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Dynamics of bacterial communities and interaction networks in thawed fish fillets during chilled storage in air

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ABSTRACT

Thawed hake (*Merluccius capensis* and *M. paradoxus*) and plaice (*Pleuronectes platessa*) fillets were used as a model to evaluate the effect of storage temperature (0 or 10 °C) and biological variability (fish species, lot to lot) on bacterial growth kinetics and microbial successions. Both culture dependent methods (plate counts on non-selective and selective media) and culture independent methods (qPCR and 16S rRNA gene metabarcoding) were used. Bacterial counts exceeded 10^7 cfu/g within 2–3 days at 10 °C and 7–8 days at 0 °C. Plate counts on three media (Plate Count Agar +0.5% NaCl, Iron Agar Lyngby and *Pseudomonas* Selective medium) and 16S rRNA gene counts estimated by qPCR were highly correlated. Growth was modelled using the D-model and specific growth rate ranged between 0.97 and 1.24 d^{-1} and 3.54 and 5.90 d^{-1} at 0 and 10 °C, respectively. The initial composition of the microbiota showed lot-to-lot variation, but significant differences between the two fish species were detected. Alpha diversity significantly decreased during storage. When bacterial counts exceeded 10^7 cfu/g, the microbiota was dominated by members of the genera *Pseudomonas*, *Psychrobacter*, *Acinetobacter*, *Serratia*, *Flavobacterium*, *Acinetobacter*, *Carnobacterium*, *Brochothrix* and *Vagococcus*. However, *Photobacterium* and *Shewanella*, two genera frequently associated with fish spoilage, were either absent or minor components of the microbiota. As expected, storage temperature significantly affected the abundance of several species. The inference of microbial association networks with three different approaches (an ensemble approach using the CoNet app, Sparse Correlations for Compositional data, and SParse InversE Covariance Estimation for Ecological Association Inference) allowed the detection of both a core microbiota, which was present throughout storage, and a number of taxa, which became dominant at the end of spoilage and were characterized by a disproportionate amount of negative interactions.

1. Introduction

Fish and seafood are economically important food commodities. Total fish and seafood production in the European Union reached 6.45×10^6 metric tons in 2017, 3% of the world production (EUMOFA, 2017). EU self-sufficiency for fish and seafood was 46% in 2015, and a large amount of the apparent food consumption in the EU (12.8×10^6 tons) came from imports (EUMOFA, 2017), often in the form of frozen products. Because of their high a_W and pH close to neutrality, fresh fish and seafood products are highly perishable and a variety of methods are used for their short- and long-term preservation (Kasuga, 2010). The spoilage dynamics of chilled fish and seafood products has been reviewed (Gram and Dalgaard, 2002). The initial contamination is extremely variable and depends on several factors, including the species, the feeding habits, and the area of capture. Further contamination occurs during fishing, storage, transport and processing (Kasuga, 2010). During storage at temperatures allowing bacterial growth (chilled or super-chilled storage in air or modified atmosphere packaging, MAP), a limited number of species prevail and make up the Specific Spoilage Association/Specific Spoilage Organism, which is ultimately responsible for the production of the sensory changes which lead to spoilage (Gram and Huss, 1996; Gram and

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Dalgaard, 2002). Knowledge on the evolution of the microbiota of fish and sea food during storage is of primary importance for the understanding of the microbial ecology of these products and for rationally developing new methods for preservation (Gram and Dalgaard, 2002).

Both culture-dependent and -independent methods have shown that psychrotrophic members of the genera *Pseudomonas* and *Shewanella* prevail during chilled storage of fish products in air. However, these genera may be replaced by *Photobacterium*, *Brochothrix* and lactic acid bacteria (LAB) in products stored in MAP or in vacuum (Calliauw et al., 2016; Dalgaard, 1995a; Hovda et al., 2007a, 2007b; Macé et al., 2012; Parlapani et al., 2013, 2018; Powell and Tamplin, 2012; Reynisson et al., 2009, 2010; Rudi et al., 2004). Several other genera have been associated with the dominant microbiota of spoiling or spoiled fish, such as *Acinetobacter* (Calliauw et al., 2016), *Aeromonas* (Parlapani et al., 2013), *Chryseobacterium* (Reynisson et al., 2009), *Flavobacterium* (Reynisson et al., 2009, 2010), *Lactococcus* (Macé et al., 2012), *Pseudoalteromonas* (Broekaert et al., 2011), *Psychrobacter* (Parlapani et al., 2018; Reynisson et al., 2010), *Serratia* (Tong Thi et al., 2013), *Streptococcus* (Rudi et al., 2004), and *Vagococcus* (Calliauw et al., 2016). However, their spoilage potential is not always clear.

Due to its high-throughput and sensitivity, 16S rRNA gene metabarcoding has become the method of choice for the study of the bacterial communities in foods (De Filippis et al., 2018). However, to date, relatively few studies have used this technique for the characterization of the bacterial spoilage of fish and seafood (Chaillou et al., 2015; Jia et al., 2018; Kuuliala et al., 2018; Parlapani et al., 2018). Even if they are often descriptive in nature and are based on a limited number of samples, these studies confirm that the initial microbiota may be affected by the area of fishing (Parlapani et al., 2018), that an initially complex microbiota is replaced by a simplified community dominated by a few species at spoilage (Chaillou et al., 2015; Kuuliala et al., 2018; Parlapani et al., 2018), and that both storage temperature (Kuuliala et al., 2018; Parlapani et al., 2018) and MAP (Kuuliala et al., 2018) strongly affect the composition of the microbiota of spoiled products. Finally, microbial interactions during food spoilage are critical in shaping the microbiota (Gram et al., 2002; Parente et al., 2018) and spoilage dynamics may change due to antagonistic, mutualistic or commensalistic relationships among the members of the microbiota (Gram and Dalgaard, 2002; Joffraud et al., 2006; Jørgensen et al., 2000), but microbial association networks in fish and fish products have never been studied.

In this study, we used thawed hake (*Merluccius capensis* and *M. paradoxus*) and plaice (*Pleuronectes platessa*) fillets as a model to evaluate the contribution of the variability in the initial microbiota and the effect of storage temperature on the dynamics of microbial communities in fish, using both culture-dependent and –independent techniques. In addition, we used three different approaches to infer microbial association networks during chilled storage, as an additional tool to identify taxa or groups of taxa which play a significant role in spoilage and their interactions.

2. Materials and methods

2.1. Sampling and experimental design

Packages of frozen fish fillets (Findus, Italy) where bought from local supermarkets. Two different products were used: hake fillets (South African hake - *Merluccius capensis*, and deep-water Cape hake - *M. paradoxus*) from Southern Atlantic, FAO fishing zone 47-02D (Hake, H); plaice fillets (European plaice - *Pleuronectes platessa*) from North-Eastern Atlantic/North Sea, FAO fishing zone 27-16W (Plaice, P). Three lots for each product were used in three experimental runs. For each run, the content of fish packages was aseptically transferred to a sterile Whirlpak bag and thawed by immersion in a shaking water bath at 30 °C. Thawing was usually completed in < 30 min. and temperature of samples never exceeded 0 °C. The content from 4 to 5 packages was aseptically mixed, 10 g pieces were placed in sterile Whirlpak bags and incubated at 0 (melting ice) or 10 °C in air (mild abuse temperature). At the beginning of storage and at selected time-points (1–4 days at 10 °C and 1–11 days at 0 °C), four random bags for each temperature were removed from incubation. pH was measured using a spear-tip electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a pH-meter (Orion 420A plus, Thermo Fisher Scientific, Rodano, Italy) in two bags. Sterile diluent (peptone saline, Bacteriological peptone 1 g/L, NaCl 8.5 g/L) was immediately added to two other bags to obtain a 10^{-1} dilution. After homogenization (1 min., Stomacher 400 Lab Blender, International PBI, Milan, Italy) in sterile strainer bags, further decimal dilutions were performed in the same diluent, and 20 mL of the strained homogenate were used for DNA extraction (see below).

Bacterial counts were performed by spiral plating (WASP Spiral Plater, bioMérieux Italia SpA, Bagno a Ripoli, Firenze, Italy) using PCA + 0.5% NaCl (PCA), *Pseudomonas* agar base with CFC supplement (Pseudomonas Selective Medium, PSM), Iron Agar Lyngby (IAL) supplemented with L-cysteine (both incubated at 25 °C for 48 h) and TCBS Cholera medium (TCBS, 35 °C, 48 h). Colonies were enumerated using a digital colony counter (EasyCount 2, bioMérieux Italia). Growth was modelled using the D-model (Baranyi and Roberts, 1994). DmFit 3.5 for Excel (https://browser.combase.cc/DMFit3_5.zip) was used to estimate the parameters of the growth curve. Data from two replicate plates and two technical replicates were averaged.

2.2. DNA extraction, quantification of 16S rRNA gene, and 16S rRNA gene metabarcoding

Strained homogenates from two technical replicates for each sampling point were mixed and centrifuged at 12,000 × *g* for 5 min at 4 °C (MicroCL 17R centrifuge, Thermo Scientific, Wilmington, DE). The pellets were washed twice with STE buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 8; 12,000 × *g*, 5 min, 4 °C) and stored at −80 °C until nucleic acid extraction. Pellets were thawed at 0 °C and resuspended in 450 µL of the PowerFood Microbial DNA isolation kit (QIAGEN Srl, Milan, Italy) lysis buffer. The initial lysis step was performed in a FastPrep24 instrument (MP Biomedicals SARL, Illkirch, France), with five 60 s cycles at 6.5 m/s, separated by a cooling step in ice for 5 min. Lysis and purification were performed following the manufacturer's instructions. Genomic DNA was eluted in 75 µL for all samples and stored at −80 °C until used. The quality and quantity of DNA was measured by using a NanoDrop®1000c spectrophotometer (Thermo Scientific).

The V3-V4 region of the 16S rRNA gene was amplified using recently described primers and conditions (Berni Canani et al., 2017). Library multiplexing, pooling and sequencing were carried out following the Illumina 16S Metagenomic Sequencing Library Preparation protocol on a MiSeq platform with the MiSeq Reagent kit v3. Sequences were deposited in the NCBI Sequence Read Archive (SRP155010). Genomic DNA from a mock community (ZymoBIOMICS Microbial Community Standard, ZYMO Research, Irvine, California, USA) was sequenced as a control.

Quantitation of the 16S rRNA gene was performed by qPCR using the Femto™ Bacterial DNA Quantification Kit (ZYMO Research), following the manufacturer's instructions. Results were expressed in both ng/µL and log(copies)/µL using *E. coli* strain JM109 DNA as a standard.

2.3. Bioinformatics analysis

Demultiplexed fastq files were processed with R 3.5.0 (R Core Team, 2018) and Amplicon Sequence Variants inference was carried out with a pipeline based on DADA2 (Callahan et al., 2016). Briefly, after filtering and trimming, error rate estimation, and dereplication, forward and reverse sequences were concatenated, obtaining sequences of 397 bp. After bimera removal, the sequence table was used to assign taxonomy using the RDP train set v16. Phylogenetic trees were inferred

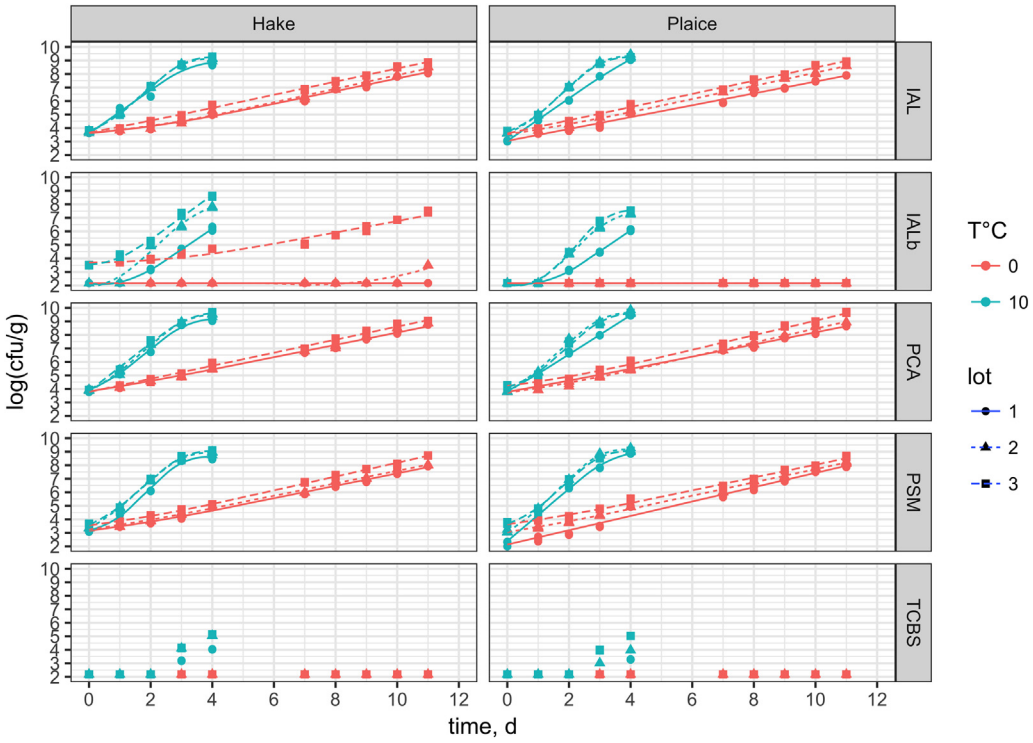

Fig. 1. Evolution of microbial counts in thawed fish fillets (hake or plaice) stored in air at 0 or 10 °C. Counts were carried out by spiral plating on Iron Agar Lyngby (the results for both total colonies, IAL, and black colonies, IALb, are shown), PCA + 0.5% NaCl (PCA), *Pseudomonas* Selective Medium (PSM) and TCBS cholera medium. Each point is the mean of two technical replicates. Continuous lines show fitted values for the D-model (Barany and Roberts, 1994).

using the phangorn package after alignment with the DECIPHER package as described in Callahan et al. (2016). OTU table, taxonomic table, sample table and phylogenetic tree were combined in a phyloseq class object (McMurdie and Holmes, 2013), which was used for further analysis. Since the use of concatenation of forward and reverse sequences and the use of RDP prevented the identification of any ASV at the species level, the latter was carried out by matching species identifications obtained by using the Greengenes 13.8 train set whenever both RDP and Greengenes resulted in the same identification at the genus level, by extracting a FASTA file with the ASVs for which a species identification was found, and using it with blastn (Altschul et al., 1990) against the 16S ribosomal RNA sequences (*Archea* and *Bacteria*) database. Only the species identification which matched with those with the highest score obtained by blastn were retained. ASVs were then aggregated at the lowest taxonomic identification possible (i.e. when species identification was not available aggregation was at the genus level, etc.).

2.4. Statistical analysis

Statistical and graphical analysis of the data was performed using R version 3.5.0 (R Core Team, 2018). Modelling of growth curves was performed as described above. The effect of temperature and fish species (as fixed factors) on specific growth rate and initial contamination was evaluated using a mixed model with package lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017), using the combination of lot and product as a random factor. Alpha-diversity analysis, ordination (constrained canonical coordinate analysis) and generation of bar plots were performed using functions of package phyloseq (McMurdie and Holmes, 2013). Ordination was performed with function ordinate() using both metric (Principal Coordinate Axis, PCoA) and non-metric multidimensional scaling and with constrained Canonical Coordinate Analysis (using temperature, time and fish species as a factor and lot as a condition). Ordination was performed using either weighted UniFrac distance on phyloseq objects (after prevalence and abundance filtering but prior to taxonomic agglomeration) or Bray-Curtis distance (distance was calculated using absolute abundance with Wisconsin double standardization and square root transformation) on data after taxonomic agglomeration and prevalence and abundance filtering. The effect of fish species, temperature and time on the composition of the microbiota was evaluated using package DESeq2 1.20 (Love et al., 2014). In addition, some graphical analyses were performed after transforming relative abundances of taxa in 16S gene counts using the data obtained from qPCR.

Microbial association network inference was performed using the methods described in Parente et al. (2018).

2.5. Chemicals and media

Unless otherwise noted all chemicals were obtained from Sigma-Aldrich (now Merck, Milan, Italy) and all media were obtained from Oxoid (Thermo Fisher Scientific, Rodano, Italia).

3. Results and discussion

3.1. Evolution of pH and bacterial counts during storage

The initial pH of hake fillets was 6.81 ± 0.03 while it was significantly more variable (6.92 ± 0.15) in plaice. The time course of pH during storage at 0 and 10 °C is shown in Supplementary Fig. 1. The time-course for pH was quite similar for the three lots of hake, but it was different for plaice, where, although the three lots showed a similar trend, differences in initial pH still resulted in differences of 0.1–0.2 pH units at the end of storage at 0 °C. The initial pH decrease at 0 °C (0.2 pH units for hake, 0.1 for plaice, within 6 days) may be due to post-mortem changes related to rigor mortis, the rapid increase in pH at 10 °C or the slower increase occurring at 0 °C after 6 days might be due to the production of ammonia and volatile bases due to autolysis and subsequent proteolysis (Gram and Huss, 1996; Gram and Dalgaard, 2002).

The evolution of microbial counts is shown in Fig. 1. Total counts on IAL, PCA or PSM showed similar trends with some lot-to-lot variation, which was more evident for plaice. Counts were systematically higher on PCA, and lower on PSM. However, counts on the three media were highly correlated (Pearson's product moment coefficient r between 0.93

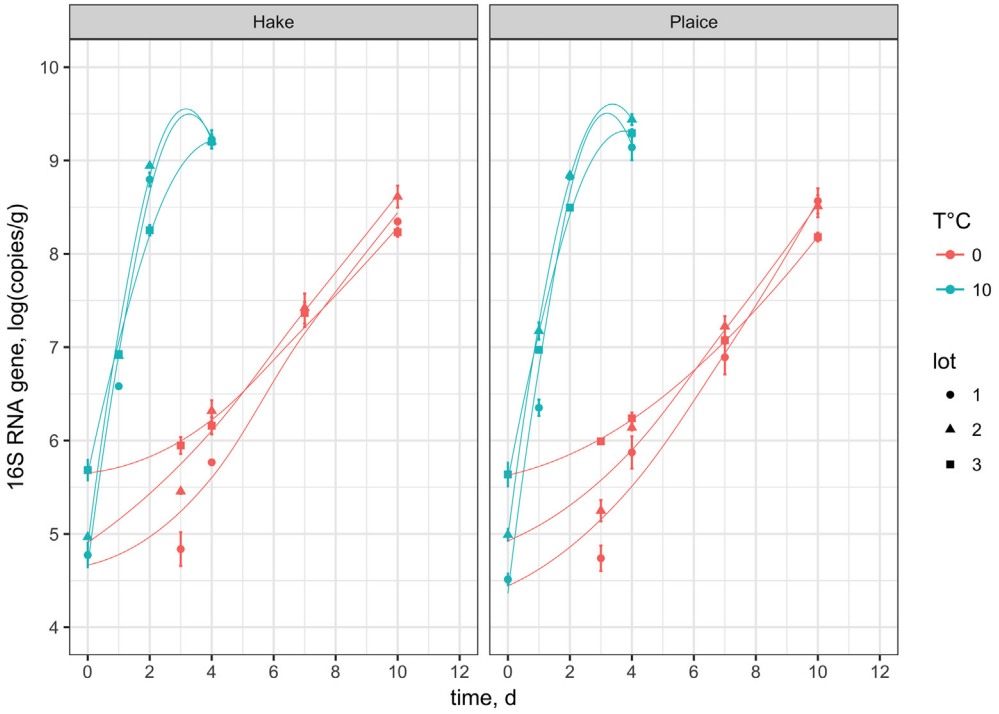

Fig. 2. Evolution of log(copies)/g of the 16S rRNA gene (estimated by qPCR) in thawed fish fillets stored in air at 0 and 10 °C. The continuous trend lines were obtained using a loess smoother. Means of 3 replicates with standard deviation bars are shown.

and 0.99; see Supplementary Fig. 2). The analysis of linear models with log(cfu/g) on PCA, fish species and temperature as independent variables and log(cfu/g) on either PCM or IAL as dependent variables showed that both product (hake vs. plaice) and temperature significantly affected the relationship (although product × temperature interaction was not significant). This is probably due to an indirect effect, since both product and temperature affect the composition of the microbiota (see below), and specific taxa may show different ability to grow on agar media (including PCA, IAL and PSM; Broekaert et al., 2011).

To obtain a cultivation independent estimate of contamination and growth, quantitative PCR was used to estimate the number of copies of the 16S rRNA gene on DNA extracted from selected samples. The results are shown in Fig. 2. Pearson's r correlation between gene counts and colony counts was between 0.92 and 0.95 and 16S rRNA gene counts were approximately 5 times higher compared to average colony counts. This is not surprising, due to the presence of non-cultivable or dead bacteria, as well as of multiple copies (usually > 4) of the 16S rRNA gene (Větrovský and Baldrian, 2013).

Initial contamination was relatively low, ranging between 3.8–4.2, 2.2–3.7 and 3.04–3.80 log(cfu/g) for PCA, PSM and IAL, respectively. Plate counts and 16S rRNA gene counts at day 0 were significantly ($p < 0.01$) different among different lots.

Black (H_2S producing) colonies were always observed on IAL at 10 °C, with large lot-to-lot variation for hake. H_2S producing colonies were always below the detection level for plaice at 0 °C, while their number steadily increased in lot three of hake.

Counts on TCBS were always below detection limit at 0–2 days and at 0 °C during storage, but increased to 4–5 log(cfu/g) after 4 days at 10 °C. The low counts for *Vibrio* are in agreement with the absence or very low abundance of this genus as shown by 16S rRNA gene metabarcoding.

Counts higher than 7 log(cfu/g) on PCA were obtained after 2 days at 10 °C and > 7–8 days at 10 °C. More than 10^6 cfu/g of H_2S producing bacteria were usually found after 3–4 days (depending on initial contamination) at 10 °C, but only one lot of hake showed high counts of H_2S producing bacteria after 9 days at 0 °C.

We did not investigate the sensory quality of the fillets during spoilage and therefore we cannot establish a relationship between sensory quality and microbial counts. Counts reported in the literature for spoiled ice stored fish are highly variable and range from values as low as 10^6 cfu/g to 10^9 cfu/g, due to the different spoilage potential of SSO members and to the perception threshold of relevant chemical spoilage markers (Dalgaard, 1995a; Hovda et al., 2007a, 2007b; Kuuliala et al., 2018; Macé et al., 2012, 2013; Parlapani et al., 2013).

Recently, three class sampling plans for heterotrophic plate counts of fish have been proposed (Svanevik et al., 2015) and the values for m (5×10^5 cfu/g) and M (5.5×10^6 cfu/g) are close to those proposed by the ICMSF (ICMSF, 1986; m = 5×10^5 cfu/g, M = 1×10^7 cfu/g). By judging from total count on IAL or PCA, the products were microbiologically unacceptable (counts higher than 5–10 × 10^6 cfu/g) within 2–3 days at 10 °C and 8–9 days at 0 °C.

3.2. Modelling bacterial growth in fish fillets

The fit of the D model (Baranyi and Roberts, 1994) is shown as continuous lines in Fig. 1, while estimated values for the lag and maximum specific growth rates (μ_{max}) are shown in Table 1. Estimated lag times were usually lower at 10 °C than at 0 °C but the quality of estimates was poor (as showed by large standard errors), probably due to the low number of experimental points during the lag phase. μ_{max} values were approximately 4 times higher at 10 °C compared to 0 °C on all media.

A mixed effect model with product and temperature nested within lot was used to test the null hypothesis that maximum specific growth rate on each media (PCA, PSM, IAL) was not affected by the fish species nor the temperature. As expected, the null hypothesis that temperature did not have a significant effect was rejected ($p < 1{\cdot}10^{-6}$), while the effect of fish species was never significant.

The media used in this study have no selectivity (PCA or IAL) or selectivity for members of a single genus (Pseudomonas Selective Medium), and therefore the growth curves reflect the growth of a

Table 1
Estimates and standard errors of the duration of the lag phase and of the maximum specific growth rate (μ_{max}) for thawed fish fillets (hake and plaice) stored in air at 0 and 10 °C. Growth curves were obtained with counts on Iron Agar Lingby (total colonies IALtot; black colonies IALb), PCA + 0.5% NaCl (PCA) and *Pseudomonas* Selective medium (PSM).

			Storage temperature					
			0 °C			10 °C		
Product	Lot	Medium	lag (d)	μ_{max} (d^{-1})	R^2	lag (d)	μ_{max} (d^{-1})	R^2
Hake	1	IALb	–	–	–	1.4 ± 0.1	3.54 ± 0.08	0.99
Hake	2	IALb	–	–	–	0.8 ± 0.2	4.85 ± 0.56	0.98
Hake	3	IALb	2.7 ± 1.3	0.97 ± 0.29	0.96	0.9 ± 0.1	3.80 ± 0.16	0.99
Hake	1	IALtot	1.4 ± 0.3	1.08 ± 0.03	0.99	–	3.67 ± 0.10	0.99
Hake	2	IALtot	1.5 ± 0.3	1.16 ± 0.03	0.99	0.3 ± 0.1	4.30 ± 0.08	0.99
Hake	3	IALtot	0.3 ± 0.2	1.12 ± 0.03	0.99	0.3 ± 0.1	4.36 ± 0.15	0.99
Hake	1	PCA	0.5 ± 0.3	1.05 ± 0.03	0.99	0.2 ± 0.1	3.78 ± 0.13	0.99
Hake	2	PCA	1.2 ± 0.2	1.16 ± 0.02	0.99	0.2 ± 0.1	4.25 ± 0.19	0.99
Hake	3	PCA	–	1.11 ± 0.01	0.99	–	3.97 ± 0.12	0.99
Hake	1	PSM	0.8 ± 0.3	1.06 ± 0.03	0.99	0.6 ± 0.1	4.94 ± 0.18	0.99
Hake	2	PSM	0.6 ± 0.2	1.06 ± 0.03	0.99	0.1 ± 0.1	4.24 ± 0.12	0.99
Hake	3	PSM	1.0 ± 0.2	1.18 ± 0.02	0.99	0.3 ± 0.1	4.26 ± 0.20	0.99
Plaice	1	IALb	–	–	–	1.5 ± 0.1	3.54 ± 0.07	0.99
Plaice	2	IALb	–	–	–	1.0 ± 0.1	5.10 ± 0.29	0.99
Plaice	3	IALb	–	–	–	1.1 ± 0.1	5.90 ± 0.23	0.99
Plaice	1	IALtot		1.01 ± 0.02	0.99	–	3.77 ± 0.05	0.99
Plaice	2	IALtot	0.6 ± 0.2	1.12 ± 0.02	0.99	0.3 ± 0.0	4.67 ± 0.08	0.99
Plaice	3	IALtot		1.12 ± 0.01	0.99	0.4 ± 0.1	4.55 ± 0.09	0.99
Plaice	1	PCA	0.2 ± 0.3	1.04 ± 0.03	0.99	0.3 ± 0.1	3.69 ± 0.09	0.99
Plaice	2	PCA	0.9 ± 0.2	1.19 ± 0.02	0.99	–	4.18 ± 0.13	0.99
Plaice	3	PCA	1.1 ± 0.3	1.24 ± 0.03	0.99	0.4 ± 0.2	4.41 ± 0.33	0.99
Plaice	1	PSM		1.22 ± 0.03	0.98	–	4.15 ± 0.09	0.99
Plaice	2	PSM	0.6 ± 0.2	1.13 ± 0.03	0.99	0.2 ± 0.0	4.85 ± 0.09	0.99
Plaice	3	PSM	0.8 ± 0.3	1.09 ± 0.04	0.99	0.4 ± 0.1	4.43 ± 0.18	0.99

consortium of bacteria. The growth of fish spoilage microorganisms including *Photobacterium*, *Pseudomonas* and *Shewanella* (Dalgaard, 1995b; Dalgaard et al., 1997; Koutsoumanis and Nychas, 2000) has been modelled successfully. The values for μ_{max} obtained in our study for counts on PCA, counts on PSM and for black colonies on IAL are generally in good agreement with those obtained on the same medium for both fish stored in air by Taoukis et al. (1999) and those reported by Koutsoumanis and Nychas (2000) on PSM for gilthead sea-bream.

3.3. Microbial community analysis by 16S rRNA gene metabarcoding

A total of 2,421,247 raw reads were obtained by Illumina MiSeq sequencing of the 48 fish and two mock community samples. From 17% to 56% sequences were lost after filtering and chimera removal. Excluding the mock community, 2854 Amplicon Sequence Variants (ASV) were inferred, belonging to 17 phyla, 28 classes, 60 orders, 133 families and 318 genera. The pipeline used in this study was able to satisfactorily describe the composition of the mock community (data not shown). After filtering low prevalence (occurring in < 5% of the samples) and low abundance ASVs (occurring at a maximum relative abundance < 0.1%) 67% of the ASVs (representing 1.3% of total sequences) were removed. ASVs were aggregated at the lowest taxonomic identification level possible (i.e. when species identification was not available aggregation was at the genus level, etc.). This resulted in 479 different taxa.

Alpha diversity estimates were obtained after taxonomic aggregation. Chao1 and, to a lesser extent, Shannon indices decreased at the end of storage, and the decrease was faster at 10 °C (Supplementary Fig. 3). Again, lot to lot variation was evident at day 0. Reduction of diversity during spoilage or fermentation is common (Chaillou et al., 2015; De Filippis et al., 2018; Parente et al., 2016) and reflects the change from the complex microbial community of fresh raw foods to the less complex communities occurring of foods in which a specific fermentation or spoilage process has taken place. A similar pattern was found in other studies using 16S metabarcoding approaches for fish (Chaillou et al., 2015; Kuuliala et al., 2018), although other authors reported little change over time (Parlapani et al., 2018).

After prevalence and abundance filtering, 67 out of 479 taxa were retained, which represented 96.4% of the original sequences. Most taxa belonged to 4 phyla only (*Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*).

The relative abundance of taxa belonging to different classes is shown in Supplementary Fig. 4, while the abundance of the eleven most abundant genera is shown in Fig. 3.

Gammaproteobacteria (the most abundant class in almost all samples and at the end of storage), *Betaproteobacteria*, *Bacilli* and *Actinobacteria* were present in all samples, while *Flavobacteriia*, *Alphaproteobacteria* and *Clostridia* had lower abundance and prevalence. The three most abundant genera were *Pseudomonas* (12.7% of total sequences), *Psychrobacter* (7.9%) and *Acinetobacter* (6.9%), followed by *Janthinobacterium* (4.2%), *Serratia* (2.3%), *Carnobacterium* (1.8%), *Brochothrix* (1.6%), *Chryseobacterium* (1.4%), *Vagococcus* (1.2%) and *Arthrobacter* (1.0%). Other genera, including some which have been often associated with fish (including plaice, Broekaert et al., 2011) and seafood spoilage, like *Photobacterium* (0.7%) and *Shewanella* (0.2%), were less abundant and prevalent. These results are in excellent agreement with those obtained using both cultivation dependent and independent techniques for chilled stored fish. Members of the genus *Pseudomonas* are frequently found both at the beginning of shelf life and as dominant members of the SSO for products stored in ice (Broekaert et al., 2011; Chaillou et al., 2015; Hovda et al., 2007a; Kuuliala et al., 2018; Parlapani et al., 2013, 2018; Pimentel et al., 2017; Reynisson et al., 2009).

Members of the genus *Psychrobacter* are frequent in fish stored at near zero temperature and are sometimes dominant at the moment of spoilage (Broekaert et al., 2011; Kuuliala et al., 2018; Parlapani et al., 2018; Reynisson et al., 2010; Svanevik and Lunestad, 2011; Wilson et al., 2008).

Acinetobacter has been associated with fish microbiota, both at the beginning and at the end of refrigerated storage (Broekaert et al., 2011;

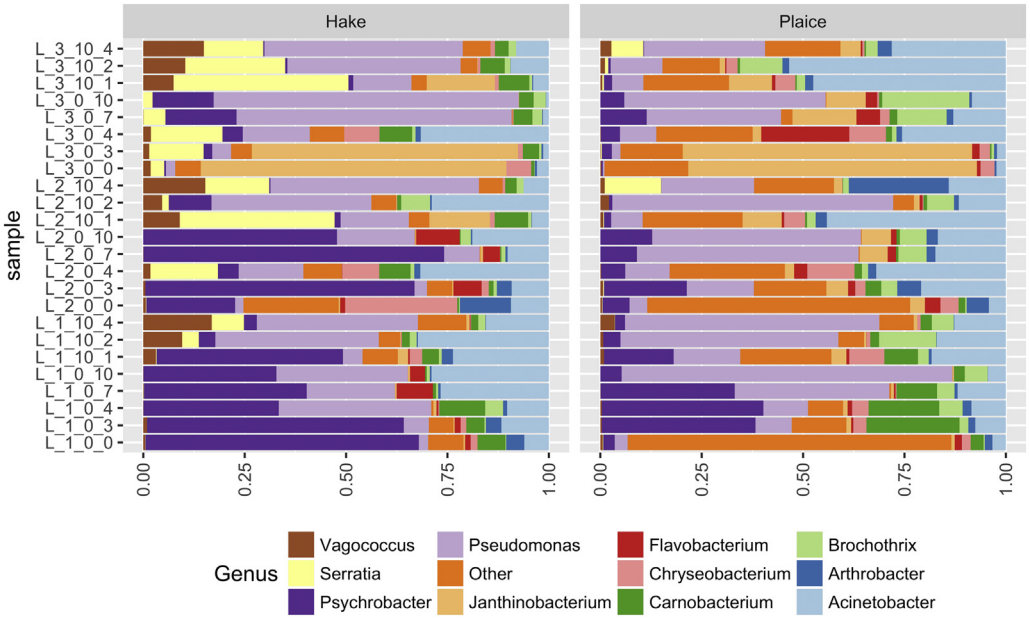

Fig. 3. Relative abundance of the top eleven most abundant genera (all the other genera are pooled and showed as "Other") in thawed fish fillets (hake or plaice) stored in air at 0 or 10 °C. Coding of samples is as follows: L_x_y_z, where x is the lot number, y the temperature and z the time of storage in days.

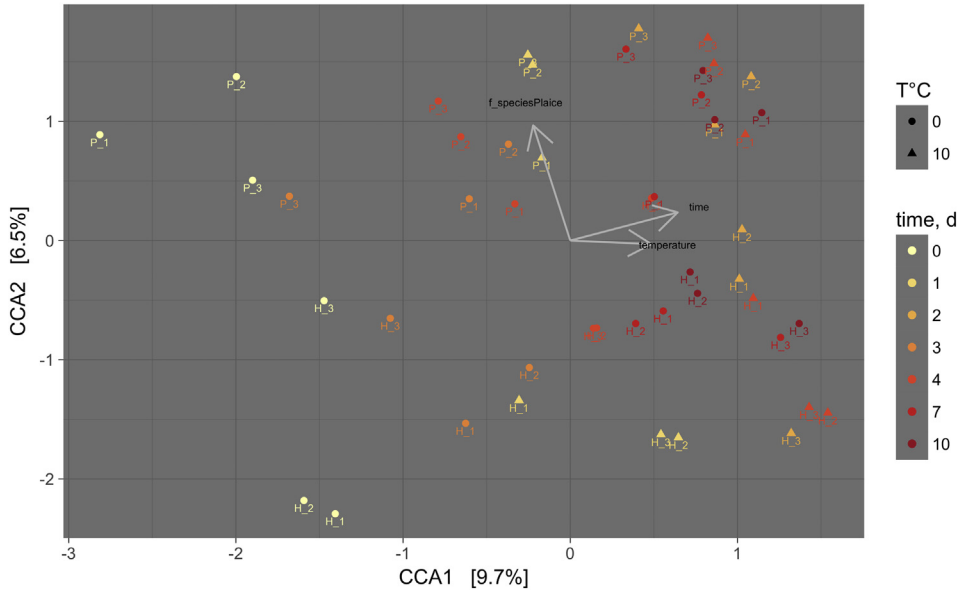

Fig. 4. Distance relationships (Bray-Curtis) among samples of thawed fish fillets (hake or plaice) stored in air at 0 or 10 °C. The ordination was obtained using constrained Canonical Coordinate Analysis. Only the first two constrained axes are shown.

Kuuliala et al., 2018; Parlapani et al., 2013; Tong Thi et al., 2013). *Serratia*, as other *Enterobacteriaceae,* occurs as a member of the microbiota of salmon (Chaillou et al., 2015; Macé et al., 2013), sea bass (Pimentel et al., 2017) or pangasium (Tong Thi et al., 2013). *Brochothrix*, *Carnobacterium* and *Vagococcus* are more frequently associated to fish stored in MAP (Chaillou et al., 2015; Kuuliala et al., 2018; Macé et al., 2013; Powell and Tamplin, 2012). The occurrence of *Chryseobacterium* and *Flavobacterium* as relatively abundant members of the microbiota especially at the beginning of storage is not infrequent (Chaillou et al., 2015; Kuuliala et al., 2018; Reynisson et al., 2009, 2010). *Actinomycetales* like *Arthrobacter* are often found as members of the initial microbiota of fish (Chaillou et al., 2015; Parlapani et al., 2018).

The low abundance of *Photobacterium*, both at the beginning and at the end of storage may be due to the sensitivity of members of this genus to freezing (Guldager et al., 1998) and to the higher competitiveness of other genera during storage in air (Gram and Dalgaard, 2002), but the reasons for the low prevalence and abundance of *Shewanella* are not clear. *Janthinobacterium* is a genus that has been associated with both freshwaters and fresh products of both vegetable and animal origin. Occurrence of *J. lividum*, even with relatively high prevalence, is not infrequent in fish (Broekaert et al., 2011; Powell and Tamplin, 2012; Reynisson et al., 2009).

Although lot-to-lot variation, especially in the composition of the bacterial community at time 0, is evident, a few genera showed an easily detectable pattern. *Acinetobacter* and *Vagococcus* were more abundant in samples stored at 10 °C. Although *Pseudomonas* was invariably present, with increasing abundance during storage both at 0 and 10 °C, its abundance showed high lot-to-lot variability. On the other hand, *Psychrobacter* was clearly more abundant in samples stored at

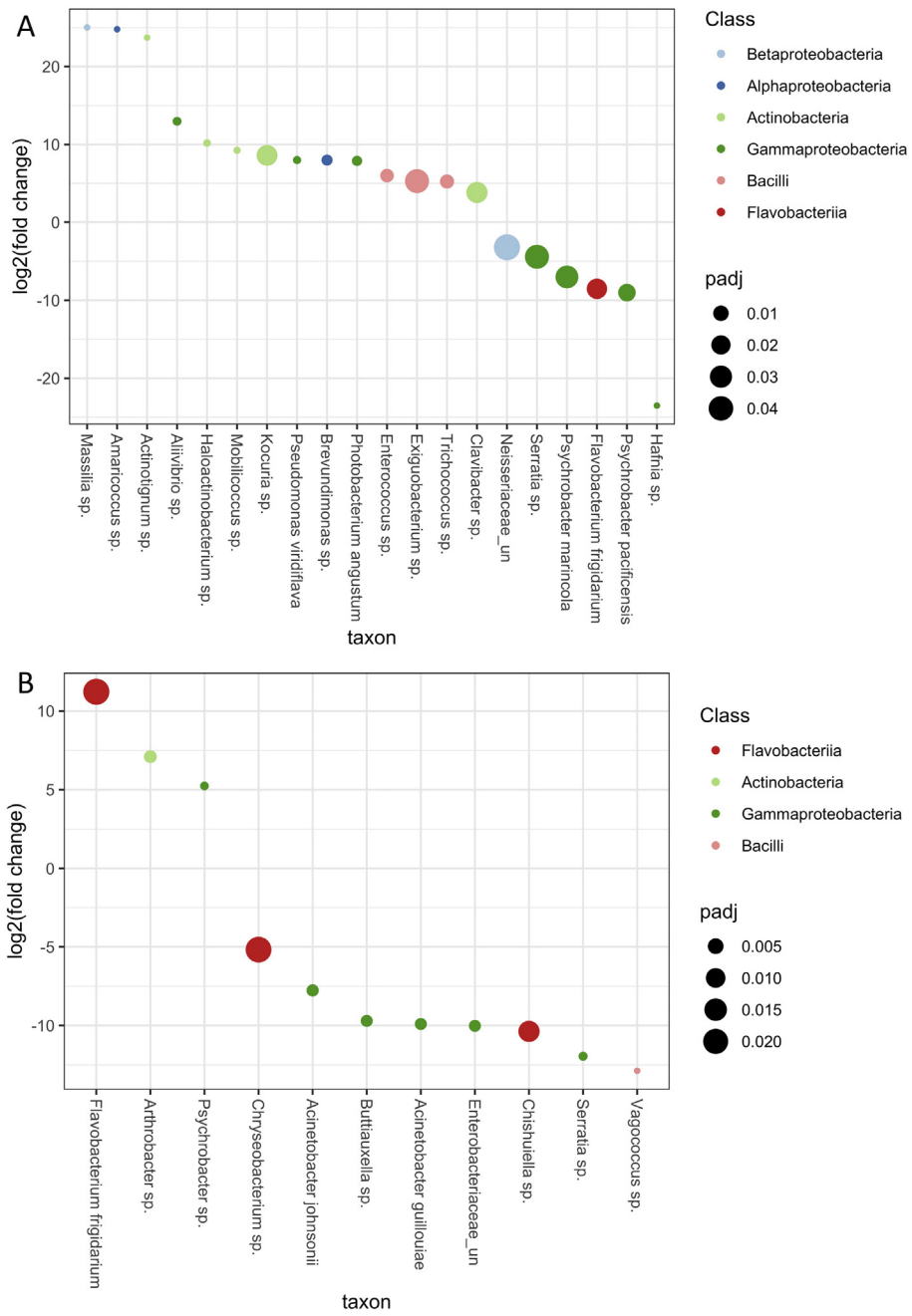

Fig. 5. Taxa showing significantly (adjusted probability < 0.05) different abundances in an experiment in which thawed fish fillets (hake or plaice) were stored in air at 0 or 10 °C. The size of symbols is made proportional to the adjusted probability (p_{adj}) for the Wald test for the null hypothesis of 0 log(fold change). A: Plaice vs. Hake, time 0; B: 0 (10 days) vs. 10 °C (4 days).

0 °C. *Serratia* was present more occasionally and was more prevalent in hake compared to plaice. *Janthinobacterium* was especially abundant at days 0 and 3 in one lot (3) for both hake and plaice.

Beta diversity was analysed using both descriptive and inferential methods. First, an attempt to ordination was carried out using the matrix of Bray-Curtis distance with metric (PCoA) or non-metric multidimensional scaling (NMDS). Ordination was poor with no clear pattern related to fish species, temperature or duration of storage (data not shown). Similar results were obtained using weighted UniFrac distance (data not shown). This may be due to the large lot-to-lot variation that masked any other pattern. Therefore, a constrained Canonical Coordinate Analysis was carried out. The results are shown in Fig. 4. The constrained ordination explained only 21.8% of the variance. However, all three factors (temperature, time and fish species) had a significant effect as shown by PERMANOVA. The first axis was associated to time and temperature, while the second axis was associated to fish species.

Differential analysis of sequence count data was then performed. Since the experimental design was unbalanced, different subsets of data were extracted and analysed to evaluate which taxa were significantly affected by fish species, temperature or time.

Firstly, data at day 0 were extracted to evaluate the effect of fish species on the composition of initial microbiota. The results are shown in Fig. 5A. Several taxa (20) showed differential abundances as a function of fish species, with 14 being systematically more abundant in plaice compared with hake, with $\log_2$(fold change) from 3.8 to 25.0, and 6 being systematically less abundant in plaice, with $\log_2$(fold change) from −3.2 to −23.5.

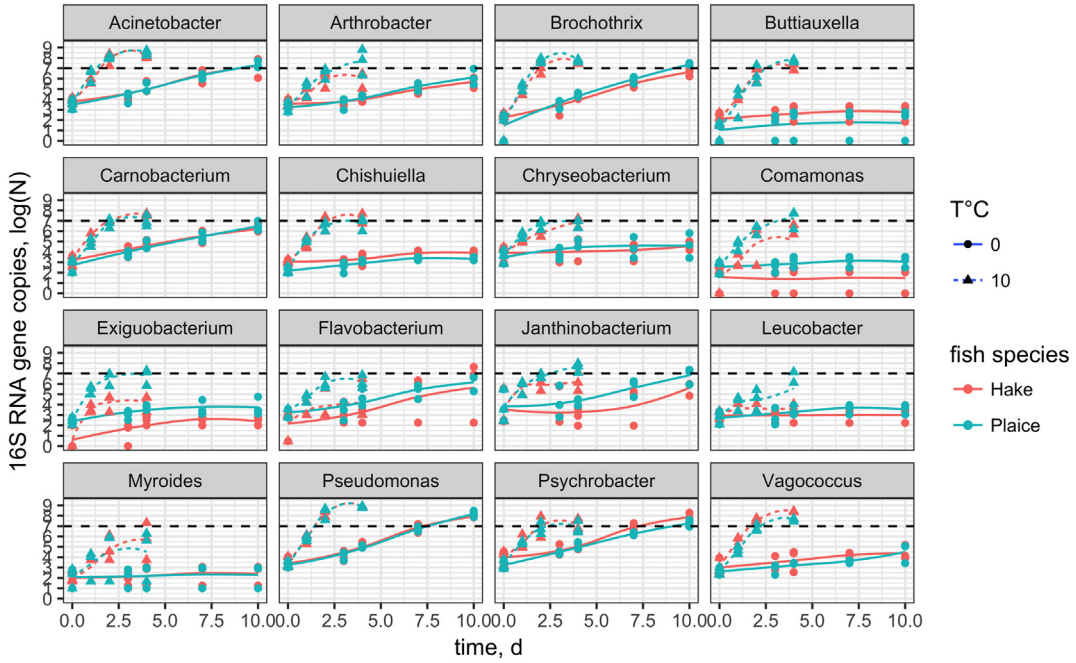

Fig. 6. Estimated evolution of 16S rRNA gene copies for selected bacterial taxa in thawed fish fillets (hake or plaice) stored in air at 0 or 10 °C. The horizontal dashed line shows an arbitrary threshold for bacteriological/quality spoilage while the continuous lines were obtained using a loess smoother (pooling together the results from three different lots).

Several authors have detected large differences in the initial contamination pattern of the flesh or surfaces of fish as affected by fish species and geographical location. Geographical differences among different catches of the same species have been found both by low-throughput (Powell and Tamplin, 2012; Tong Thi et al., 2013; Wilson et al., 2008) and high-throughput methods (Parlapani et al., 2018; Pimentel et al., 2017), and it has been suggested that 16S meta-barcoding can be used for traceability of fish products (Parlapani et al., 2018; Pimentel et al., 2017). Using a high number of samples analysed by culture independent methods (tRFLP, 16S clone libraries) and inferential analyses (linear discriminant analysis) (Wilson et al., 2008) found that the bacterial communities from fish mucus of Atlantic cod caught in three zones of the Northern Atlantic could be discriminated and that the communities of fish caught in North Sea showed significant variation over time. Significant pack to pack and lot to lot variation was found using tRFLP and clone libraries in Atlantic salmon steaks (Powell and Tamplin, 2012). Differences in the composition of the microbiota of flesh of farmed gilt head sea bream from two geographically different areas of the Mediterranean Sea were found by 16S meta-barcoding and descriptive/explorative statistical methods (Parlapani et al., 2018); initial differences in composition resulted in different SSO prevailing at time of spoilage even when the same temperature was used in storage. An approach combining DGGE and 16S meta-barcoding for the study of the bacterial communities of skin mucus of farmed sea bass hinted that fish coming from different farms can be traced with high spatial resolution (Pimentel et al., 2017). Finally, differences have been found in the contamination pattern of fish and surfaces for the same fish (Fogarty et al., 2019; Reynisson et al., 2010).

In our work, fish species, geographic location and processing plant were confounded, and it is therefore impossible to determine which is the cause of the differential abundances in Fig. 5A. Evaluating presence/absence by the use of Venn diagrams may only give a partial idea of the differences in microbiota. In fact, only eight of the taxa listed in Fig. 5A were present in plaice but not in hake (*Actinotignum* sp., *Aliivibrio* sp., *Amaricoccus* sp., *Haloactinobacterium* sp., *Kocuria* sp., *Massilia* sp., *Mobilicoccus* sp., *Pseudomonas viridiflava*).

The effect of temperature (independent of fish species) was tested by comparing the microbial communities after 10 days at 0 °C or 4 days at 10 °C, because they showed similar colony counts and could represent a similar moment in the spoilage history. Fish species was nested within lot. The results are shown in Fig. 5B. *Psychrobacter* sp., *Arthrobacter* sp. and *Flavobacterium frigidarium* showed a significantly higher relative abundance at 0 °C, while two species belonging to the genus *Acinetobacter*, two *Flavobacteriia* (*Chryseobacterium* and *Chishuiella*), *Enterobacteriaceae* (*Serratia*, *Buttiauxella*) and *Vagococcus* sp. showed lower abundance. Several authors have investigated, using culture dependent or independent methods, the effect of storage temperature on the composition of microbial communities in fish during preservation, and our results are in good agreement with published studies. In Atlantic cod fillets stored in MAP at 4 or 8 °C (Kuuliala et al., 2018), even if *Photobacterium* dominated the spoilage microbiota under all conditions, *Acinetobacter* and *Brochothrix* were more abundant at 8 °C. *Psychrobacter* and *Flavobacterium* are the dominating members of the microbiota of gutted haddock stored in ice (Reynisson et al., 2010). In sea bream from the Ionian Sea (Parlapani et al., 2018) the abundance of *Psychrobacter* was higher at lower temperature (2 °C), while *Carnobacterium* increased at higher temperature. We found that some *Enterobacteriaceae*, including *Buttiauxella*, and *Serratia*, were more abundant at 10 °C compared to 0 °C. *Buttiauxella* has occasionally been found in fish (Macé et al., 2012). *Serratia* was found at higher abundance is sea bream samples stored at 4 °C or 8 °C, and was able to spoil salmon at 8 °C (Macé et al., 2012, 2013) and halibut stored in MAP at 4 °C (Hovda et al., 2007b). Members of the genus *Chishuiella* (*Flavobacteriaceae*) were relatively rare in our study (0.7% of total sequences) but, in both hake and plaice, their abundance was higher at 10 °C. They have been isolated from freshwater (Zhang et al., 2014), were reported to grow in the range 10–33 °C and are members of the microbiota of the silver carp (Jia et al., 2018). *Chryseobacterium* has been frequently found in fish (Chaillou et al., 2015; Kuuliala et al., 2018; Reynisson et al., 2009, 2010), but we are not aware of data on the differential effect of temperature on the abundance of this species. In our study, its relative abundance decreased over time at 0 °C and, to a lesser extent at 10 °C, and this may explain an indirect effect of temperature on its abundance. The same was true for *Arthrobacter*, a well-known member of the core

Table 2
Microbial interactions detected by an ensemble of three methods (CoNet, SparCC and SPIEC-EASI, see text for details) in the bacterial communities of thawed fish fillets (hake and plaice) stored at 0 or 10 °C.

Interaction	Interaction type
Acinetobacter guillouiae ⟷ *Acinetobacter johnsonii*	Copresence
Brochothrix sp. ⟷ *Pseudomonas* sp.	Copresence
Buttiauxella sp. ⟷ *Vagococcus* sp.	Copresence
Carnobacterium sp. ⟷ *Psychrobacter pulmonis*	Copresence
Chishuiella sp. ⟷ *Vagococcus* sp.	Copresence
Chryseobacterium sp. ⟷ *Soonwooa* sp.	Copresence
Clavibacter sp. ⟷ *Corynebacterium* sp.	Copresence
Clavibacter sp. ⟷ *Leuconostoc mesenteroides*	Copresence
Clavibacter sp. ⟷ *Rothia nasimurium*	Copresence
Comamonas sp. ⟷ *Pseudomonas viridiflava*	Copresence
Corynebacterium sp. ⟷ *Gallicola* sp.	Copresence
Corynebacterium sp. ⟷ *Leucobacter* sp.	Copresence
Corynebacterium sp. ⟷ *Rhodococcus* sp.	Copresence
Corynebacterium sp. ⟷ *Rothia nasimurium*	Copresence
Empedobacter sp. ⟷ *Soonwooa* sp.	Copresence
Enterococcus sp. ⟷ *Exiguobacterium* sp.	Copresence
Epilithonimonas sp. ⟷ *Myroides odoratimimus*	Copresence
Exiguobacterium sp. ⟷ *Lactococcus* sp.	Copresence
Exiguobacterium sp. ⟷ *Macrococcus* sp.	Copresence
Flavobacterium frigidarium ⟷ *Psychrobacter marincola*	Copresence
Hafnia sp. ⟷ *Serratia* sp.	Copresence
Kurthia sp. ⟷ *Pseudomonas viridiflava*	Copresence
Pseudomonas fragi ⟷ *Pseudomonas* sp.	Copresence
Pseudomonas umsongensis ⟷ *Pseudomonas veronii*	Copresence
Psychrobacter marincola ⟷ *Psychrobacter pacificensis*	Copresence
Psychrobacter marincola ⟷ *Psychrobacter* sp.	Copresence
Streptococcus sp. ⟷ *Trichococcus* sp.	Copresence
Acinetobacter guillouiae ⟷ *Aliivibrio* sp.	Mutual exclusion
Acinetobacter guillouiae ⟷ *Photobacterium angustum*	Mutual exclusion
Acinetobacter johnsonii ⟷ *Psychrobacter marincola*	Mutual exclusion
Acinetobacter johnsonii ⟷ *Psychrobacter* sp.	Mutual exclusion
Arthrobacter psychrolactophilus ⟷ *Vagococcus* sp.	Mutual exclusion
Buttiauxella sp. ⟷ *Flavobacterium* sp.	Mutual exclusion
Clavibacter sp. ⟷ *Kurthia* sp.	Mutual exclusion
Clavibacter sp. ⟷ *Yersinia* sp.	Mutual exclusion
Comamonas sp. ⟷ *Hafnia* sp.	Mutual exclusion
Exiguobacterium sp. ⟷ *Psychrobacter pacificensis*	Mutual exclusion
Flavobacterium sp. ⟷ *Serratia* sp.	Mutual exclusion
Flavobacterium succinicans ⟷ *Vagococcus sp.*	Mutual exclusion
Leuconostoc mesenteroides ⟷ *Pseudomonas veronii*	Mutual exclusion
Pseudomonas fragi ⟷ *Rhodococcus* sp.	Mutual exclusion
Pseudomonas viridiflava ⟷ *Psychrobacter* sp.	Mutual exclusion
Rothia nasimurium ⟷ *Shewanella* sp.	Mutual exclusion

microbiota of fish (Chaillou et al., 2015). A systematic increase of relative abundance at 10 °C was found for *Vagococcus*, a pathogenic and spoilage microorganism (Calliauw et al., 2016; Macé et al., 2012; Powell and Tamplin, 2012; Svanevik and Lunestad, 2011; Wallbanks et al., 1990).

3.4. Visualizing the growth kinetics of individual members of the microbiota

In this work both data on relative abundance of taxa and 16S gene counts data estimated by qPCR were available. The former suffers from the usual biases of sequence count data: the relative abundances of individual OTUs are not independent and the meaning of the absence of a given taxon is uncertain (Layeghifard et al., 2017). Correlation between gene counts and bacterial counts may be complicated by lack of cultivability and by the large variability in the number of 16S gene copies/cell in different taxa (Větrovský and Baldrian, 2013).

Even with this in mind we used the relative abundance data and the qPCR data to obtain an estimate of the kinetics of growth of different taxa. When at any given time $t_i > 0$ the abundance of a taxon was 0, while its abundance at t_{i-1} was larger than 0, the value at time t_{i-1} was used. The results for selected genera (chosen on the basis of their high relative abundance or because their relative abundance was affected by time, temperature or fish species) are shown in Fig. 6. As shown before, lot-to-lot variation is evident for several genera. Some genera showed little or no increase in estimated gene copies at 0 °C, and this is in agreement with their differential response to temperature (see for example *Vagococcus*, *Buttiauxella* and *Chishuiella*). Only some genera were able to grow to an estimated number of gene copies $> 10^7$ at one or both of the storage temperature. *Acinetobacter*, *Arthrobacter*, *Brochothrix*, *Carnobacterium*, all of which are known member of psychrotrophic spoilage communities of fish and seafood, grew to high numbers at 10 °C but were unable to grow over 10^7 copies at 0 °C, except in a few cases. On the other hand, both *Pseudomonas* and *Psychrobacter* were able to grow to $> 10^7$ est. gene copies/g within 2 days at 10 °C and 7–10 days at 0 °C. Due to the low number of data points at each temperature we were unable to model the growth curves for estimated 16S gene counts. qPCR and relative abundance data estimated by 16S metabarcoding have also been combined by other authors (Cauchie et al., 2017; Fougy et al., 2016; Rouger et al., 2018). Those who used genus or species-specific qPCR for selected organisms (Cauchie et al., 2017; Fougy et al., 2016; Rouger et al., 2018), found a good agreement between quantitative estimates of absolute abundances obtained by qPCR and 16S rRNA gene metabarcoding.

3.5. Inference of microbial association networks

Network analysis tools are increasingly being used in the analysis of microbial ecosystems because of their potential in clarifying the interaction between group of species and in assisting in the identification of keystone species. The potential of network analysis tools for microbial community analysis, and the methods used for inference of association networks have been recently reviewed (Layeghifard et al., 2017). We inferred microbial association networks using three different methods (the CoNet app of Cytoscape, Faust and Raes, 2016; SparCC, Sparse Correlations for Compositional data, Friedman and Alm, 2012; and SPIEC-EASI, SParse InversE Covariance Estimation for Ecological Association Inference, Kurtz et al., 2015) as described in Parente et al. (2018). The microbial association network inferred using CoNet is shown as an example in Supplementary Fig. 5. The three tools inferred different numbers of associations, as found by Parente et al. (2018), with SparCC detecting the largest number of significant associations (either positive, copresence, or negative, mutual exclusion) and SPIEC-EASI the lowest. A Venn diagram showing the number of associations inferred with one or more methods is shown in Supplementary Fig. 6. In agreement with our previous findings, all interactions detected by SPIEC-EASI were also detected by one of the other methods. The interactions shared by the three methods are shown in Table 2.

Network statistics (size, average path length, clustering coefficient, positive edge proportion, etc.; Layeghifard et al., 2017; Parente et al., 2018) provide summary information on the structure of association network and are useful in the comparison of networks inferred for different ecosystems. The CoNet association network statistics were well within the range of those detected in a previous study for a wide range of foods (Parente et al., 2018): network size, both in terms of nodes (69) and edges (885) was intermediate between that of fresh and spoiled foods of animal origin; positive edge proportion (0.61) and normalized clustering coefficient were within the interquartile range for other food biomes (Parente et al., 2018) while average path length was relatively low (1.64) and density high (0.38).

A plot showing the relationship between positive degree and degree for the microbial association network is shown in Fig. 7. The taxa with unusually low ratio between total degree and positive degree (i.e. taxa with a high number of negative, mutual exclusion interactions, and a low number of positive, co-occurrence interactions) are known in network terminology as negative hubs and. They appear in the left upper side of the graph and include generally microbes associated with the spoiled samples. The taxa along the diagonal line (and thus with a ratio between total and positive degree close to the average) are, on the other hand, associated with the core microbiota of both products.

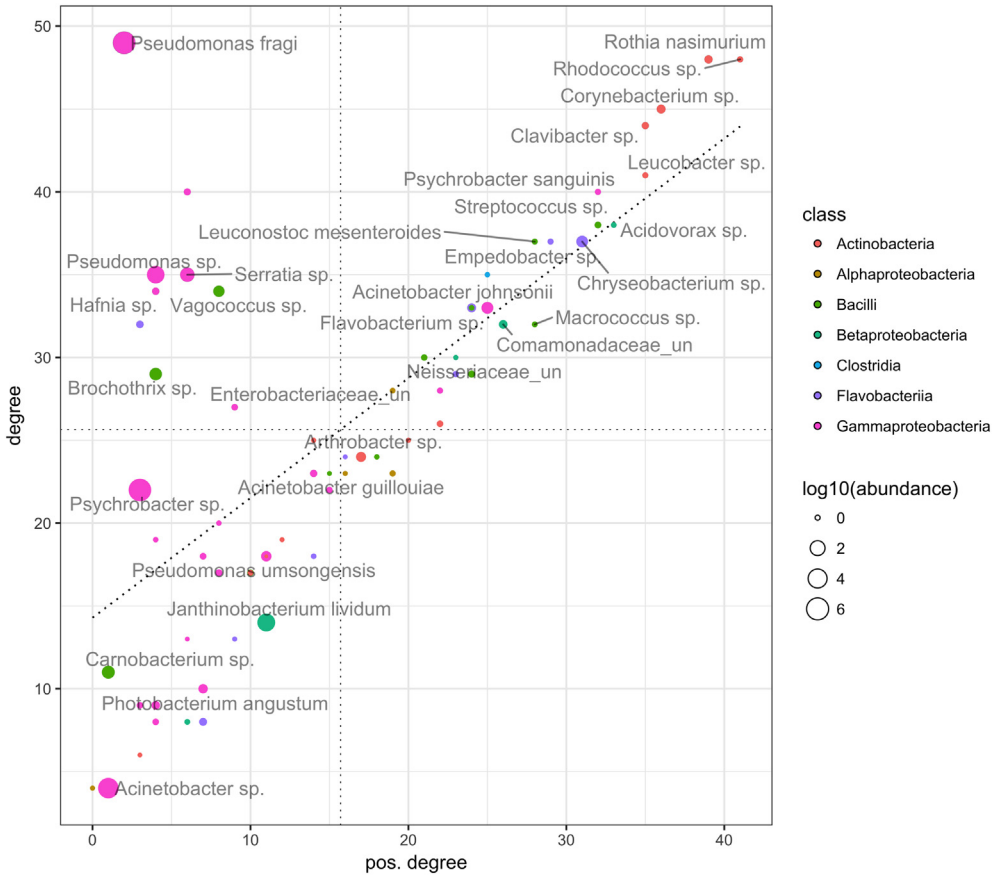

Fig. 7. Relationship between positive degree and degree for nodes in the microbial association network for thawed fish (hake or plaice) fillets stored in air at 0 or 10 °C. The microbial association network was inferred using the CoNet app of Cytoscape. Relative abundance of OTUs is shown as size of symbols. Only the labels of the nodes which are in the top 20% range for the properties shown in the graph are shown. The horizontal and vertical dotted lines indicate the average degree and positive degree, respectively, while the diagonal dotted line indicates the average ratio of positive to total degree.

Microbial interactions are known to affect spoilage and fermentation of fish products (Gram and Melchiorsen, 1996; Joffraud et al., 2006; Jørgensen et al., 2000; Macé et al., 2013). Inference of microbial interactions in a variety of food ecosystems (Parente et al., 2016; Parente et al., 2018), including fish and seafood products (Chaillou et al., 2015), may provide useful insights in the factors that shape the composition of microbial communities Layeghifard et al., 2017). The results we found in this study are in good agreement with what we found for a variety of other food biomes (Parente et al., 2018). The node degree plot largely supported the conclusions drawn by other inferential and descriptive methods on the composition of the core community and on the occurrence of keystone species (mainly negative hubs) involved in spoilage. Several interactions (27 co-occurrence and 16 mutual exclusion relationships) were supported by three different approaches (Table 2). However, they appear to result more from habitat filtering (i.e. changes in community structure due to changes in the habitat; here changes were due to temperature of storage) and host specificity (i.e. negative and positive interaction patterns related to the differential association of taxa to a host, here hake and plaice) than from true interactions. Indeed, when habitat filtering is significant, positive interactions may lose interpretability (Berry and Widder, 2014) and some true negative interactions may be difficult to detect with the methods we used (Weiss et al., 2016).

4. Conclusions

The results of this study largely confirm the findings of previous studies on chill stored fish, in terms of kinetics of growth, pattern of contamination, and effect of storage temperature. However, we found large lot-to-lot variation that partly obscured differences due to other factors (fish species, storage temperature). Therefore, the number of replicates should be increased to take this variability into account. Nevertheless, using inferential methods, we were able to detect bacterial taxa whose abundance was significantly different in the two products analysed in this study.

Both *Shewanella* and *Photobacterium*, two important members of the Specific Spoilage association of chilled fish (Gram and Dalgaard, 2002) had low prevalence and abundance at the end of storage, even if they were present at time 0. While for *Photobacterium* this may be due to the effect of freezing and thawing, it is unclear why *Shewanella* was unable to grow during refrigerated storage. On the other hand, other species less frequently associated with fish spoilage (*Vagococcus*, *Buttiauxella*), were found to be able to reach high levels at 10 °C, and their role in fish spoilage may need further investigation. We also demonstrated that combining 16S rRNA gene counts obtained by qPCR and HTS data may provide interesting insights on the effect of selected ecological parameters (temperature) on microbial successions, compared to the use of compositional data alone.

Finally, we used network analysis tools to infer the microbial association network. While the inferred co-presence and mutual exclusion relationships appear to be due more to habitat specificity and habitat filtering than to real microbe-microbe interactions, this technique provided independent measures of the “importance” of individual taxa (such as number of interactions and positive edge proportion), which were in good agreement with results obtained by other methods for the analysis of alpha and beta diversity.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.01.008.

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