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Quantitative PCR assays as a monitoring tool for bacterial genera in fresh fish fillets

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ABSTRACT

Fresh fish fillets are a valuable but highly perishable food, and their rapid microbial deterioration is a drawback for food safety and sustainability of aquaculture, food and retail industries. Quantitative PCR (qPCR) assays based on *16S* rRNA gene (*16S*) sequences were developed for the most abundant bacteria genera detected by metagenomics in fresh or processed fish fillets. The efficiency and specificity of six qPCR assays (for *16S* of all bacteria or genera *Shewanella, Pseudomonas, Carnobacterium, Janthinobacterium* and *Massilia*) was verified using *in silico* predictions, cloning, sequencing and phylogenetic analyses of amplicons obtained from refrigerated control or high-pressure processed (HPP) European sea bass (*Dicentrarchus labrax*) fillets. In HPP sea bass fillets, significant decreases in total bacteria *16S* and of *Shewanella, Pseudomonas, Carnobacterium* and *Janthinobacterium* *16S* compared to control fillets were confirmed by qPCR, after 11 days of refrigerated storage. The qPCR assays were successfully applied to monitor microbial contamination during refrigerated storage of fresh fillets from commercial (retail) sea bass and gilthead sea bream (*Sparus aurata*). Significant increases in total bacterial and *Shewanella, Pseudomonas, Carnobacterium* and *Janthinobacterium* contamination were detected after 7–14 days. *16S* copy number for total bacteria and the four target genera positively correlated with total viable counts using culture enumeration. *16S* of *Massilia*, that is abundant in fresh fish fillets, did not significantly change during storage. The six validated qPCR assays developed are proposed as specific, sensitive, culture-independent methods for monitoring quality or processing outcomes for fish fillets during cold chain storage.

1. Introduction

Fish and seafood products are highly appreciated by consumers due to their health promoting properties and high nutritional value, with low content of saturated fat and carbohydrates and supplying long-chain omega-3 fatty acids and minerals (Larsen et al., 2011). Fish production and consumption have been increasing, likely due to the significant growth of the aquaculture sector and higher availability of fresh and processed fish in supermarkets (FAO, 2022). However, fresh fish is an extremely perishable food product, and its chemical and microbial deterioration is a drawback for the safety and sustainability of aquaculture and associated food and retail industries (Tahiluddin et al., 2022). More than 35% of annual fishery and aquaculture production are lost or wasted, and measures are needed to achieve the United Nations Sustainable Development Goal 12.3, aimed at reducing global food loss and waste by 50% in 2030 (FAO, 2022; Xue et al., 2017). To contribute to this while assuring food safety, priority should be given to develop or improve preservation and quality control methodologies for fish products (Hassoun et al., 2022; Tavares et al., 2021). Combined with improved packaging, novel non-thermal processing techniques like

Abbreviations: ***16S***, *16S* ribosomal RNA gene; **bp**, base pairs; **cfu**, colony-forming units; **Ct**, quantitative PCR amplification cycle threshold value; **HPP**, high-pressure processing; **LPSN**, List of Prokaryotic names with Standing in Nomenclature; **qPCR**, quantitative real-time PCR; **TVC**, total viable counts.

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high-pressure processing (HPP) can contribute to fish product quality and safety, while maintaining high sensory and nutritional qualities (e.g. Gómez-Estaca et al., 2018). HPP has been applied to fish or sea food products with positive effects on their shelf life, although the species, storage and processing conditions may affect shelf life extension and organoleptic properties, and optimization may be required by species and product type (de Oliveira et al., 2017; Ganjeh et al., 2024; Roobab et al., 2022; Wang et al., 2016).

HPP decreases the total bacterial load of fish products and their contamination by selected bacterial species, mainly associated with pathogenicity, spoilage or histamine-production (e.g. de Alba et al., 2019; Tsevdou et al., 2023; Tsironi et al., 2019; Ucak et al., 2018). High-throughput metagenomics approaches like *16S* ribosomal RNA (*16S* rRNA) gene amplicon sequencing ("*16S* metabarcoding") can generate global profiles of food associated microbiota without their cultivation and can be used to detail HPP microbiological impacts (Chaillou et al., 2015; Ferone et al., 2020; Hassoun et al., 2022; Sequino et al., 2022; Tsironi et al., 2019).

Concerning the quality and safety monitoring of fresh or processed sea food products throughout the supply chain, the protocols routinely used by most companies or external quality control programs are based on the microbiological enumeration of bacterial total viable counts (TVC) in selective or non-selective media. Classical culture methods have low sensitivity and specificity, fail to cover many bacterial species and have a long time-to-detection that can limit the quality control of short shelf life products like fish (Jian et al., 2020; Martínez et al., 2011). Monitoring specific bacterial types by quantitative PCR (qPCR) allows culture-independent, rapid, sensitive, specific quantification of microbes in food matrices (Ferone et al., 2020; Martínez et al., 2011), but this approach is still less frequently applied in industry and is generally limited to few selected foodborne pathogenic species. Nevertheless, many studies have reported the development of qPCR assays to quantify specific bacterial groups (such as pathogens and spoilage-associated bacteria) in fish and sea food, providing accessible tools to monitor quality or safety in the food supply chain (Jérôme et al., 2022; Kimura et al., 2001; Macé et al., 2013; Mamlouk et al., 2012; Reynisson et al., 2008; Rodriguez-Lazaro et al., 2005).

The aims of this study were i) to develop qPCR assays for abundant bacterial genera (selected from available *16S* metabarcoding datasets) in fresh fish fillets; ii) to evaluate their applicability as candidate biomarkers for quality and deterioration of fresh fish, by monitoring bacteria in industrial (control or HPP-European sea bass) or retail (sea bass and gilthead sea bream) fillets during refrigerated storage for up to 14 days.

2. Materials and methods

2.1. Quantitative PCR assay design

Instruments, operating conditions, raw materials, dataset accession numbers, reagents and the main methods used in this study are provided in detail in Supplementary file S1. As a starting point to select candidate fish quality biomarkers for qPCR assay development (Fig. 1), we performed data surveys of *in-house* 16S metabarcoding profiles previously obtained from sea bass fillets from a Greek aquaculture company. Fillets had been stored at 2 °C in aerated plastic pouches (control) or after vacuum-packaging and HPP (600 MPa) for 5 min, 25 °C (Anjos et al., 2019; Tsironi et al., 2019; Suppl.file S1). Representative *16S* sequences of all species belonging to the five target genera were downloaded from the LPSN database (2020). Multiple sequence alignments generated by ClustalX 2.1 (Larkin et al., 2007) included LPSN representative species and *16S* metabarcoding sequences obtained from sea bass fillets (Suppl. Figs. S1–S4). Primers (Table 1) were designed to target the maximum number of bacterial species and genus-specific metabarcoding sequences and avoid cross-amplification of related genera. Designed or literature-available primer pairs were evaluated in PrimerPremier 5.0 (Zhai et al., 2008) and specificities were predicted *in silico* by: 1) calculating target genus coverage (ratio between the number of species with 100% match for both primers and the number of LPSN eligible species, based on Figs. S1–S4 alignments and Table 2); and 2) TestPrime1.0 (2012) automatic genus coverage in the SILVA database (Quast et al., 2012)-Suppl.Table S1.

Specificity and coverage were also evaluated for universal *16S* primers 341F/805R (V3–V4 regions), previously used to generate the *16S* metabarcoding datasets (Illumina, 2013; Tsironi et al., 2019), and 515FY/806RN(V4), also commonly used for bacterial metabarcoding (EMP, 2018), here optimized for qPCR of total bacterial DNA loads in fish fillets (Table 1).

2.2. Testing primers by PCR in commercial aquaculture sea bass fillets

Each primer pair was evaluated by conventional PCR in genomic DNA samples from refrigerated unprocessed or HPP-sea bass fillets (Tsironi et al., 2019), sampled and extracted as in Anjos et al. (2019) and Suppl. file S1. PCR reactions of 25 μL contained 10–50 ng of DNA and 500 nM of each primer, being tested at a range of annealing temperatures until the optimal was determined (Suppl. file S1; Table 1). PCR products were analysed on 2% agarose gels and amplicons of the expected size were purified using an Illustra GFX-PCR DNA&Gel Band Purification Kit (GE Healthcare, Germany). Purified products were sequenced (Sanger method) at CCMAR-CTS (2024) to confirm sequence identity and primer specificity.

2.3. Cloning and phylogenetic analyses

To confirm the main bacterial genera/species in sea bass fillets, longer fragments between 340 and 697 base pairs (bp) were amplified, combining novel primers with 16S qPCR primers (Table 1), using similar PCR conditions and control/HPP sea bass fillet DNAs. Amplicons of the expected size were purified from 2% agarose gels and 15–100 ng were ligated overnight at 4 °C into the pGEM-T Easy vector (Promega), followed by transformation of DH5α *Escherichia coli* competent cells. Up to ten positive (white) clones were selected per target bacterial genus, inoculated in 100 μL of LB broth with 75 μg/mL ampicillin and incubated for 2 h at 37 °C. Inserts were amplified by PCR with universal M13 primers (Promega cat.Q5401) and identities were confirmed by bidirectional Sanger sequencing, revision of chromatograms and BlastN (Altschul et al., 1997) against the NCBI (2020) nt database. The phylogeny of non-redundant, validated colony sequences for each target genera (GenBank OQ154852-OQ154866) was confirmed using SILVA Aligner (2012) with default options (Najafpour et al., 2022).

2.4. Real-time qPCR in control or HPP fish fillets

Optimal *16S* qPCR reactions, cycling and efficiencies were determined for all primer pairs (Table 1) as in Najafpour et al. (2022) and detailed in Suppl. file S1. qPCR reactions included 5 μL of Forget-Me-Not™EvaGreen® qPCR Master Mix, 300 nM of each primer and 10 ng of fish muscle DNA and were run for 40 cycles (Bio-Rad CFX96Touch Real-Time thermoclycler) in 96-well PCR plates with duplicate reactions for all tested DNAs, non-template controls and the standard curve containing 10^7-10^2 copies of each purified genus-specific *16S* amplicon. Curves were used to estimate qPCR efficiencies (Table 1) and calculate *16S* levels of each target bacterial group in test samples, based on the equation between template quantities and amplification cycle threshold values (Ct) (Martyniuk et al., 2009; Suppl. file S1).

Each qPCR primer pair was evaluated using control/HPP sea bass fillet DNAs with higher levels of each genus detected by *16S* metabarcoding, verifying 1) the production of single peaks in melt curves; 2) a single band of the expected size in 2% agarose gels; 3) amplicon identity, using cloning and sequencing; 4) high efficiencies (Table 1); 5) no amplification in non-template controls. For closely related genera

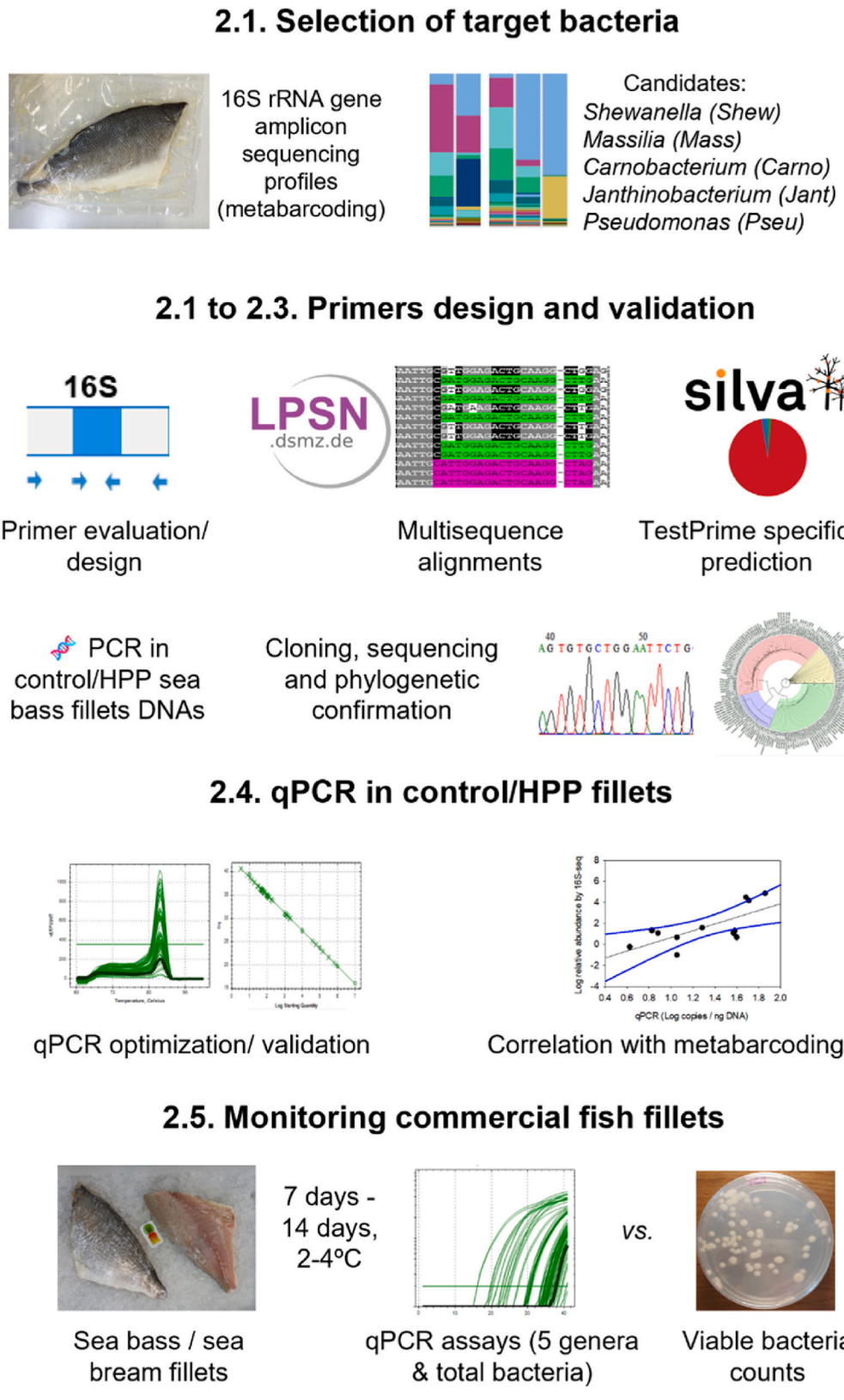

Fig. 1. Schematical representation of the workflow followed in this study to design, test and use the *16S* rRNA gene-based quantitative PCR assays to determine the total bacterial DNA loads and abundant bacterial genera DNAs in fish fillets. qPCR = quantitative PCR; HPP = high-pressure processing. To facilitate comprehension of the link between the scheme and approaches used it is organized according to the numbered sections of the Methods, where more details can be found. In 2.1, previous metabarcoding datasets (accession numbers in the Data Statement; Anjos et al., 2019; Tsironi et al., 2019) were used as a starting point to select the candidate target bacterial genera for qPCR assay development in this study.

Table 1
List of primers optimized for the amplification of all bacteria or specific bacterial genus from fish fillets, based on *16S* rRNA gene sequences.

Aim	Genus/target	Reference	Primer name	Sequence (5′to 3′)[a]	Size (bp) [b]	Ta (°C) [c]	Eff. (%)[d]	R^2
***16S* rRNA gene amplicon sequencing**	*All bacteria* (universal *16S*)	Klindworth et al. (2013)	341F 805R	CCTACGGGNGGCWGCAG GACTACHVGGGTATCTAATCC	450	55	–	–
Quantitative (q) PCR	*All bacteria* (universal *16S)*	Apprill et al., 2015; Parada et al., (2016)	515FY 806RN	GTGYCAGCMGCCGCGGTAA GGACTACNVGGGTWTCTAAT	291	50	89.4	0.99
	Shewanella 16S	Li et al. (2018)	640F 815R	RACTAGAGTCTTGTAGAGG AAGDYACCAAAYTCCGAGTA	195	61	82.5	0.99
	Massilia 16S	This paper	MassF2 MassR3	GAAACGGTAGRGGCTAATATCCT CAAGCCTTGCAGTCTCCATC	205	59	97.1	1.00
	Carnobacterium 16S	This paper and Cailliez-Grimal et al., (2005)	CarF2 CarR1(CB1)	TACGGGAGGCAGCAGTAGGGAATC CCGTCAGGGGATGAGCAGTTAC	143	61	94.6	1.00
	Janthinobacterium16S	This paper	JantF2 JantR3	AGAAACGGTGAGRGCTAATATCTC CTAGCCTTGCAGTCTCCAATG	206	60	97.7	1.00
	Pseudomonas 16S	Najafpour et al. (2022)	PuF1 PuR2	ACCGCATACGTCCTACGG CGAAGACCTTCTTCACACACG	250	61	91.1	1.00
Cloning and sequence (identification of main species)	*Shewanella 16S*	This paper and Li et al., (2018)	Shewa93F-697 815R	GGATAACAGTTGGAAACGACT AAGDYACCAAAYTCCGAGTA	697	59	–	–
	Massilia 16S	This paper	MassF3-451 Mass_R3	GATCGCAAGACCTCATGCTCC CAAGCCTTGCAGTCTCCATC	451	59	–	–
	Carnobacterium 16S	Najafpour et al. (2022)	CarF1 (CB2R) CarR1 (CB1)	ACATTCGGAAACGGATGCTAAT CCGTCAGGGGATGAGCAGTTAC	340	61	–	–
	Janthinobactrium 16S	This paper	JantF3-451 JantR3	GATCGCAAGACCTCATGCTCG CTAGCCTTGCAGTCTCCAATG	451	59	–	–

[a] Primers degenerate bases code: R = A or G; D = A or G or T; Y= C or T.
[b] Amplicon size in base pairs (bp).
[c] Optimized annealing temperature used for each pair of primers.
[d] Primer pair efficiency. In the primer names, "Mass" is used to denote *Massilia*, "Car" for *Carnobacterium*, "Jant" for *Janthinobacterium*, "Pu" for *Pseudomonas* and "Shewa" for *Shewanella*.

Massilia and *Janthinobacteria*, each primer pair was tested on their respective standard curves (Mass205.B or Jant206.B dilutions) and confirmed no significant cross-amplification (Ct was at least 20 cycles higher than using the specific standard-curve). Optimized qPCR assay conditions were used to quantify total or genus-specific bacterial *16S* levels in DNAs from unprocessed/HPP sea bass fillets of aquaculture origin (3 sea bass/group, Anjos et al. (2019)) stored for up to 11 days at 2 °C.

2.5. Monitoring retail fish fillets under refrigerated storage

Four commercial sea bass (from an offshore Greek aquaculture) or four sea bream (from a tank-based aquaculture in Turkey) were purchased at a local supermarket (Montenegro, Portugal), filleted, and transferred to CCMAR in polystyrene boxes with ice and stored between 2-4 °C. Duplicate samples of 100–160 mg of white muscle were immediately excised at the surface (0.2–0.3 cm deep) of the central part of one fillet per fish, using sterilized dissection material in a laminar flow cabinet. Samples were weighed, frozen on dry ice and stored at −80 °C until DNA extraction of 1–2 samples/fillet (within 2 weeks). Approx. 5 g of white muscle were excised from the same fillet region, added to 9 mL of sterile Ringer solution (NaCl 2.25 g/L; KCl 0.105; $CaCl_2$(6H2O) 0.12; $NaHCO_2$ 0.05) per g of tissue in sterilized plastic bags. Fillet samples were hand-homogenized by squeezing the bag for 2 min and placing on ice. For TVC determination, ten-fold serial dilutions of each homogenate (10^0 to 10^5) were prepared in Ringer and 3–4 dilutions were plated on duplicate Plate Count Agar (PCA) per individual and time. Plates were incubated at 25 °C for 48 h, colonies were enumerated and expressed as the average Log10 of counted colony-forming units (cfu) per gram of tissue (Log cfu/g), calculated from duplicate plates of selected dilution (s).

After the initial sampling (day 1), fillets were stored in polystyrene boxes separated by plastic layers and refrigerated at 2-4 °C in a domestic fridge until sampling at days 7 and 14. Muscle samples were excised from an adjacent area to the first sample in each fillet and stored until DNA extraction or directly used for TVC determination.

Total DNA was extracted from 100 to 160 mg of fish fillet samples using the DNeasy Blood&Tissue Kit (Qiagen) with small modifications (optional lysozyme treatment and mechanical disruption with glass beads), detailed in Suppl.file S1 and Anjos et al. (2019). DNA extracts were eluted in 30 μL of 10 mM Tris-HCl pH 8 and their quality and integrity were analysed using a Nanodrop spectrophotometer and 1% agarose gel electrophoresis. DNAs were diluted to 5 ng/μL and 2 μL were used in duplicate in each qPCR assay (section 2.2), and the results expressed as copies of bacterial *16S* rRNA gene/ng of muscle DNA.

2.6. Statistics

Statistical analyses were run using SigmaStat3.50 (SYSTAT) on Log10 transformed data for bacterial TVC (cfu/g), qPCR levels of all or specific bacterial genera DNAs (*16S* copies/ng DNA) or bacterial DNA levels estimated by *16S* metabarcoding (sum of relative proportions of all sequences assigned to each bacterial genus), with $p < 0.05$ as the significance threshold. After verifying normality and equal variance (Shapiro-Wilk and Brown-Forsythe tests), differences in bacterial *16S* levels determined by qPCR between control/HPP treatments or between storage times (section 2.5) were evaluated by two-way ANOVA followed by a post-hoc Holm-Sidak test. Differences in TVC or qPCR *16S* levels

Table 2
In silico specificity predictions for the optimized primers with LPSN representative species.

Primer names/target gene [a]	Genus in alignment [b]	Nr. species [c]	Eligible [d]	Matches [e]	Coverage [f]	Species that may not be amplified [g]
640F/815R (*Shewanella 16S*)	*Shewanella* (Suppl.Fig. S1)	85	79	71	89.9	*S.amazonensis, S.avicenniae, S.mangrovi, S.dokdonensis, S. fodinae, S. glacialipiscicola, S.irciniae, S. benthica.*
PuF1/PuR2 (*Pseudomonas 16S*)	*Pseudomonas* (Suppl. Fig.S2)	320	311	282	90.7	*P. turukhanskensis, P. beteli, P. geniculata, P. cissicola, P. oryziphila, P. hibiscicola, P. carboxydohydrogena, P. siderocapsa, P. boreopolis, P. vranovensis, P. saliphila, P. savastanoi, P. guariconensis, P. wadenswilerensis, P. profundi, P. mendocina, P. parafulva, P. tructae, P. sihuiensis, P. aeruginosa, P. bharatica, P. endophytica, P. oryzihabitans, P. kirkiae, P. rhizoryzae, P. cerasi, P. luteola, P. entomophila, P. khazarica.*
CarF2/CarR1(CB1) (*Carnobacterium 16S*)	*Carnobacterium* (Suppl.Fig.S3)	13	13	12	92.3	*C. mobile*
Najafpour et al. 2022 MassF/MassR [A](*Massilia 16S*)	*Massilia* *Janthinobacterium* (Suppl.Fig.S4)	64 9	64 9	62 0/9 [B]	96.9 0/100 [B]	*M. horti, M.brevitalea* N.a.
MassF2/MassR3 novel primers (*Massilia 16S*)	*Massilia* (Suppl.Fig.S4)	64	64	1	1.6	*M.aerilata, M.agilis, M.agri, M.albidiflava, M.alkalitolerans, M. antarctica, M.antibiotica, M.aquatica, M.arenae, M.arenosa, M. armeniaca, M.aromaticivorans, M.arvi, M.atriviolacea, M.aurea, M. brevitalea, M.buxea, M.cellulosiltyt, M.chloroacetimidivorans, M. consociata, M.dura, M.eburnea, M.eurypsychrophila, M.flava, M. forsythiae, M.frigida, M.ginsengisoli, M.glaciei, M.guangdongensis, M. haematophila, M.horti, M.humi, M.jejuensis, M.kyonggiensis, M.lurida, M.lutea, M.mucilaginosa, M.namucuonensis, M.neuiana, M.niabensis, M. niastensis, M.norwichensis, M.oculi, M. phosphatilytica, M.pinisoli, M. plicata, M.polaris, M.psychrophila, M.puerhi, M.putida, M.rhizosphaerae, M.rivuli, M.rubra, M.soli, M.solisilvae, M.terrae, M.tieshanensis, M. timonae, M.umbonata, M.varians, M.violacea, M.violaceinigra, M. yuzhufengensis.*
	Janthinobacterium (Suppl.Fig.S4)	9	9	0	0	*J.agaricidamnosum, J.aquaticum, "J.kumbetense", J.lividum, "J. psychrotolerans", J.rivuli, "J.svalbardensis", "J.tructae", J. violaceinigrum.*
JantF2/JantR3 (*Janthinobacterium 16S*)	*Janthinobacterium* (Suppl.Fig.S4)	9	9	5	55.6	*J.agaricidamnosum, J.violaceinigrum, "J.psychrotolerans", "J. svalbardensis".*
515F–Y/806R–N (all bacteria, qPCR)	*Shewanella* (Suppl. Fig. S1)	85	82	82	100.0	N.a.
	Pseudomonas (Suppl. Fig.S2)	320	307	301	98.0	*P. turukhanskensis, P. otitidis, P. persica, "P. capeferrum", P. reinekei, P. furukawaii.*
	Carnobacterium (Suppl.Fig.S3)	13	12	12	100.0	N.a.
	Massilia (Suppl.Fig.S4)	64	64	61	95.3	*M.yuzhufengensis, M.aurea, M.ginsengisoli.*
	Janthinobacterium (Suppl.Fig.S4)	9	9	9	100.0	N.a.
341F_Klw/805R_Klw (all bacteria, metabarcoding)	*Shewanella* (Suppl.Fig. S1)	85	82	80	97.6	*S. sedimentimangrovi, S. vesiculosa.*
	Pseudomonas (Suppl. Fig.S2)	320	304	299	98.4	*P. turukhanskensis, P. otitidis, P. persica, "P. capeferrum", P. reinekei.*
	Carnobacterium (Suppl.Fig.S3)	13	12	12	100.0	N.a.
	Massilia (Suppl.Fig.S4)	64	64	64	100.0	N.a.
	Janthinobacterium (Suppl.Fig.S4)	9	9	9	100.0	N.a.

The specificity of all pairs of primers [a] (see Table 1 for details) was evaluated *in silico* based on their match to the LPSN (List of Prokaryotic names with Standing in Nomenclature) representative species of each genus [b] (multiple sequence alignments with primer matches highlighted in Figs. S1–S4). In the primer names, "Mass" is used to denote *Massilia*, "Car" for *Carnobacterium*, "Jant" for *Janthinobacterium*, "Pu" for *Pseudomonas* and "Shewa" for *Shewanella.*
Columns represent the total nr. of species for each genus present in the LPSN database [c], those eligible for evaluation as they had available sequences in the analysed primer target region [d], the species matched by both primers with 100% sequence identity [e], the calculated coverage of the primers = ratio between the number of matched and eligible species [f] and the list of species unlikely to be amplified with the primers = those with at least one mismatch in either the forward or reverse primer [g]. Note: genus names are abbreviated as M. (*Massilia*), C. (*Carnobacterium*), J. (*Janthinobacterium*), P. (*Pseudomonas*) and S. (*Shewanella)*. The names of some species are indicated between "" according to their designation at LPSN, meaning that their names are not validly described. N.A. = not applicable (used when all species of the genus were completely matched by both primers).
[A] Indicates the evaluation of *Massilia* genus-specific primers previously optimized for fish eggs in Najafpour et al., 2022.
[B] Indicates the number of matches or coverage if one mismatch was allowed, evaluated for the previous *Massilia* genus-targeted primers (Najafpour et al., 2022).

between storage times (section 2.5) were assessed by one-way ANOVA and the Holm-Sidak test. For cases not passing normality (TVC in sea bream), non-parametric Kruskal-Wallis ANOVA on ranks and Dunn's test were used. Pearson correlations were run between Log10 transformed measurements in each experiment and only correlations with a coefficient (r) > 0.5 or < -0.5 and with $p < [0.05/(\text{nr. of comparisons})]$ were considered significant (Bonferroni correction, see Table 3).

Table 3
Correlations between measurements in the refrigerated storage experiments (Log10 applied to all variables).

A- European sea bass	*Universal 16S*	*Shewanella 16S*	*Carnobacterium 16S*	*Pseudomonas 16S*	*Massilia 16S*	*Janthinobacterium 16S*
Bacterial TVC (cfu/g)	r=0.96	r=0.94	r=0.99	r=0.98	r=-0.20	r=0.96
	p=6.7E-07	p=5.1E-06	p=2.2E-09	p=2.5E-08	p=5.3E-01	p=4.5E-07
Universal 16S (total bacterial DNA)		r=0.97	r=0.98	r=0.98	r=-0.16	r=0.98
		p=1.7E-07	p=8.7E-09	p=7.8E-08	p=6.2E-01	p=8.3E-09
Shewanella			r=0.96	r=0.99	r=-0.13	r=0.94
			p=1.0E-06	p=2.5E-09	p=6.8E-01	p=7.1E-06
Carnobacterium				r=0.98	r=-0.18	r=0.98
				p=1.2E-08	p=5.7E-01	p=5.3E-08
Pseudomonas					r=-0.17	r=0.96
					p=6.0E-01	p=9.4E-07
Massilia						r=-0.17
						p=6.0E-01
B- Gilthead sea bream	***Universal 16S***	***Shewanella 16S***	***Carnobacterium 16S***	***Pseudomonas 16S***	***Massilia 16S***	***Janthinobacterium 16S***
Bacterial counts, TVC (cfu/g)	r=0.97	r=0.97	r=0.93	r=0.98	r=-0.57	r=0.95
	p=2.1E-07	p=9.6E-08	p=1.2E-05	p=4.5E-08	5.50E-02	p=2.0E-06
Universal 16S (total bacterial DNA)		r=1.00	r=0.98	r=1.00	r=-0.52	r=0.96
		p=4.5E-12	P=5.0E-08	p=4.8E-13	p=8.2E-02	p=5.0E-07
Shewanella 16S			r=0.96	r=1.00	r=-0.54	r=0.95
			p=5.9E-07	p=5.2E-12	p=6.9E-02	p=2.2E-06
Carnobacterium 16S				r=0.96	r=-0.46	r=0.95
				p=3.9E-07	p=1.4E-01	p=1.9E-06
Pseudomonas 16S					r=-0.52	r=0.97
					p=8.2E-02	p=2.9E-07
Massilia 16S						r=-0.47
						p=1.2E-01

In the central cells of the table, r = Pearson correlation coefficients and *p = probability* value; grey indicates non-significant correlations with p<0.008 (Bonferroni correction applied to the 0.05 significance threshold, see methods section). TVC = total viable counts, expressed in cfu = colony-forming units. *16S* = detection of *16S* rRNA gene copies, using quantitative PCR.

3. Results and discussion

3.1. Dominant bacterial genera identified in fish fillets

Five relevant genera were selected as targets for *16S*-based qPCR development and monitoring (Fig. 1), based on their high or altered proportions in the *16S* metabarcoding bacterial community profiles previously characterized in control or HPP refrigerated sea bass fillets from Greek aquaculture origin (Tsironi et al., 2019 and unpublished results, accessions in the Data Statement). Fresh fillets (stored 1 day at 2 °C) were mainly colonized by bacteria belonging to *Shewanella* or *Massilia*, accounting for up to 72%–88% of total bacteriomes. Unprocessed fillets stored for 11 days at 2 °C had higher proportions of *Pseudomonas* or *Carnobacterium* (up to 28%–72%) and increased *Janthinobacterium* proportions were found in HPP fillets.

Shewanella, Pseudomonas and *Carnobacterium* are common food spoilers, reported in fresh unprocessed or processed fillets from marine or freshwater fish and during refrigerated storage (Chaillou et al., 2015; Jérôme et al., 2022; Møretrø et al., 2016; Tsironi et al., 2019; Zotta et al., 2019). Members of the genus *Massilia* are widespread bacteria detected in environmental soils and waters, drinking and waste waters, plants and in wild or aquaculture fish gut, larvae and refrigerated fillets (Bikouli et al., 2021; Huyben et al., 2020; Kämpfer et al., 2011; Li et al., 2014; Najafpour et al., 2022; Ofek et al., 2012; Zotta et al., 2019). The closely-related genus *Janthinobacterium* is commonly isolated from soil and environmental freshwaters and was detected at low levels in the microbiota of seaweeds, vegetables, meat and fish products (salmon, cod, hake and plaice refrigerated fillets) and on surfaces of a variety of food facilities, being associated with vegetable spoilage in synergy with other spoilage bacteria (Chaillou et al., 2015; Leff & Fierer, 2013; Lincoln et al., 1999; Picon et al., 2021; Reynisson et al., 2009; Xu et al. 2022, 2023; Zotta et al., 2019).

3.2. qPCR assays design, optimization and evaluation

Two of the genus-specific *16S* rRNA gene primer pairs available in the literature passed our evaluation of qPCR specificity/performance, using *in silico* methods and experimental confirmation in sea bass fillets as detailed in sections 2.1 to 2.4. The Shewa640F/815R primers targeting 195-bp of the *Shewanella 16S* V4 region, designed by Li et al. (2018) for their detection in environmental and wastewater samples, had a perfect sequence match to 71 out of 79 eligible *16S* sequences for *Shewanella* species in the curated LPSN database (June 2022), corresponding to an 89.9% coverage in this region (Fig. S1, Table 2). The primers also shared 100% identity with the *16S* metabarcoding-obtained sequence "ShewAY573039.M" from control and HPP sea bass fillets, with no significant match for sequences of other identified abundant genera, indicating good specificity (Fig. S1). The TestPrime automatic prediction using the SILVA138.1 *16S* database release for the 640F/815R primers gave 84.2% coverage of the genus *Shewanella* (Table S1), a similar level of magnitude to the 87% predicted by Li et al. (2018) and higher than that of the available primers 20F/220R (Himmelheber et al., 2009).

The corresponding PCR product was amplified from pooled DNAs of

control sea bass fillets stored for 11 days at 2 °C (C-11d) with expected size and sequence ("Shew195.B", GenBank deposit OQ154854) that was clearly assigned to the genus *Shewanella* by public database searches and phylogenetic analysis clustering (Table S2 and Fig. 2). The optimized qPCR assay complied with quality criteria defined in 2.4 (namely with the amplification of a single, specific product from control/HPP sea bass fillet DNA and an efficiency over 85%, Table 1), and was applied to quantify *Shewanella 16S* DNA abundance in fish fillets from aquaculture or retail sources (sections 3.5 - 3.6).

The PuF1/PuR2 primers previously optimized to quantify *Pseudomonas*-genus members DNA in fish eggs and larvae from Mediterranean aquaculture sites (Najafpour et al. 2022, 2023) matched 282 LPSN representative species of this large genus (Fig. S2, Table 2) with 90.7% alignment coverage. The TestPrime predicted coverage was 67.5%,

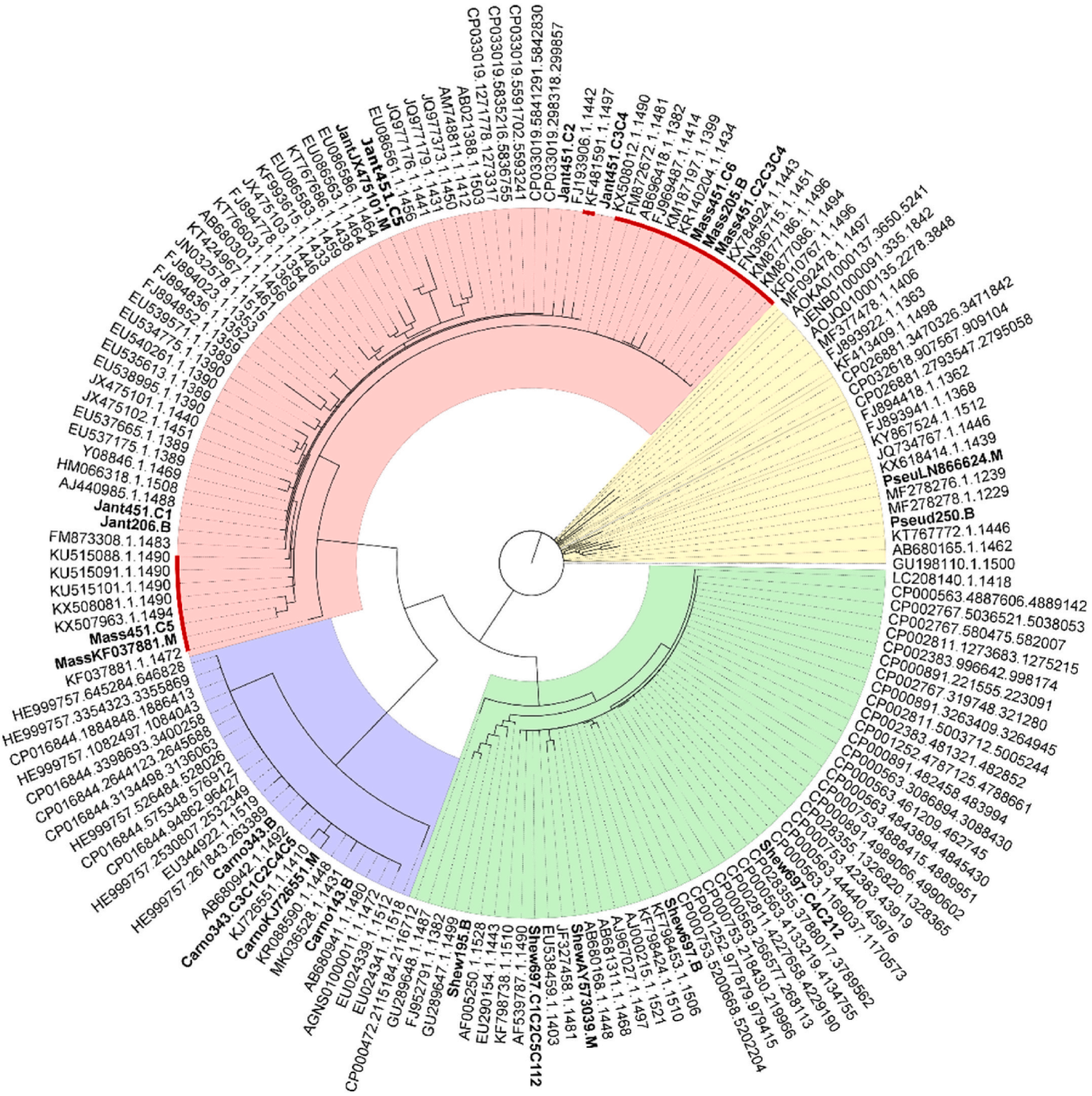

Fig. 2. Phylogenetic tree clustering the quantitative PCR amplicons for the *16S* rRNA gene of the five target genera with sequences from the SILVA database. The clustering was used to confirm primer specificity. The phylogenetic tree was built with the Aligner tool (https://www.arb-silva.de/aligner/) using the default options as previously described (Najafpour et al., 2022); all isolated qPCR amplicons (indicated by.B after their designation) and clones (indicated by.C) from sea bass fillets are highlighted in bold and their accession numbers are listed in Table S2 (clones that had identical sequences were submitted under the same accession number); the number of neighbours (SILVA sequences) per query sequence was set to 10 and their identities can be elucidated using their accession numbers at https://www.arb-silva.de/search/. Colours highlight the clades of SILVA sequences annotated as *Carnobacterium* (purple), *Shewanella* (green), *Pseudomonas* (yellow) or as *Massilia/Janthinobacterium* (common clade in pink, where sequences annotated as *Massilia* are highlighted with a red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

probably reflecting a biased representation among the 7333 *Pseudomonas 16S* sequences in the redundant SILVA database (Table S1). The PuR2 primer perfectly matched the *16S* metabarcoding sequence PseuLN866624.M and no significant match occurred with other abundant genera; the forward primer PuF1 was not within the sequence of the previous metabarcoding experiments but was predicted to anneal to 290 of 311 *Pseudomonas* LPSN representative sequences (Fig. S2, Table 2). The PuF1/PuR2 amplicon "Pseud250.B″ obtained from HPP sea bass fillets after 11 days of refrigeration (HPP-11d) was confirmed to be *Pseudomonas* (Fig. 2, Table S2), the genus-specific *16S* qPCR assay was compliant with the defined quality criteria (Table 1) and was used for commercial samples analyses (sections 3.5 and 3.6).

The best performance for *Carnobacterium 16S* qPCR was obtained combining the novel CarF2 primer with the reverse CarR1-CB1 primer (Table 1) previously used to detect the *Carnobacterium* genus in meat and cheese samples (Cailliez-Grimal et al. 2005, 2007; Nissen et al., 1994). The CarF2/CarR1 pair that targeted a 143-bp amplicon in the *16S* rRNA gene V3 region of *Carnobacterium*-genus members, had a perfect match to 12/13 LPSN *Carnobacterium* species sequences and to "CarnoKJ726551.M″, and not to the other abundant *16S* metabarcoding sequences (Fig. S3, Table 2), with 82% of automatic coverage prediction (Table S2). The single amplicon "Carno143.B″ from pooled DNAs of HPP-11d refrigerated sea bass fillets was clearly assigned to the genus *Carnobacterium* (Fig. 2, Table S2), the qPCR assay presented 94.6% efficiency, passed all quality controls and was also applied to commercial samples.

The MassF/MassR *16S* primers previously used to quantify genus *Massilia* members in sea bass/sea bream eggs and larvae from Mediterranean aquacultures (Najafpour et al. 2022, 2023) were predicted to cross-react with the related genus *Janthinobacterium* also detected in sea bass fillets (Tsironi et al., 2019). MassR matched the *16S* rRNA gene of 97% *Massilia* species but also all nine *Janthinobacterium* LPSN representative species, and MassF could match all *Massilia* and *Janthinobacterium* species if one mismatch was allowed (Fig. S4, Table 2).

To improve the specific detection of abundant *Massilia* species in fish fillets, novel primers (MassF2/MassR3) were designed to target the metabarcoding MassKF037881.M sequence isolated from control/HPP sea bass fillets at a 205-bp region (between V3–V4) of the *16S* rRNA gene that had lower conservation between these related genera (Fig. S4, Table 1). The same region was targeted by JantF2/R3 primers, which had higher specificity and performance than several primers designed to distinguish *Janthinobacterium 16S* sequences from *Massilia* members. Sensitive amplification and high efficiencies were verified in sea bass fillets using the novel *Massilia* or *Janthinobacterium 16S* primer pairs (Table 1), while the use of related-genus primers (e.g. MassF2/R3 primers with the Jant206.B standard curve) confirmed the absence of significant cross-reaction between the qPCR assays of the two related genera.

This increased discrimination was however accompanied by a lower species coverage within each genus (Fig. S4). Three *Massilia* representative species (*M. oculi, haematophila* and *suwonensis*) had 100% identity with the MassF2 primer (and *M. puerhi, niabensis, atriviolacea* and *psychrophila* if one mismatch was allowed) while MassR2 matched 10 species (33 if one mismatch was allowed), and 5 out of 6 of the *Massilia* amplicons from sea bass muscle gave a 100% match to the MassF2/R3 primers (Fig. S4). All nine *Janthinobacterium* species and the six *16S* amplicons had 100% sequence identity with the JanR3 primer, while five *Janthinobacterium* species and 5/6 of the sea bass muscle amplicon sequences shared 100% identity with both JantF2/R3 primers (Fig. S4, Table 2). Specificity of these primer pairs was further confirmed by sequencing the 205-bp amplicons isolated from HPP-11d refrigerated sea bass fillets and their clustering in the phylogenetic tree (Table 2, Fig. 2).

Massilia and *Janthinobacterium* are closely related genera from the family Oxalobacteraceae (order Burkholderiales, subclass Betaproteobacteria) of Gram-negative, mainly aerobic, nonspore-forming bacteria (Baldani et al., 2014). *Massilia* has recently been placed in the *Telluria* genus (Bowman, 2023), but designation as *Massilia* continues to be valid and included at LPSN.

Given the reported meso-to psychrophilic characteristics of family Oxalobacteraceae and wide occurrence in soil, aquatic environments, plant and animal samples (Baldani et al., 2014; Chaillou et al., 2015; Kämpfer et al., 2011; Ofek et al., 2012), the co-detection in refrigerated sea bass fillets in both *16S* rRNA gene metabarcoding and qPCR is not surprising. Previous co-occurrence of *Massilia* and *Janthinobacterium* genus-members was detected by *16S* metabarcoding in refrigerated cod and plaice (Reynisson et al., 2009; Zotta et al., 2019). In aquaculture sea bream and sea bass eggs, larvae and water, *Massilia* bacteria (but not *Janthinobacterium*) were detected in *16S* metabarcoding surveys and a *16S*-based genus-specific qPCR assay (primers MassF/R, Tables 1 and 2) was developed (Najafpour et al., 2021, 2022, 2023). In sea bass fillets, our *in silico* and *in situ* evaluations indicated MassF/R cross-react with the *16S* of *Janthinobacterium* bacteria when they are present in samples and thus novel qPCR pairs MassF2/R3 and JantF2/R3 were designed and validated. These qPCR assays had efficiencies above 97% and were highly discriminative between the two genera (although they may miss some species within the respective genera and underestimate their abundance). They could amplify the most abundant *Massilia* or *Janthinobacterium* sequences previously detected in unprocessed or HPP sea bass fillets (Tables 1 and 2, Suppl.Tables S1–S2 and Fig. 2) by *16S* metabarcoding (Tsironi et al., 2019) and were thus used to monitor *Massilia* and *Janthinobacterium 16S* DNAs during refrigerated storage of commercial fillets (sections 3.5 and 3.6).

3.3. Universal 16S qPCR to assess total bacterial DNA loads in fish fillets

Universal primers 515FY/806RN targeting the *16S* rRNA gene V4 region of all bacteria (Table 1), previously used by the Earth Microbiome Project (EMP, 2018) to estimate bacterial diversity by metabarcoding were evaluated for the qPCR detection of *16S* of all bacteria in fish fillets.

High coverage of 515FY/806RN was predicted for the five abundant target genera in the LPSN alignments (95.3–100% coverage) with only 9 representative species not fully matched among the 488 evaluated, and the automatic method predicted 73.2–89% coverage of the five genera (Figs. S1–S4; Table 2 and Suppl. File S1).

The universal *16S* rRNA gene qPCR assay (Table 1) was previously applied with success to compare total bacterial loads between fish eggs and larvae from different aquaculture sites, ages or treatments (Najafpour et al. 2021, 2023). In this study, the 515FY/806RN qPCR assay had good efficiencies using refrigerated sea bass fillet DNAs (Table 1), the outcome of amplicon cloning and sequencing confirmed the dominance of *Massilia*, *Shewanella* and *Carnobacterium* bacteria, and the primers were used for commercial samples (sections 3.5 and 3.6).

3.4. Cloning and phylogenetic analysis to confirm the main bacteria in sea bass fillets

Amplification of longer fragments (250-697-bp, Table 1), cloning, sequence and phylogenetic analyses confirmed the main bacterial genera present in control and HPP sea bass fillets (Fig. 2). All five *Carnobacterium* isolated from HPP-11d refrigerated sea bass fillet pooled DNAs had a similar sequence (GenBank Najafpour et al. 2021, 2023) and clustered with the metabarcoding sequence CarnoKJ726551.M, amplicon Carno143.B and to SILVA database entries of *16S* rRNA genes assigned to C. *maltaromaticum* or *Carnobacterium* sp. (Fig. 2). All *Pseudomonas* sequences amplified from HPP-11d sea bass fillets clustered closely with *P. fluorescens*; amplicons of *Carnobacterium* and *Pseudomonas* presented single products when directly sequenced that together with their phylogenetic clustering suggests the presence of one main species for each of these genera in sea bass fillets (Fig. 2).

Shewanella amplicons from HPP-11d sea bass fillets represented two main sequence clusters sharing 98% sequence identity (Fig. 2, Table S2).

The *Shewanella* amplicons clustered with available sequences assigned to *S. baltica*, *S. putrefaciens* or *Shewanella* sp., confirming the specificity of the primers for this genus but not allowing species discrimination or suggesting a mixture of species were present in the fillet samples.

Sequencing of several clones for the 451-bp amplicons isolated from control-11d (*Massilia*) or HPP-11d (*Janthinobacterium*) revealed they shared 98%–99% sequence identity within each genus (approx. 94% when comparing the two genera) and clustered in a large, related clade (Fig. 2 and Table S2), in agreement with their reported phylogenetic relatedness (Baldani et al., 2014). The *16S* rRNA sequence entries

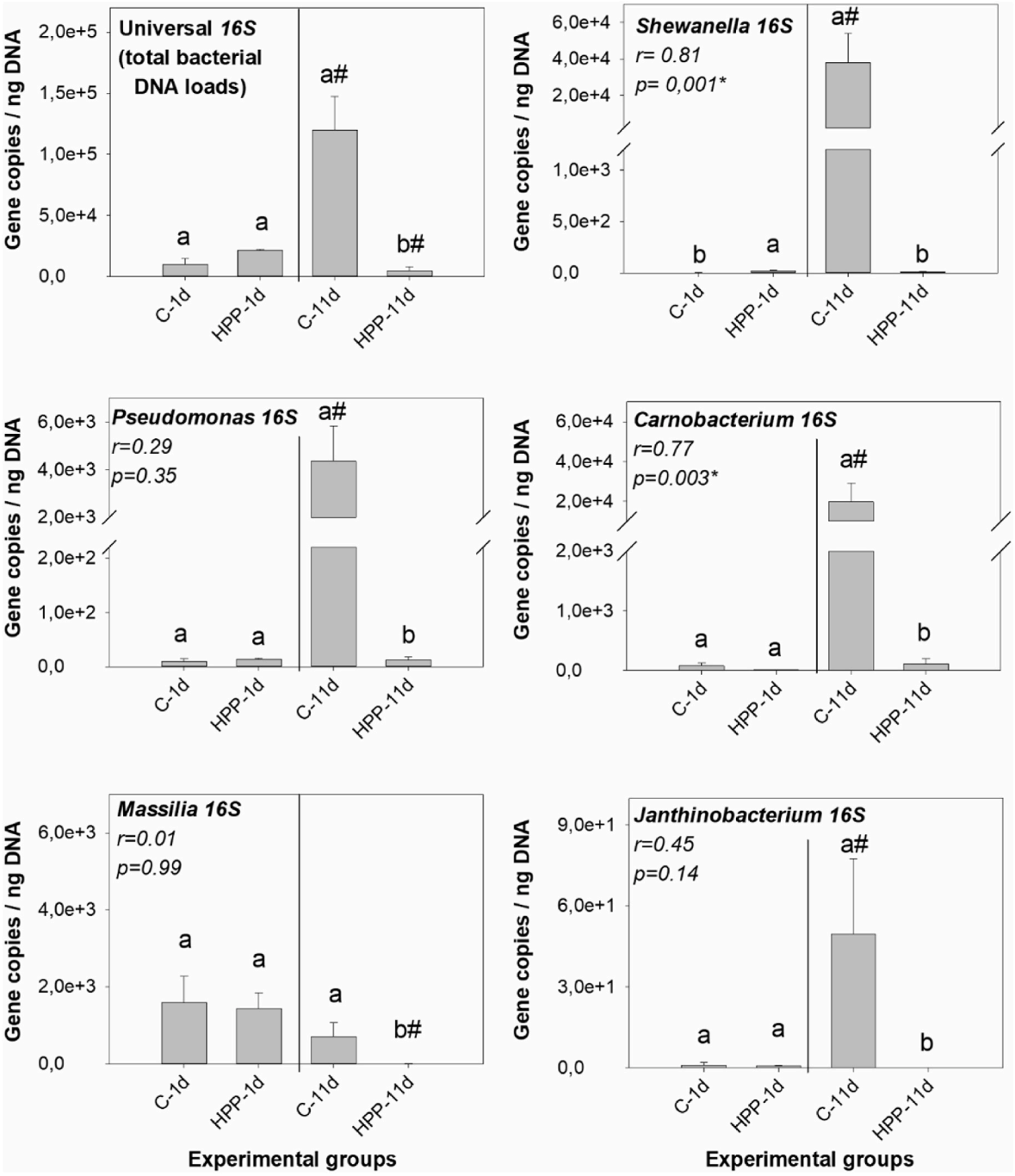

Fig. 3. Quantitative (q) PCR detection of total bacterial load DNAs (using universal primers for the *16S* rRNA gene as target) and of DNAs of the five selected bacterial genera in control or HPP (high-pressure processed) sea bass fillets (using *16S*-based genus-specific primers), with the correlation with the metabarcoding results. The results are represented as the mean ± SEM of the copy number of each bacterial group quantified using the optimized qPCR assays for each target (Table 1); these values were normalized by the amount of sea bass fillet DNA used per reaction (10 ng) and are represented as total or genus-specific *16S* gene copies/ng DNA, for n = 3 individual sea bass fillets from each experimental group: control (non-processed, C) fillets and fillets processed by 600 MPa for 5 min at 25 °C (HPP) as described in Anjos et al., 2019, stored for 1 or 11 days (d) under controlled isothermal (2 °C) conditions. Different letters within each sampling time indicate significant differences between the control and HPP fillets (starting with groups with higher compared value) at this time, and # means significant changes in each group (C or HPP) at 11 days compared to 1 day, tested by two-way ANOVA ($p < 0.05$). Pearson correlation coefficients (r) and *p* values are presented for the correlations between the qPCR copy numbers determined by qPCR assays and the relative abundance of the genera in the total bacterial community profile estimated by *16S* rRNA gene metabarcoding, in the same sea bass fillet DNAs.

(SILVA Aligner, 2012) clustered in this big clade were annotated as *Janthinobacterium* sp/*uncultured, J. lividum* or *J. agaricidamnosum* and included the respective *16S* metabarcoding sequences and qPCR amplicons. Sequences that clustered with the sea bass fillets *Massilia* amplicons were mainly annotated as "*Massilia* genus" members without species discrimination.

3.5. Bacterial detection by 16S-qPCR and metabarcoding in control and HPP sea bass fillets

The six optimized qPCR assays were applied to compare total and genus-specific bacterial DNA between control and HPP sea bass fillets stored for 1–11 days at 2 °C (Fig. 3). Significant increases in total bacterial and *Shewanella*, *Pseudomonas*, *Carnobacterium* and *Janthinobacterium 16S* levels were detected in control fillets after 11 days of refrigeration. This indicates a likely association with spoilage and coincides with the evolution of fillet deterioration, indicated by altered sensorial characteristics (appearance, odour, taste and texture) and increased bacterial TVC observed in control fillets stored for 11 days in the previous experiment (Tsironi et al., 2019). In contrast, *Massilia 16S* levels in control sea bass fillets were stable over the refrigerated storage but were significantly reduced by HPP compared to the control fillets after 11 days, which could make this genus a useful proxy for common microbiota in fish fillets. A significant decrease in the *16S* levels of all microbiological markers was detected by qPCR in the HPP treatment compared to control fillets after 11 days of refrigeration, revealing the inhibitory effects of this processing method on microbial growth and explaining the increased shelf life of processed fish shown for several species (e.g. Parlapani et al., 2014; Ramirez-Suarez & Morrissey, 2006; Rode & Hovda, 2016; Roobab et al., 2022; Suemitsu & Cristianini, 2019; Tsevdou et al., 2023; Tsironi et al., 2015; Wang et al., 2016).

Significant correlations between the *16S* qPCR copies and metabarcoding sequence proportions were obtained for *Shewanella* and *Carnobacterium*, but not for *Massilia*, *Pseudomonas* or *Janthinobacterium* (Fig. 3). The lack of correlation is unsurprising: metabarcoding is often expressed in relative proportions while qPCR can provide absolute quantification and may be more efficient and specific (Jian et al., 2020; Zemb et al., 2020). The lower correlations in *Janthinobacterium* may be due to the lower sensitivity of metabarcoding and qPCR targeted specific abundant groups of *Massilia* and *Janthinobacterium* in fish muscle, while metagenomics is expected to be more comprehensive (Sequino et al., 2022). Another factor that may explain the discrepancy is the specificity of genus-specific primers and the small amplicon size that improves sensitivity and efficiency of qPCR relative to the highly degenerate universal primers used for metabarcoding (Najafpour et al., 2022; Yu et al., 2022). Although *16S*-based amplicon metagenomics is a powerful culture-independent affordable technology to identify and profile novel microbial targets, many factors still affect its accuracy, coverage and reproducibility, including the presence of PCR inhibitors in contaminated foods, PCR and primer selection biases, sequencing and annotation quality and dependence on public databases (Cao et al., 2017; Liu et al., 2021; Rausch et al., 2019). A good strategy is to use *16S* metabarcoding to identify the bacterial targets most abundant in fish samples complementing it with other molecular-(e.g. specific qPCR assays) or culture-based (enumeration of viable bacteria) approaches to evaluate microbial dynamics between groups, treatments or refrigerated storage, as reported here and in previous studies (Carballo et al., 2019; Dreier et al., 2022; Hassoun et al., 2022; Najafpour et al., 2023; Pinto et al., 2019; Rieder et al., 2023; Sequino et al., 2022; Tsironi et al., 2019).

Despite the low percentages of *Pseudomonas* detected by metabarcoding in all groups, qPCR results (Fig. 3) were consistent with previous culture enumerations (reporting increases in viable *Pseudomonas* in sea bass fillets at 11 compared to one day of refrigerated storage and very low levels in HPP (Tsironi et al., 2019)) and with the reported sensitivity of this psychrotrophic spoilage-associated genera to HPP and/or osmotic dehydration in meat and fish products (Andreou et al., 2018; Tsevdou et al., 2023; Tsironi et al., 2015).

3.6. Application of bacterial qPCR to monitor refrigerated storage of commercial fish fillets

The optimized qPCR assay using universal *16S* primers 515-FY/806R–N (Table 1) confirmed significant increases in total bacterial DNA loads at the surface of both sea bream and sea bass fillets of retail origin when they were stored for 7 days at 2-4 °C, becoming higher after 14 days (Fig. 4). Similar results were obtained using classical culture enumeration, with highly significant Pearson correlations found for both fish species between the two approaches (Fig. 4). TVC levels reached 6.4–8.0 Log cfu/g in sea bass and sea bream fillets (respectively) stored for 7 days and 8.0 Log cfu/g for both species after 14 days, which demonstrate deterioration of microbiological quality above the general acceptability level of 7 Log cfu/g (ICMSF, 1986) and similar to the TVC levels detected in sea bass/sea bream fillets at the end of their shelf life (10–14 days at recommended commercial refrigeration of 0-2 °C and 4–5 days at 5 °C; e.g. Tsironi et al., 2019; Parlapani et al., 2014; Tsironi and Taoukis, 2014; Tsevdou et al., 2023).

The application of the optimized qPCR assays also revealed significant increases in *Shewanella*, *Carnobacterium*, *Pseudomonas* and *Janthinobacterium 16S levels* on sea bream and sea bass fillet surfaces after 7 days of refrigerated storage at 3 ± 0.5 °C, which were further increased by day 14 in the case of *Carnobacterium*, *Pseudomonas* and *Janthinobacterium* (Fig. 5). *Massilia 16S* levels did not significantly change over time compared to the initial fillets (Fig. 5). Finally, strong ($r > 0.9$) and significant ($p < 1.2\ E^{-05}$) positive correlations were found between the *Shewanella, Carnobacterium, Pseudomonas* and *Janthinobacterium 16S* qPCR assay copy numbers and TVC determinations and with bacterial loads quantified with the universal *16S* rRNA gene assay (Table 3), confirming they had a similar growth and contribution as the total bacterial load increased, except for *Massilia*.

3.7. Challenges and relevance of the developed qPCR assays for total and food-borne bacteria

In line with the identified needs for sensitive, culture-independent and less labour-intensive assays to monitor contamination of fish products by specific bacterial groups in the cold chain, and aiming to contribute to a global decrease in loss and waste in the food and retail industries (FAO, 2022; Jian et al., 2020), we developed and validated in commercial samples six qPCR assays for total and food-borne bacteria. Using information from *16S* rRNA metabarcoding and existing primer pairs we designed or optimized highly efficient universal *16S*-based qPCR assays for total bacterial DNA load, genus-specific qPCR assays for *Shewanella, Carnobacterium* and *Pseudomonas* DNAs and specific qPCRs to distinguish and amplify the *16S* rRNA gene for the main species of related genera *Massilia* and *Janthinobacterium* in fish fillets (Table 1). For the latter genera we propose as a strategy for fish processing and retail quality control that an initial fast assessment of *Massilia* and *Janthinobacterium* be made with F2/R3 qPCR followed by Sanger amplicon sequencing. In cases where both genera are present, we recommend using the two novel F2/R3 qPCR assays directed at the main species identified in fish fillets (although they may slightly underestimate genus-level detection). If the tested fish samples are mainly contaminated with *Massilia,* previously developed (MassF/R) qPCR assays (Najafpour et al., 2021) can be used, guaranteeing a wider coverage of *Massilia* species.

The main challenges encountered in the design and validation of these qPCR assays were the underrepresentation of some genera or redundancies in public databases such as SILVA or GreenGenes, although presumably the most abundant bacteria are represented and the recent exponential increase in available *16S* rRNA gene sequences due to high-throughput sequencing is likely to overcome this limitation (Glöckner, 2019; Park & Won, 2018). Improvements in the availability of curated

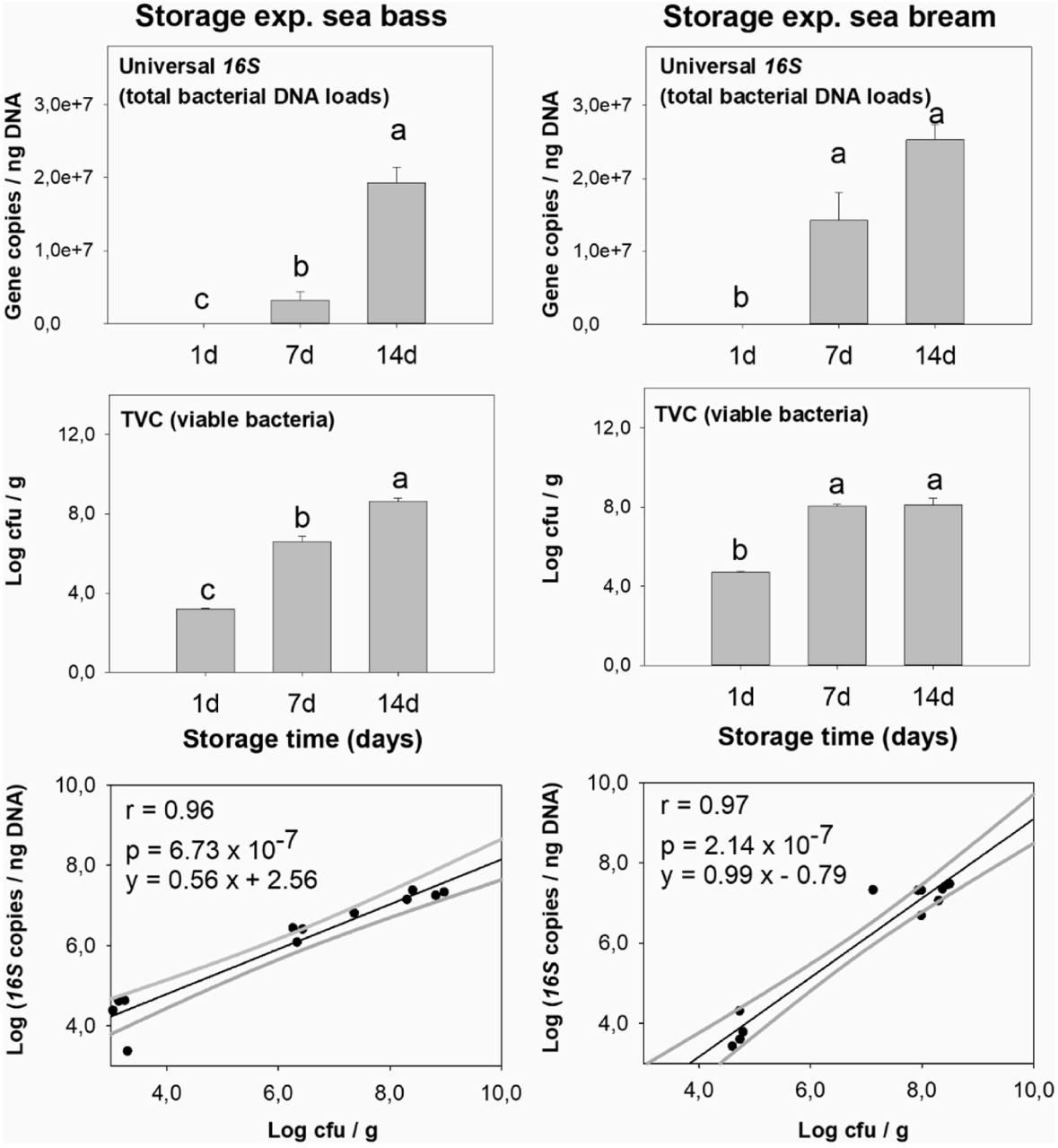

Fig. 4. Quantitative (q) PCR detection of total bacterial load DNAs in commercial fillets from sea bass and sea bream during 14 days of refrigerated storage at 3 ± 0.5 °C, compared to classical culture enumeration of viable bacteria. Results of total bacterial load DNAs are represented in the top panel as the mean ± SEM of the total bacteria copy numbers, quantified using qPCR with *16S* universal primers 515-FY/806R–N (Table 1) and normalized by the amount of fish DNA used per reaction (10 ng), represented as total *16S* gene copies/ng DNA, for n = 4 sea bass or sea bream fillets. In the second panel, total viable counts (TVC) of bacteria in PCA cultures are represented in Log cfu/g for each storage time. Different letters indicate significant differences between times, evaluated by one way ANOVA ($p < 0.05$). The last panel shows the correlation between total bacterial loads estimated using the universal *16S* qPCR assays and by culture enumeration. The Pearson correlation coefficients (r), *p* values, equation of the linear regressions and number of samples are indicated.

databases such as the LPSN (Parte et al., 2020) facilitates primer design and improves estimation of primer specificities and coverage for a given genus.

Complementing *16S* metabarcoding and qPCR with molecular and phylogenetic analyses, we confirmed that filleted and refrigerated sea bass or sea bream from commercial aquaculture and retail origins were mainly contaminated with low levels of *Massilia, Shewanella, Janthinobacterium,* or *Carnobacterium.* Microbial load increased during refrigerated storage (2-4 °C) of the fillets except for *Massilia* and was strongly correlated with fillet quality deterioration and an alteration in sensorial characteristics (Tsironi et al., 2019). Most of these genera have been detected in refrigerated raw fish or meat (Chaillou et al., 2015; Reynisson et al., 2009; Tahiluddin et al., 2022; Zotta et al., 2019). Their occurrence in fish fillets may have resulted from contamination with soil or water or from live fish (likely for *Massilia, Janthinobacterium, Shewanella* or *Pseudomonas*) and/or from cross-contamination in humid production and processing environments due to low hygiene or disinfection during fish handling (likely for *Pseudomonas*, *Carnobacterium* and *Shewanella*) (Chaillou et al., 2015; Møretrø et al., 2016). *Pseudomonas, Shewanella* and *Carnobacterium* are commonly associated with spoilage of refrigerated meat, fish or seafood products (Bagge-Ravn et al., 2003; Chaillou et al., 2015; Jérôme et al., 2022; Møretrø et al., 2016; Pilet

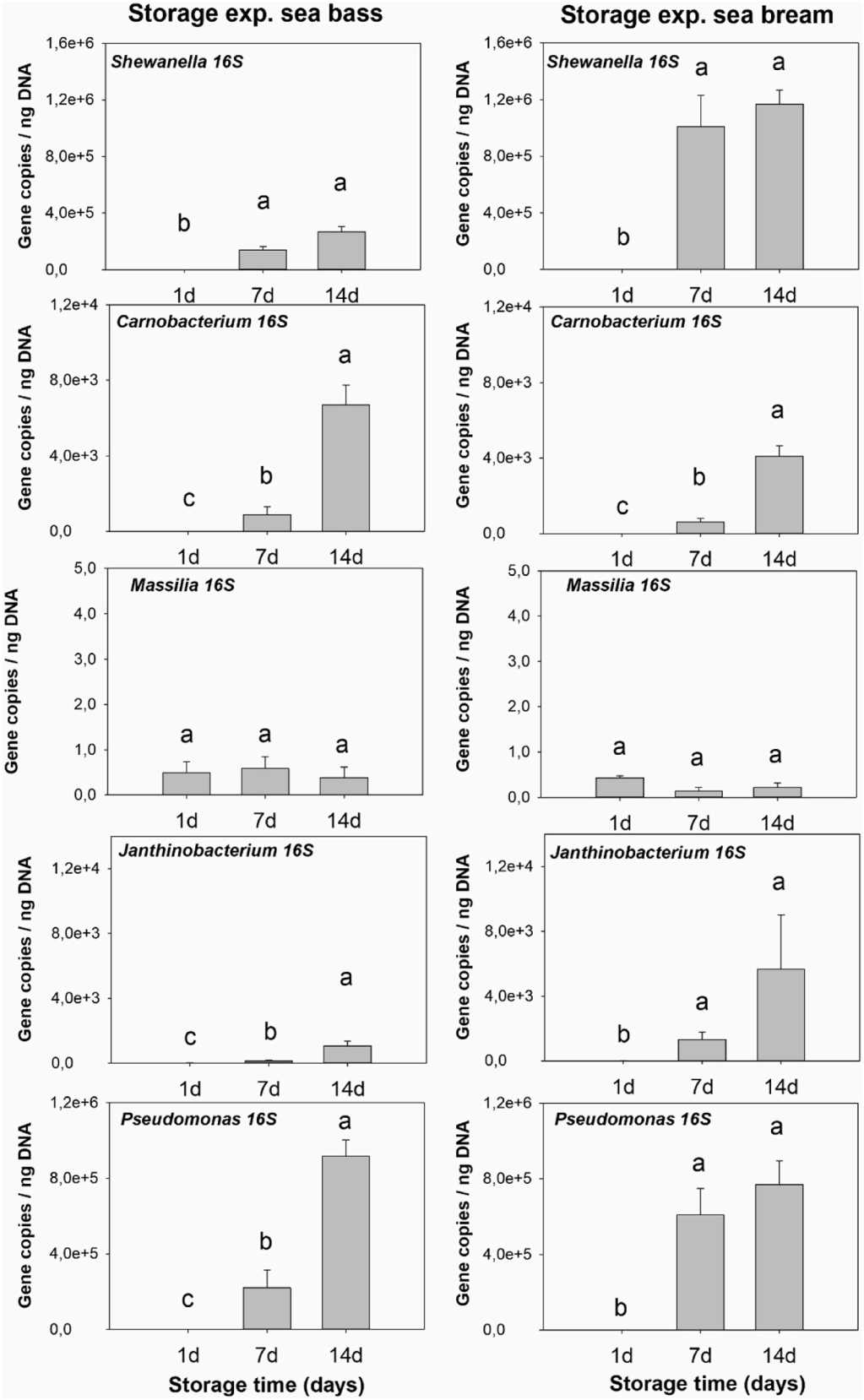

Fig. 5. Quantitative (q) PCR detection of the five selected bacterial genera DNAs in commercial fillets of sea bass and sea bream across 14 days of refrigerated storage at 2 °C, based on *16S* rRNA gene detection. Results are represented as the mean ± SEM of the copy number of each bacterial group *16S* gene quantified using the optimized qPCR assays (Table 1) and normalized by the amount of fish DNA used per reaction (10 ng), for n = 4 individual sea bass or sea bream fillets. Different letters indicate significant differences between sampling times (starting with groups with higher compared value), evaluated by one way ANOVA ($p < 0.05$).

et al., 1995; Zhang et al., 2019), agreeing with our conventional plate count and qPCR results for both sea bass and sea bream refrigerated fillets.

Although the short lengths of the *16S*-metabarcoding or qPCR amplicons did not allow species identification, cloning and phylogenetic analysis suggested the prevalent contaminations were *Carnobacterium maltaromaticum*, *Pseudomonas fluorescens, Shewanella baltica* or *S. putrefaciens*, while *Massilia* and *Janthinobacterium* sequences shared high identity with *M. brevitalea, M. suwonensis, J. lividum* and *J. agaricidamnosum*. Contrary to some species in these genera (e.g. *Pseudomonas aeruginosa* causing blood or lung infections) these species are not considered pathogenic for humans or fish, except for *S. putrefaciens* that can be a rare opportunistic pathogen in humans and *J. lividum* occasionally reported as an opportunistic pathogen of wild rainbow trout (Müller et al., 2023; Oh et al., 2019). Several food spoilers were identified including *P. fluorescens* reported to spoil proteinaceous foods including refrigerated seafood through enzymatic, pigment and biofilm production (Liu et al., 2019). *C. maltaromaticum* is tolerant to refrigeration, freezing, vacuum packing and HPP and has been reported to spoil dairy, meat, fish or sea food, producing odours and off-flavours. However, since *C. maltaromaticum* can potentially inhibit the growth of pathogenic bacteria like *Listeria monocytogenes* in fish and meat products, its presence has been considered beneficial (Leisner et al., 2007). The detection of *S. baltica* or *S. putrefaciens* was unsurprising as they are typical bacteria dominating spoilage in refrigerated fish and seafood (Wang et al., 2019; Zhang et al., 2022). Finally, *J. lividum* is normally associated with dairy products and is thought to have protective properties against viral, fungal or bacterial contamination (Ambrožič Avguštin et al., 2013; Pantanella et al., 2007). The significant reduction in *Shewanella, Carnobacterium, Pseudomonas* and *Janthinobacterium* DNAs in HPP compared to control sea bass fillets after 11 days refrigeration corroborates the reported microbial growth inhibition and explains the increased shelf life of HP processed fish (Ramirez-Suarez & Morrissey, 2006; Rode & Hovda, 2016; Suemitsu & Cristianini, 2019; Tsevdou et al., 2023; Tsironi et al., 2015).

In this study, the developed *16S*-based qPCR assays directed at total or specific bacterial genera DNAs were successfully applied to fresh, refrigerated sea bream and sea bass fillets and to HPP sea bass of aquaculture and commercial origin. It will be interesting in the future to apply them to monitor quality and freshness of commercial samples of other raw or processed fish products and species, and in larger scale experiments. The approach and qPCRs can be scaled up for high-throughput screening of different stages of the cold chain or for optimization of processing conditions, complementing time and labour-intensive classical microbiological enumeration. Application of the qPCR assays for routine food screening may include standardization tests for copy number detection, robotization, direct PCR on food product homogenates, multiplexing or digital PCR development (Jian et al., 2020; Martínez et al., 2011). These qPCR assays could also form the basis of biosensors designed to detect specific spoilage products or microorganisms, as is already under development for some pathogenic food bacteria (e.g.Ali et al., 2020).

4. Conclusions

Six qPCR assays directed at total bacteria or at specific bacterial genera (*Shewanella, Pseudomonas, Carnobacterium, Janthinobacterium* and *Massilia*) DNAs were developed and successfully applied to fresh or processed fish fillets. This set of qPCR assays provide sensitive, specific, culture-independent and cost-effective tools that can be used to monitor quality (in particularly freshness) or processing outcomes of fish fillets to complement current classical microbiological approaches.

Competing interests or conflicts of interests

The authors have no competing interests or conflicts of interests to declare.

Data availability

The accessions of metabarcoding data used to select candidate bacterial genera targets and obtained clone sequences are provided (Data Statement). Additional data can be provided on request.

Data statement

The raw reads from the *16S*-based metabarcoding datasets used as a starting point for qPCR assay development (section 2.1) were deposited at NCBI SRA (Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra/) under project accessions PRJNA517618 and PRJNA798764. The non-redundant, validated 16S-rRNA partial gene sequences from the clones isolated in this study for each target genera for phylogenetic analysis (section 2.3) were deposited at NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with accession numbers OQ154852 to OQ154866.

CRediT authorship contribution statement

P.I.S. Pinto: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **B. Najafpour:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **P. Lima:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **P. Machado:** Writing – review & editing, Methodology, Investigation. **T. Aires:** Writing – review & editing, Investigation, Data curation. **A. Engelen:** Writing – review & editing, Investigation, Data curation. **T. Tsironi:** Writing – review & editing, Formal analysis. **L. Anjos:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **D.M. Power:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

Authors declare they have not used generative AI and AI-assisted technologies in the writing process.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.104928.

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