Research Article

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Microbiological changes, shelf life and identification of initial and spoilage microbiota of sea bream fillets stored under various conditions using 16S rRNA gene analysis

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Abstract

BACKGROUND: Sea bream fillets are one of the most important value-added products of the seafood market. Fresh seafood spoils mainly owing to bacterial action. In this study an exploration of initial and spoilage microbiota of sea bream fillets stored under air and commercial modified atmosphere packaging (MAP) at 0 and 5 °C was conducted by 16S rRNA gene sequence analysis of isolates grown on plates. Sensory evaluation and enumeration of total viable counts and spoilage microorganisms were also conducted to determine shelf life and bacterial growth respectively.

RESULTS: Different temperatures and atmospheres affected growth and synthesis of spoilage microbiota as well as shelf life. Shelf life under air at 0 and 5 °C was 14 and 5 days respectively, while under MAP it was 20 and 8 days respectively. Initial microbiota were dominated by *Pseudomonas fluorescens*, *Psychrobacter* and *Macrococcus caseolyticus*. Different temperatures and atmospheres affected the synthesis of spoilage microbiota. At the end of shelf life, different phylotypes of *Pseudomonas* closely related to *Pseudomonas fragi* were found to dominate in most cases, while *Pseudomonas veronii* dominated in fillets under MAP at 0 °C. Furthermore, in fillets under MAP at 5 °C, new dominant species such as *Carnobacterium maltaromaticum*, *Carnobacterium divergens* and *Vagococcus fluvialis* were revealed.

CONCLUSION: Different temperature and atmospheric conditions affected bacterial growth, shelf life and the synthesis of spoilage microbiota. Molecular identification revealed species and strains of microorganisms that have not been reported before for sea bream fillets stored under various conditions, thus providing valuable information regarding microbiological spoilage.

Keywords: fish; seafood; sea bream; spoilage microbiota; 16S rRNA; PCR

INTRODUCTION

Sea bream (*Sparus aurata*) is one of the most important cultured fish species in the Mediterranean region and especially Greece. Sea bream production in Greece was estimated at 60 249 tons in 2009, with Greece being the leading producer in the world with 44.3% of the total production.[1] Chilled sea bream fillets packed in modified atmosphere packaging (MAP) are a value-added product that exhibits increasing demand in the international market (DIAS Aquaculture SA, personal communication).

Microbial spoilage is the major cause of fresh seafood quality deterioration.[2] Spoilage is caused by a fraction of the total initial microbiota called specific spoilage organisms (SSO).[3] The succession of spoilage microbiota is greatly influenced by temperature and packaging atmosphere.[2] It is well established that MAP prolongs the shelf life of fishery products.[4] MAP gaseous composition not only extends shelf life but also affects spoilage microbiota. Primarily *Pseudomonas* spp. and secondarily *Shewanella putrefaciens* have been found to be the main spoilage microorganisms of fish from the Mediterranean region (FAO 37) stored aerobically at low temperatures,[5–9] while other species such as lactic acid bacteria usually predominate in Mediterranean fish packed in atmospheres rich in CO_2.[6,10] The determination of species and/or strains involved in fish/seafood spoilage is the first step before the determination of spoilage potential and activity (e.g. volatile metabolites, off-odour production, etc.) of isolates.

The identification of seafood microbiota is mainly carried out by phenotypic tests (morphological, biochemical) after the isolation of microorganisms using various non-selective and/or selective growth media. In the past, several researchers have studied seafood microbiota on either selective or general growth media based on phenotypic characteristics of each isolated colony.[11–16] However, phenotypic tests lack the discriminatory power of molecular techniques.[7,17] The application of molecular techniques in foods has changed how microbial communities are explored.[18,19] 16S rRNA gene sequence analysis is currently the most common

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approach for studying seafood microbiota grown on plates.[9,20–24] Microorganisms grown on laboratory media are easily isolated and identified using only a small amount of DNA. Sequencing analysis of the 16S rRNA gene gives far more precise phylogenetic information than other techniques that have been used for the differentiation of cultivated microorganisms in seafood.[25]

To our knowledge, no previous study has investigated the initial and spoilage microbiota of sea bream fillets stored under either air or MAP using 16S rRNA gene sequence analysis. The aim of the present investigation was to (1) determine the microbiological changes and shelf life and (2) identify the initial and spoilage microbiota using 16S rRNA gene analysis of sea bream fillets stored under air and commercial MAP (60% CO_2/10% O_2/30% N_2) at 0 and 5 °C in order to provide valuable information regarding sea bream spoilage.

EXPERIMENTAL

Sea bream fillet provision and storage

Sea bream fillets weighing approximately 120 g each, prepared from sea bream caught in the geographical area designated FAO 37, 3.1 (Aegean Sea) in March 2012, were obtained from DIAS Aquaculture SA (Athens, Greece). Fillets were packaged in polystyrene boxes (Sirap Gema SpA, Verolanuova, Italy) under air or MAP. The MAP gas composition was 60% CO_2/10% O_2/30% N_2, which is one of the commercial gas compositions used by the Hellenic seafood industry for sea bream fillets, while the MAP film material was BDF 8050 F (Cryovac-Sealed Air Ltd, Athens, Greece). Samples were transferred to the laboratory within 4 h after packaging, using insulated boxes with melted ice, and stored in incubators operating at 0 and 5 °C.

Sensory analysis

Sensory evaluation was carried out by five panellists. The sensory attributes evaluated were appearance (translucent, glossy, natural colour, opaque, dull, discoloured) and odour (marine, fresh, neutral, sour, stale, spoiled, putrid). Each sensory attribute was rated using a nine-point descriptive hedonic scale (9 being the highest quality score and 1 the lowest). A score of 5 was taken as the average score for minimum acceptability.[26] The primary aim of the sensory evaluation was to determine the rejection time point.

Microbiological analysis

All microbiological media were supplied by LAB M (Heywood, Lancashire, UK) apart from streptomycin sulfate, thallous acetate and cycloheximide (actidione) agar (STAA), which were supplied by Biolife Italiana srl (Milan, Italy). Iron agar (IA) was prepared according to Gram *et al.*[14] by mixing 20 g L^{-1} peptone, 3 g L^{-1} meat extract, 3 g L^{-1} yeast extract, 3 g L^{-1} ferric citrate, 0.3 g L^{-1} sodium thiosulfate, 5 g L^{-1} NaCl, 0.6 g L^{-1} L-cysteine and 14 g L^{-1} agar and adjusting the pH 7.4.

At every sampling point, 25 g fillet samples were transferred aseptically to Stomacher bags with 225 mL of Maximum Recovery Diluent (MRD; 1 g L^{-1} peptone, 8.5 g L^{-1} NaCl) and homogenized for 2 min using a Stomacher (Bug Mixer, Interscience, London, UK). Samples of 0.1 mL of serial dilutions in MRD were spread on the surface of dried media in Petri dishes for enumeration of (a) total viable counts (TVC) on tryptone soy agar (TSA) after incubation at 25 °C for 48–72 h, (b) *Pseudomonas* spp. on cetrimide/fucidin/cephaloridine (CFC) agar after incubation at 25 °C for 48 h and (c) *Brochotrix thermosphacta* on STAA after incubation at 25 °C for 48–72 h. Samples of 1 mL of serial dilutions in MRD were used for the pour plate technique for enumeration of (a) H_2S-producing bacteria (presumptive *Shewanella*) on IA, counting only black colonies, after incubation at 25 °C for 72 h, (b) Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBGA) after incubatioin at 37 °C for 24 h and (c) lactic acid bacteria (LAB) on de Man, Rogosa and Sharpe (MRS) agar after incubation at 25 °C for 72 h.

TSA was used instead of plate count agar (PCA) or IA to monitor TVC and isolate the colonies for molecular identification. TSA gives almost tenfold higher numbers of colonies compared with other agar media used for TVC in seafood and especially sea bream.[27] TSA was also selected instead of Long and Hammer (L&H) agar owing to its ability to give higher numbers and suitability for Mediterranean fish, since *Photobacterium* does not exist in significant number.[7,28]

Identification of microbiota

A colony polymerase chain reaction (PCR) method was employed for the amplification of 16S rRNA gene fragments using bacterial cells directly as the template.[29] Approximately 900 bp of 16S rRNA gene was amplified by PCR with universal primers 27f BAC (5′-AGAGTTTGATCMTGGCTCAG-3′; M = A/C)[30] and 907r BAC (5′-CCCGTCAATTCCTTTGAGTTT-3′)[31] on a thermal cycler (MyCycler, Bio-Rad, Hercules, CA, USA). For direct PCR amplification, the PCR mixture (20 μL final volume) consisted of 4 μL of 5× buffer (Green GoTaq Flexi buffer, Promega, Madison, WI, USA), 2 μL of dNTPs (2 mmol L^{-1}), 1.2 μL of $MgCl_2$ (25 mmol L^{-1}), 0.1 μL of each primer and 0.1 μL of Taq polymerase (GoTaq DNA polymerase, Promega). A small amount of a single colony was added to the reaction mix as the DNA template using a sterile micropipette tip. The PCR conditions were pre-PCR at 95 °C for 5 min, then denaturation at 95 °C for 45 s, annealing at 52.5 °C for 45 s and elongation at 72 °C for 1 min, with final post-PCR elongation at 72 °C for 10 min. PCRs were adjusted to 28 cycles for isolated colonies of the initial and the rejection time point samples. Positive and negative controls were also included throughout the experiments. The PCR products were stained with ethidium bromide and visualized on 10 g L^{-1} agarose gel under UV light. A Montage PCR kit (Millipore, Billerica, MA, USA) was used for purification of the PCR products according to the manufacturer's instructions.

Sequence data were obtained using an ABI Prism 3730 XL capillary sequencer (VBC-Biotech, Vienna, Austria). Each sequence read was approximately 900 bp, and for each individual sample, forward and reverse reads were assembled. For the detection of closest relatives, all sequences were compared with the BLAST function. Sequence data were aligned using the ClustalX aligning utility (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and phylotypes were defined as sequences showing ≥98% homology to each other. All unique phylotypes were then compiled along with sequences obtained from GenBank (www.ncbi.nlm.nih.gov), and phylogenetic trees were constructed by the neighbour-joining method using MEGA5 software. Bootstrapping was performed with 1000 replicates to assign confidence levels to the tree topology. Sequences of dominant phylotypes found in this study were submitted to GenBank with accession numbers KJ411278–KJ411292.

Experimental design and statistical analysis

The packages with fillets were from two different batches. At every sampling point, four packages (two from each batch) were opened and one fillet from each package was taken for microbiological analyses. Bacterial counts were expressed in log colony-forming units (CFU) g^{-1} as mean ± standard deviation of four replicates.

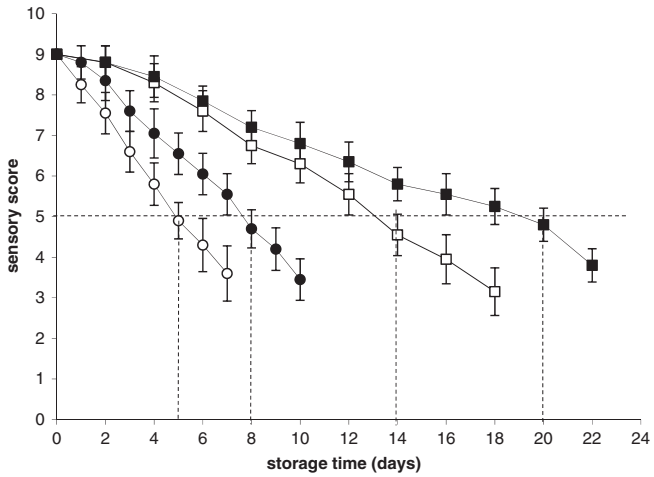

Figure 1. Sensory scores of sea bream fillets stored under aerobic conditions at 0 °C (□) and 5 °C (○) and under MAP at 0 °C (■) and 5 °C (●). The broken lines show the times of organoleptic rejection.

The microbial population changes against storage time were fitted using the Baranyi equation[32]

$$y(t) = y_{max} - \ln\left\{1 + \left[\exp\left(-y_{max} - y_0\right) - 1\right] \exp\left(\mu_m A_n(t)\right)\right\}$$

where $y(t)$ is the logarithm of the population at time t, y_{max} is the logarithm of the maximum population, y_0 is the logarithm of the initial population, μ_m is the maximum specific growth rate and $A_n(t)$ is a function related to the physiological state of the cells. DMFIT software (Institute of Food Research, Reading, UK) was used for fitting and growth rate estimation. This approach allows the estimation, comparison and evaluation of growth characteristics of various microorganisms.

Differences in means of bacterial populations, specific growth rates, lag phase duration, etc. were statistically tested by performing *t*-tests and analysis of variance (ANOVA) followed by Tukey's significant difference test, using STATISTICA 6.0 (StatSoft, Tulsa, OK, USA). A probability level of $P \leq 0.05$ was considered statistically significant.

For bacterial identification using colony PCR and 16S rRNA sequencing analysis (see above), half of the colonies from TSA plates (two plates, one from each batch) at the beginning of the experiment (day 0) and at the rejection time point for each storage condition were taken (ten TSA plates in total). The selected TSA plates were those of the highest dilution containing 30–300 colonies per plate.

RESULTS

Shelf life and microbiological growth

The shelf life of sea bream fillets stored under air determined by sensory assessment was 14 and 5 days at 0 and 5 °C respectively. The shelf life of MAP-stored fillets was extended up to 20 and 8 days at 0 and 5 °C respectively (Fig. 1). Initially, fish fillet freshness was excellent (grade 9 of hedonic scale). Fresh characteristics such as appearance (translucent, glossy, natural colour) and odour (marine, fresh) at 0 °C remained strong (score higher than grade 7.5) for 6 days for both air- and MAP-stored fillets ($P > 0.05$), while at 5 °C the characteristics remained strong for 2 and 3 days for fillets stored under air and MAP respectively (Fig. 1). At the time point of minimum acceptability (grade 5 of hedonic scale), fillets had an opaque and dull appearance and a stale odour. After this point, fillets were discoloured and their odour was putrid, so they were graded as unfit (grade <5 of hedonic scale) and rejected.

Total microbial population changes (TVC) are shown in Fig. 2, while kinetic parameters such as maximum specific growth rate, lag phase duration and initial and maximum population densities of spoilage microorganisms during storage of sea bream fillets at 0 and 5 °C under air and MAP are shown in Table 1. The total microbial population (TVC) of fillets initially was about 4 log CFU g^{-1} (Fig. 2, Table 1). Bacteria grew faster under air and higher temperature. Elevated CO_2 and reduced O_2 inhibited bacterial growth. Indeed, specific growth rates for all spoilage microorganisms studied were significantly lower at 0 °C and under MAP compared with 5 °C and aerobic storage respectively (Table 1). Maximum population densities (y_{max}) and populations at rejection (spoilage level) were also lower under MAP. Indeed, spoilage occurred when TVC under air storage reached about 8.3 and 8.6 log CFU g^{-1} at 0 and

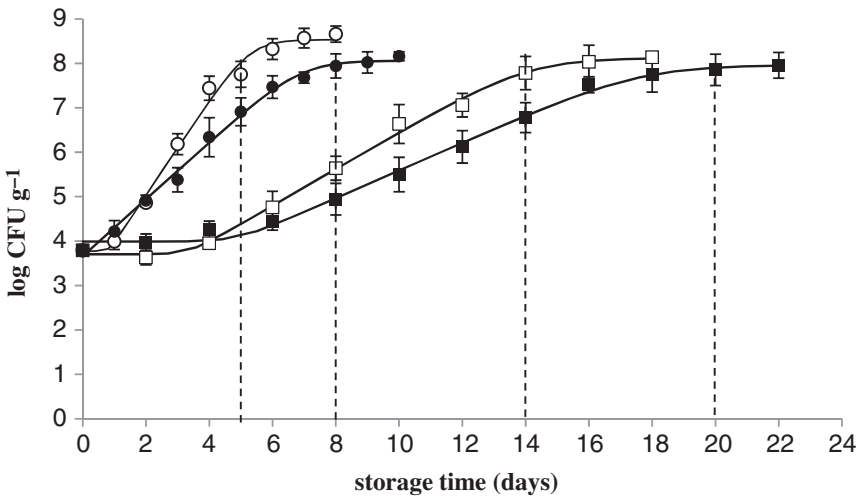

Figure 2. Total microbial population (TVC) changes of sea bream fillets stored under aerobic conditions at 0 °C (□) and 5 °C (○) and under MAP at 0 °C (■) and 5 °C (●), fitted with Baranyi equation.[32] Each experimental data point and error bar represents the mean ± standard deviation of four replicates. The broken lines show the rejection time points where the colonies on TSA plates were collected for molecular identification.

Table 1. Initial (y_0), maximum (y_{max}) and rejection time point (y_{tr}) populations, lag phase duration (Lag) and maximum specific growth rate (μ_{max}) of microorganisms of sea bream fillets stored under air and MAP at 0 and 5 °C, estimated using Baranyi equation.[32] Experimental values are also shown in parentheses

		Air					MAP				
T (°C)	Counts	y_0(log CFU g^{-1})	y_{max}(log CFU g^{-1})	y_{tr}(log CFU g^{-1})	Lag (days)	μ_{max} (day^{-1})	y_0(log CFU g^{-1})	y_{max}(log CFU g^{-1})	y_{tr}(log CFU g^{-1})	Lag (days)	μ_{max} (day^{-1})
0	TSA	4.16 ± 0.13a,a,A (4.02 ± 0.19)	8.32 ± 0.12a,a,A (8.37 ± 0.14)	8.28 ± 0.14a,a,A (8.26 ± 0.13)	2.52 ± 0.73a,a,A	0.484 ± 0.03a,a,A	4.18 ± 0.16a,a,A (4.02 ± 0.19)	7.96 ± 0.11a,b,A (7.96 ± 0.15)	7.63 ± 0.12a,b,A (7.74 ± 0.10)	2.73 ± 1.37a,a,A	0.247 ± 0.02a,b,A
	CFC	3.24 ± 0.13b,a,A (3.23 ± 0.08)	7.61 ± 0.12b,a,A (7.81 ± 0.12)	7.72 ± 0.11b,a,A (7.69 ± 0.11)	1.88 ± 0.81b,a,A	0.447 ± 0.02b,a,A	3.32 ± 0.09b,a,A (3.23 ± 0.08)	7.41 ± 0.20b,a,A (7.43 ± 0.20)	7.13 ± 0.09b,b,A (7.21 ± 0.12)	2.41 ± 0.48a,a,A	0.273 ± 0.01b,b,A
	IA	2.98 ± 0.11c,a,A (3.03 ± 0.08)	7.33 ± 0.09c,a,A (7.55 ± 0.04)	7.33 ± 0.09c,a,A (7.25 ± 0.12)	0c,a,A	0.496 ± 0.04c,a,A	3.07 ± 0.07c,a,A (3.03 ± 0.08)	7.35 ± 0.13cb,a,A (7.55 ± 0.18)	7.18 ± 0.07b,b,A (7.08 ± 0.09)	2.47 ± 0.47a,b,A	0.306 ± 0.01c,b,A
	VRBGA	2.55 ± 0.12d,a,A (2.59 ± 0.16)	6.21 ± 0.18d,a,A (6.27 ± 0.18)	6.21 ± 0.05d,a,A (6.19 ± 0.18)	0c,a,A	0.390 ± 0.01d,a,A	2.58 ± 0.14d,a,A (2.59 ± 0.16)	5.49 ± 0.15d,b,A (5.74 ± 0.14)	5.47 ± 0.15c,b,A (5.37 ± 0.15)	0.80 ± 0.93b,b,A	0.228 ± 0.01d,b,A
	MRS	3.76 ± 0.18e,a,A (3.73 ± 0.21)	5.26 ± 0.18e,a,A (5.30 ± 0.14)	5.23 ± 0.18e,a,A (5.24 ± 0.20)	2.63 ± 0.68a,a,A	0.160 ± 0.01e,a,A	3.77 ± 0.14e,a,A (3.73 ± 0.21)	5.99 ± 0.15e,b,A (6.27 ± 0.14)	5.97 ± 0.13d,b,A (5.84 ± 0.12)	8.02 ± 0.49c,b,A	0.299 ± 0.04e,b,A
	STAA	1.99 ± 0.03f,a,A (<2)	5.32 ± 0.08e,a,A (5.22 ± 0.14)	4.59 ± 0.17f,a,A (4.78 ± 0.13)	0c,a,A	0.210 ± 0.04f,a,A	2.23 ± 0.11f,b,A (<2)	6.02 ± 0.39e,b,A (6.00 ± 0.33)	5.65 ± 0.14ec,b,A (5.74 ± 0.30)	2.42 ± 0.90a,b,A	0.246 ± 0.02f,b,A
5	TSA	4.16 ± 0.14a,a,A (4.02 ± 0.19)	8.61 ± 0.03a,a,B (8.67 ± 0.03)	8.58 ± 0.04a,a,B (8.48 ± 0.07)	0.40 ± 0.06a,a,B	1.325 ± 0.04a,a,B	4.02 ± 0.18a,a,A (4.02 ± 0.19)	7.89 ± 0.09a,b,A (7.95 ± 0.08)	7.80 ± 0.08a,b,A (7.82 ± 0.12)	1.13 ± 0.28a,b,B	0.778 ± 0.26a,b,B
	CFC	3.18 ± 0.09b,a,A (3.23 ± 0.08)	8.63 ± 0.05a,a,B (8.68 ± 0.05)	8.42 ± 0.13a,a,B (8.39 ± 0.24)	0b,a,B	1.219 ± 0.06b,a,B	3.23 ± 0.06b,a,A (3.23 ± 0.08)	7.40 ± 0.12b,b,A (7.44 ± 0.09)	7.29 ± 0.10b,b,A (7.31 ± 0.13)	0.56 ± 0.70b,b,B	0.761 ± 0.25a,b,B
	IA	2.96 ± 0.09c,a,A (3.03 ± 0.08)	7.47 ± 0.11b,a,A (7.49 ± 0.11)	7.32 ± 0.16b,a,A (7.30 ± 0.15)	0b,a,A	1.060 ± 0.04c,a,B	3.02 ± 0.05c,a,A (3.03 ± 0.08)	6.58 ± 0.11c,b,B (6.67 ± 0.10)	6.52 ± 0.07c,b,B (6.54 ± 0.14)	1.83 ± 0.52c,b,B	0.989 ± 0.49b,b,B
	VRBGA	2.77 ± 0.12d,a,A (2.59 ± 0.16)	8.40 ± 0.16c,a,B (8.60 ± 0.05)	8.41 ± 0.16a,a,B (8.25 ± 0.36)	0b,a,A	1.107 ± 0.07d,a,B	2.59 ± 0.19d,a,A (2.59 ± 0.16)	7.38 ± 0.08bd,b,B (7.42 ± 0.07)	7.31 ± 0.08bd,b,B (7.28 ± 0.13)	0d,a,B	0.731 ± 0.04c,b,B
	MRS	3.72 ± 0.17e,a,A (3.73 ± 0.21)	5.50 ± 0.07d,a,B (5.59 ± 0.19)	5.44 ± 0.05c,a,B (5.35 ± 0.13)	1.13 ± 0.28c,a,B	0.539 ± 0.07e,a,B	3.74 ± 0.19e,a,A (3.73 ± 0.21)	5.95 ± 0.08e,b,A (6.00 ± 0.06)	5.95 ± 0.07e,b,A (5.93 ± 0.02)	1.75 ± 0.47c,b,B	0.624 ± 0.26d,b,B
	STAA	1.99 ± 0.12f,a,A (<2)	5.89 ± 0.04e,a,B (5.91 ± 0.05)	5.24 ± 0.26c,a,B (5.25 ± 0.31)	0b,a,A	0.705 ± 0.07f,a,B	1.98 ± 0.08f,a,B (<2)	6.04 ± 0.07e,b,A (6.11 ± 0.09)	5.98 ± 0.07e,b,B (5.89 ± 0.13)	1.33 ± 0.18a,b,B	0.718 ± 0.03e,b,B

Values are mean ± standard deviation of four replicates. Mean values with the same letter are not statistically different. The first lowercase letters correspond to comparisons within columns (different bacteria, same storage conditions using ANOVA and Tukey's test), while the second lowercase letters correspond to comparisons between columns (same bacteria, different atmosphere for same storage temperature using *t*-test). The capital letters correspond to comparisons between the same bacteria for different temperature and same atmosphere using *t*-test.

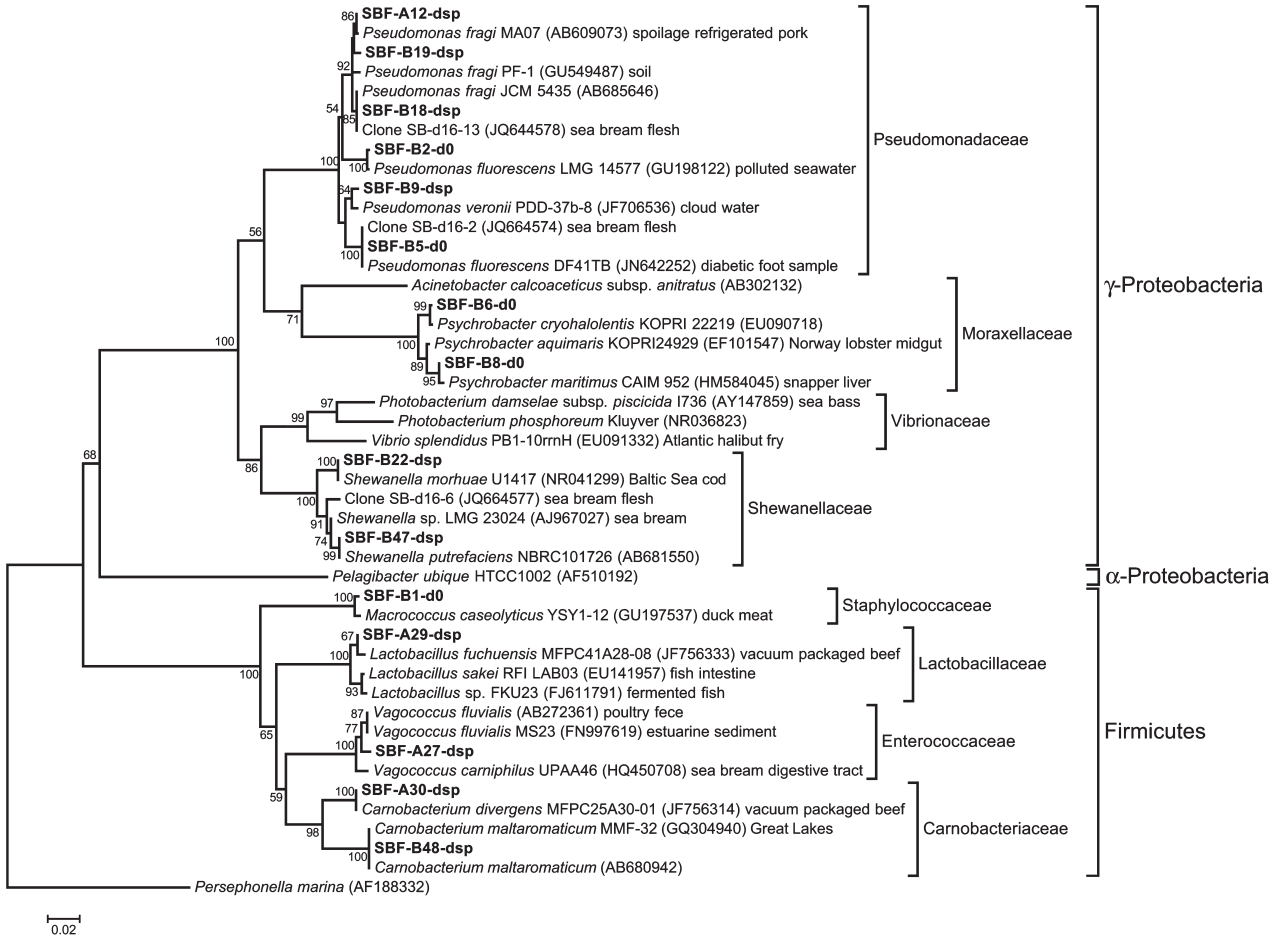

Figure 3. Phylogenetic tree of PCR-amplified bacterial 16S rRNA gene phylotypes (in bold) (~900 bp) from fresh sea bream fillets and after storage under various conditions, based on neighbour-joining method as determined by distance using Kimura's two-parameter correction. GenBank accession numbers are shown in parentheses. One thousand bootstrap analyses (distance) were conducted, and percentages above 50% are indicated at nodes. Scale bar represents 2% estimated distance.

5 °C respectively, in contrast to 7.6 and 7.8 log CFU g^{-1} at 0 and 5 °C respectively under MAP (Table 1).

Pseudomonas spp. were the predominant spoilage microorganisms reaching the highest population densities in all cases, together with H_2S-producing bacteria and Enterobacteriaceae at 0 and 5 °C respectively. The above-mentioned microorganisms predominated under both atmospheric conditions. However, LAB and *B. thermosphacta* growth was more pronounced and their growth rates and maximum populations were significantly higher under MAP than under air. LAB reached 6 log CFU g^{-1} under MAP, while the *B. thermosphacta* population was initially below the detection limit of 2 log CFU g^{-1} but finally reached levels as high as 5–6 log CFU g^{-1} (Table 1).

Identification of microbiota

One hundred and sixty-one colonies (from 322 colonies in total) were identified. Thirty-one colonies (from 62 colonies in total) were taken at day 0. The identification using 16S rRNA gene sequence analysis showed that 55% of the colonies of the initial microbiota were *Pseudomonas fluorescens* (two different phylotypes, SBF-B2-d0 and SBF-B5-d0) (Fig. 3). Thirty-two per cent of the colonies were identified as *Psychrobacter cryohalolentis* KOPRI 22219 and *Psychrobacter maritimus* CAIM 952 (Table 2, Fig. 3), while the rest of the initial microbiota presented 99.0% similarity to *Macrococcus caseolyticus* YSY1-12 (Table 2, Fig. 3).

At rejection time point, 130 colonies (from 260 colonies in total) were taken from TSA plates. For fillets under air at 0 °C, the majority of isolated colonies were determined as *Pseudomonas fragi* (Table 2). *Pseudomonas fragi* was found to belong to two different phylotypes, SBF-B18-dsp and SBF-B19-dsp, closest related to *P. fragi* JCM 5396 and *P. fragi* JCM 5435 respectively (Table 2, Fig. 3). The rest of the colonies were identified as *Shewanella morhuae* U1417 (Table 2). At 5 °C, *P. fragi* dominated exclusively in sea bream fillets (Table 2). *Pseudomonas fragi* presented two different phylotypes, SBF-B19-dsp and SBF-A12-dsp, closest related to *P. fragi* JCM 5396 and *P. fragi* MA07 respectively (Table 2, Fig. 3).

In fillets under MAP, *Pseudomonas veronii* PDD-37b-8 formed 100% of the spoilage microbiota at 0 °C (Table 2). At 5 °C under MAP, the spoilage microbiota comprised mostly LAB such as *Carnobacterium maltaromaticum* MMF-32, *Carnobacterium divergens* MFPC25A3001 and *Lactobacillus fuchuensis* MFPC41A28-08 (Table 2, Fig. 3). *Vagococcus fluvialis* MS23 was also found. *Pseudomonas fragi* was also found to co-dominate with LAB. *Pseudomonas fragi* presented two different phylotypes, the first closest related to *P. fragi* JCM 5396 and the second to *P. fragi* JCM 5435 (Table 2). *Shewanella putrefaciens* NBRC 101726 was also identified (Table 2).

Table 2. Initial (day 0) and spoilage microbiota of sea bream fillets stored under air (A) and MAP (M) at 0 and 5 °C, determined by molecular analysis. Abundance indicates the percentage of the number of colonies belonging to the corresponding phylotype out of the number of identified colonies

	Abundance (%)	Closest relative	Similarity (%)	GenBank number
Day 0	35.5	*Pseudomonas fluorescens* DF41TB	99.0	JN642252
	19.5	*Pseudomonas fluorescens* LMG 14577	97.0	GU198122
	19.0	*Psychrobacter maritimus* CAIM 952	99.0	HM584045
	13.0	*Psychrobacter cryohalolentis* KOPRI 22219	99.0	EU090718
	13.0	*Macrococcus caseolyticus* YSY1-12	99.0	GU197537
A, 0 °C	66.7	*Pseudomonas fragi* JCM 5396	99.0	AB685609
	13.3	*Pseudomonas fragi* JCM 5435	99.0	AB685646
	20.0	*Shewanella morhuae* U1417	99.0	NR041299
M, 0 °C	100	*Pseudomonas veronii* PDD-37b-8	98.0	JF706536
A, 5 °C	75.0	*Pseudomonas fragi* JCM 5396	99.0	AB685609
	25.0	*Pseudomonas fragi* MA07	98.0	AB609073
M, 5 °C	25.0	*Pseudomonas fragi* JCM 5396	99.0	AB685609
	11.4	*Pseudomonas fragi* JCM 5435	99.0	AB685646
	27.3	*Carnobacterium maltaromaticum* MMF-32	100	GQ304940
	9.1	*Lactobacillus fuchuensis* MFPC41A28-08	99.0	JF756333
	9.1	*Vagococcus fluvialis* MS23	98.0	FN997619
	9.1	*Carnobacterium divergens* MFPC25A3001	99.0	JF756314
	9.1	*Shewanella putrefaciens* NBRC 101726	99.0	AB681550

DISCUSSION

In the present study the dominant initial and spoilage microbiota of sea bream fillets stored under air and MAP at 0 and 5 °C were evaluated. The level of initial bacterial counts (4 log CFU g^{-1}) presumably indicates good hygienic practices along the production line. Sea bream fillets were rejected by sensory evaluation when TVC were between $10^{7.5}$ and $10^{8.5}$ CFU g^{-1}. According to guidelines and microbiological specifications of the ICMSF,[33] TVC of fresh fish should not exceed 10^6–10^7 CFU g^{-1}. However, spoilage of fresh fish becomes organoleptically detectable when TVC are as high as 10^8–10^9 CFU g^{-1}.[2,28]

The shelf life of fillets packed under air at 0 and 5 °C was 14 and 5 days respectively. Other researchers found that the shelf life of sea bream fillets stored at 5 °C usually varied from 4 to 7 days.[26,34] The differences might be due to different initial microbial populations through contamination by personnel, working surfaces and utensils during processing, packaging, etc. In fillets under MAP, shelf life was extended up to 20 and 8 days at 0 and 5 °C respectively. It is known that the use of CO_2 in MAP significantly inhibits bacterial growth compared with aerobic packaging.[4,35] Numerous studies reported that the shelf life of fish fillets such as hake,[36] sea bass[37] and catfish[38] stored under μαρ at low temperatures was extended by 100% or more. Regarding sea bream, Dalgaard *et al.*[39] found that the shelf life of sea bream fillets stored under 50% CO_2/50% N_2 was 14 days at 0 °C. The differences can be attributed to the various gaseous atmospheres used in MAP and also to other influencing factors such as gas/product ratio, type of packaging film, initial microbial load, etc.[4,35]

Primarily *Pseudomonas* spp. and secondarily H_2S-producing bacteria were reported to be the dominant spoilage microorganisms of fish caught from Mediterranean waters and kept in chilled storage under aerobic conditions.[5,6,8,9] Our study agrees with these results. Under MAP, *B. thermosphacta* and LAB usually predominate. Indeed, Koutsoumanis *et al.*[40] and Drosinos and Nychas[41] found that *B. thermosphacta* and *S. putrefaciens* grew under μαρ at low temperatures in red mullet (*Mullus barbatus*) and sea bream (*Sparus aurata*) respectively. In the present study, *B. thermosphacta* and LAB did not reach population densities higher than 6 log CFU g^{-1}. However, the gaseous composition in our case differed compared with the above-mentioned works. Indeed, Kostaki *et al.*[42] and Pournis *et al.*,[43] using the same gaseous composition as in our study, found that *Pseudomonas* spp. and H_2S-producing bacteria were the dominant spoilage microorganisms in organic aquacultured sea bass (*Dicentrarchus labrax*) and Mediterranean mullet (*Mullus surmuletus*) respectively, while *B. thermosphacta* and LAB reached populations no higher than 5–6 log CFU g^{-1}. It is obvious that the gas composition used in our case did not favour adequately the growth of *B. thermosphacta* and LAB.

Growth rates of fish spoilage bacteria in our work differ from those recorded in other studies. *Pseudomonas*, *Shewanella*, *Brochothrix* and LAB growth rates on Mediterranean red mullet stored at 0 and 4 °C under air and 50% CO_2 were higher than those recorded in the present study.[40] Again, *Pseudomonas* growth rates on whole sea bream stored aerobically at 0 and 5 °C were higher compared with our study.[44] On the contrary, Tsironi *et al.*[45] found that growth rates of LAB on sea bream fillets stored at 0 and 5 °C under 35% CO_2 were lower compared with the present study. It is obvious that different growth substrates (different fish species, whole fish, filleted fish, etc.) and changes in storage conditions affect growth rates of spoilage bacteria.

The initial microbiota of fish from temperate waters usually consist of various psychrotrophic Gram-negative bacteria such as *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Shewanella* and Vibrionaceae.[3,46] In this study the initial microbiota were dominated by *P. fluorescens* (two phylotypes, LMG 14577 and DF41TB), *Ps. cryohalolentis* KOPRI 22219 and *Ps. maritimus* CAIM 952. *Pseudomonas fluorescens* LMG 14577 was found in spoiled iced whole sea bream in a previous study.[7] *Psychrobacter cryohalolentis* KOPRI 22219 has also been isolated from the midgut of Atlantic cod.[47] Moreover, another phylotype of *Ps. cryohalolentis* was found to dominate in spoiled iced ray (*Raja* sp.).[48] *Psychrobacter maritimus* CAIM 952 has also been isolated from the liver of spotted rose snapper (*Lutjanus guttatus*) (Gomez-Gil B., unpublished, GenBank). Several species of the genus *Psychrobacter* were

found to dominate in spoiled iced angler fish.[21] However, Gennari *et al.*,[12] using only a classical approach, isolated *Psychrobacter* from the initial microbiota of sardines from the Adriatic Sea. *Macrococcus caseolyticus* strain YSY1-12 found in our study has been reported as part of the spoilage microbiota of water-boiled salted duck stored at 4 °C.[49] However, *M. caseolyticus*, previously classified in the genus *Staphylococcus* (*S. caseolyticus*), has also been isolated from fermented fish products,[50] fermented meat products,[51] fermented sausages,[52] raw milk and cheese.[53–55]

Many researchers have studied the spoilage microbiota of iced fish caught from the Mediterranean Sea by using a classical approach and found that *Pseudomonas* and *Shewanella* are the most predominant spoilage microorganisms grown on plates.[8,9,12] It is known that primarily *Pseudomonas* and secondarily *Shewanella* are the main spoilers of chilled sea bream.[5–9] Our results agree with those of previous works, but in many cases the molecular approach gave different information at both species and strain levels. Tryfinopoulou *et al.*[8] reported that *Pseudomonas lundensis* and *P. fluorescens* were the dominant species in spoiled chilled sea bream caught from Greek waters. In the present study, in fillets packed under aerobic conditions, *P. fragi* was found to be the predominant microorganism at the end of shelf life of chilled sea bream at both temperatures (0 and 5 °C). Although *P. fragi* was not isolated from the initial microbiota, in contrast to *P. fluorescens*, it was found to be predominant at the end of shelf life. Lebert *et al.*[56] reported that strains of *P. fragi* have shorter lag times than those of *P. fluorescens*, which presumably explains the predominance of the former over the latter during storage at low temperatures.

Among phylotypes that were found, *P. fragi* MA07 has also been isolated from spoiled refrigerated pork (Maneerat, S. and Avapak, M., unpublished, GenBank). In fillets stored at 0 °C, *P. fragi* was followed by *S. morhuae* U1417, which has also been found in iced cod from the Baltic Sea.[57,58] *Shewanella morhuae* has not been isolated so far from fish caught in Greek waters. Hitherto, *S. putrefaciens*, *Shewanella baltica* and *Shewanella oneidensis* have been found to be among the spoilage microorganisms of sea bream.[9]

In fillets stored under air at 5 °C, Enterobacteriaceae species were not found on TSA using the molecular approach, but only *P. fragi*, despite the fact that VRBGA counts were high and comparable to those on TSA. Taking into account that Enterobacteriaceae can grow easily on TSA, the high VRBGA counts might due to *Pseudomonas* spp. growth on VRBGA, as has been noted by other researchers.[59]

Although CO_2 favours the growth of LAB,[3,4,10,40] the predominant spoilage microbiota under MAP at 0 °C comprised only Gram-negative bacteria such as *P. veronii*. *Pseudomonas* might be dominant as a consequence of various factors such as high O_2 concentration (10%) and very low temperature (0 °C), as has been noted by Kostaki *et al.*[42] and Pournis *et al.*[43] using the same storage conditions.

The spoilage microbiota of fillets under MAP at 5 °C consisted mainly of Gram-positive bacteria followed by *Pseudomonas* and *S. putrefaciens*. Based on 16S rRNA gene sequence analysis, the microbiota were dominated by *C. maltaromaticum*, *C. divergens*, *L. fuchuensis*, *V. fluvialis* and Gram-negative bacteria such as *P. fragi* and *S. putrefaciens*. Although LAB such as *Carnobacterium* dominated under MAP at 5 °C, the counts on MRS were about 1.5 and 0.5 log CFU g^{-1} lower than those on CFC and IA respectively. This might be attributed to the inability of *Carnobacterium* species to grow well on MRS (pH 6.3–6.5), in contrast to TSA (pH 7.2–7.4). Indeed, Casaburi *et al.*[60] reported that *C. maltaromaticum* and *C. divergens* can grow well at higher pH values. *Carnobacterium maltaromaticum* and *C. divergens* have never before been reported as dominant spoilage microorganisms of fish from Greek waters. However, *C. maltaromaticum* has been found to form part of the spoilage microbiota of chilled seafood from other geographical regions, such as Atlantic horse mackerel[20] and salmon[61,62] under MAP and vacuum packaging. Laursen *et al.*[63] reported that both *C. divergens* and *C. maltaromaticum* are capable of growing in chilled raw and processed seafood products stored under different packaging conditions. Dalgaard *et al.*[64] found *C. divergens* as part of the dominant spoilage population of cooked and brined MAP shrimps. Jaffrès *et al.*[23] reported that bacteria such as *C. divergens*, *C. maltaromaticum* and indiscernible *Carnobacterium alterfunditum/pleistocenium* as well as *Vagococcus* (indiscernible *V. carniphilus/fluvialis*) were isolated from non-selective media such as L&H used for the determination of TVC. Accordingly, in the present work, *Vagococcus* was also found to be part of the spoilage microbiota at 5 °C. *Vagococcus* has never before been reported as a spoilage microorganism in fish from Greek waters.

By direct comparison, with a cut-off level of 98% similarity, of the environmental sequences found in spoiled sea bream flesh in our previous work[7] and the isolate sequences reported in this work, we appended three of the phylotypes to certain isolates. Phylotypes SB-d16-2 and SB-d16-13[7] were identified as *P. fluorescens* and *P. fragi* respectively, while phylotype SB-d16-6[7] was confirmed as *S. putrefaciens*. This comparison highlights these three microorganisms as key players in sea bream spoilage, as they have been found in both whole and fillet samples. Further phenotypic characterization, especially the spoilage potential and activity of these isolates, will provide insight into seafood spoilage mechanisms.

CONCLUSIONS

Different temperatures and atmospheres affected growth, shelf life and the synthesis of spoilage microbiota. 16S rRNA gene sequence analysis gave information at both species and strain levels. Moreover, new dominants were revealed by the molecular approach. *Psychrobacter* spp., as part of the initial microbiota, as well as *Carnobacterium* spp. and *Vagococcus* spp., as part of the spoilage microbiota, have never before been reported as dominant microorganisms of fish from Greek waters. The present work can be the baseline for further investigation of the spoilage potential and activity of these microorganisms in Mediterranean fish/seafood, especially sea bream.

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