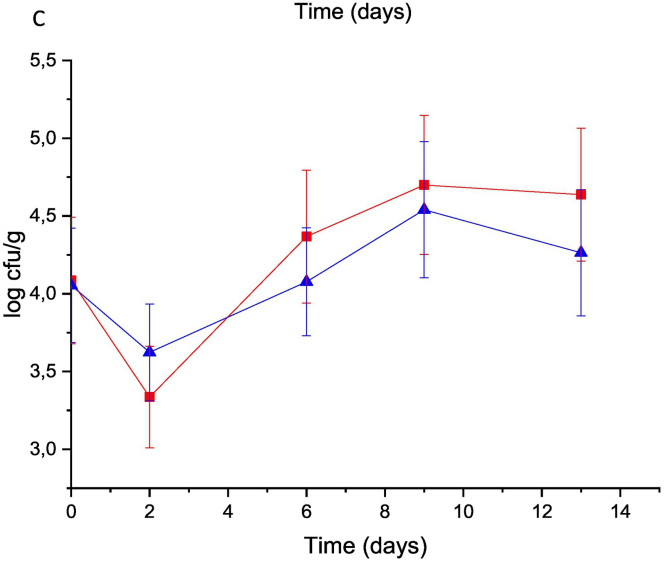
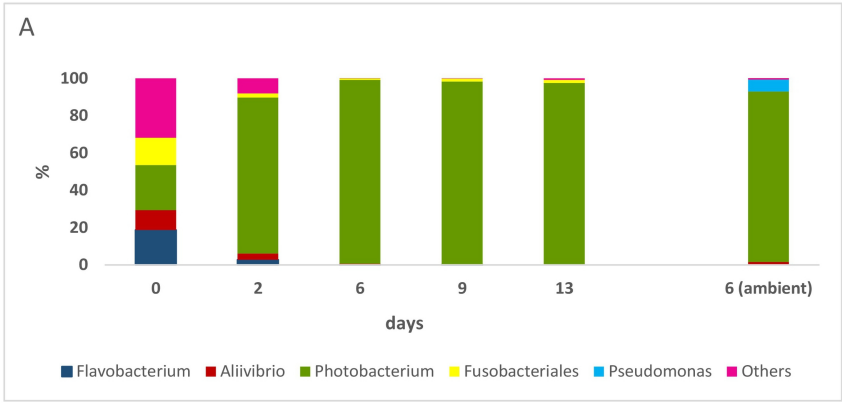


Figure 1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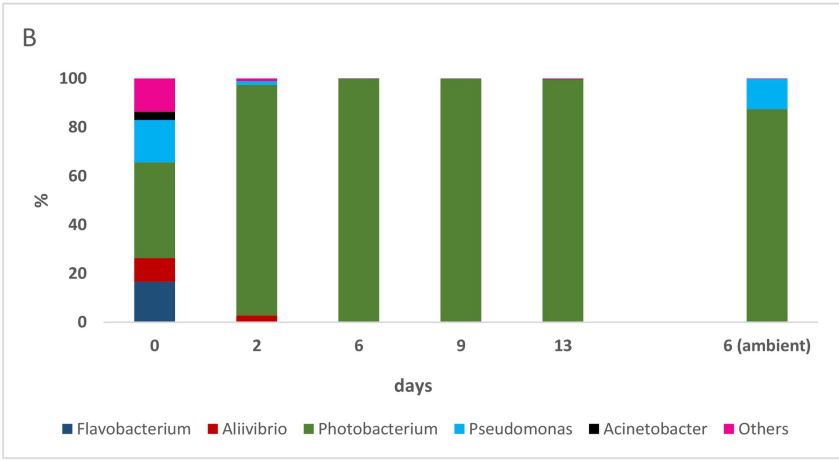
Flavobacterium
Aliivibrio
Photobacterium
Fusobacteriales
Pseudomonas
Others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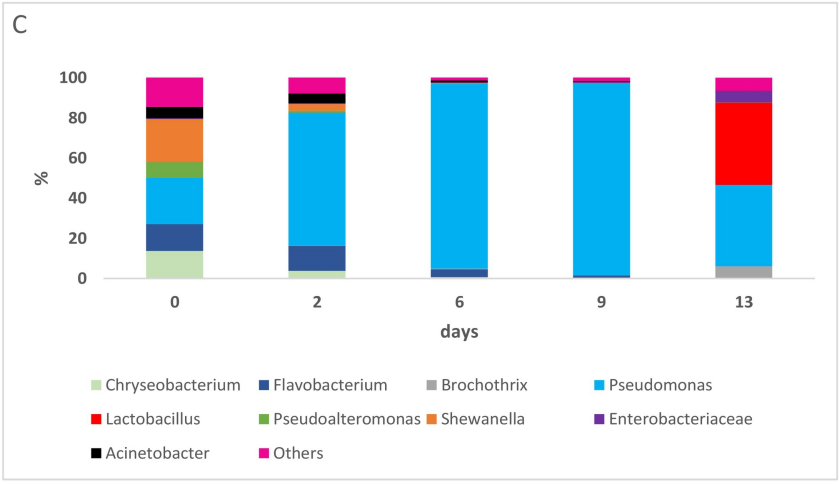
Photobacterium
Pseudomonas
Acinetobacter
Others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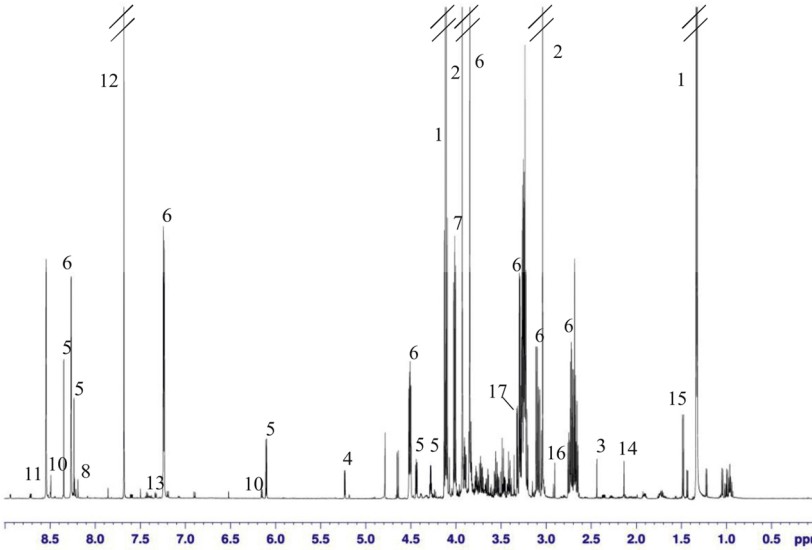
Lactobacillus
Pseudoalteromonas
Shewanella
Enterobacteriaceae
Acinetobacter
Others

Figure 2

A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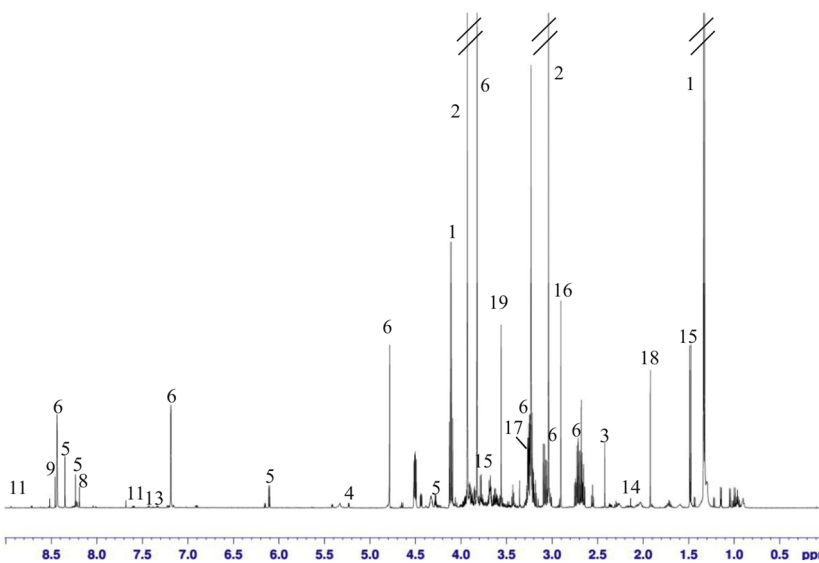
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

B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18
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8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

Figure 3