Quantifying dominant bacterial genera detected in metagenomic data from fish eggs and larvae using genus-specific primers

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Abstract
The goal of this study was to design genus-specific primers for rapid evaluation of the most abundant bacterial genera identified using amplicon-based sequencing of the 16S rRNA gene in fish-related samples and surrounding water. Efficient genus-specific primers were designed for 11 bacterial genera including Alkalimarinus, Colwellia, Enterovibrio, Marinomonas, Massilia, Oleispira, Phaeobacter, Photobacterium, Polarbacerium, Pseudomonas, and Psychrobium. The specificity of the primers was confirmed by the phylogeny of the sequenced polymerase chain reaction (PCR) amplicons that indicated primers were genus-specific except in the case of Colwellia and Phaeobacter. Copy number of the 16S rRNA gene obtained by quantitative PCR using genus-specific primers and the relative abundance obtained by 16S rRNA gene sequencing using universal primers were well correlated for the five analyzed abundant bacterial genera. Low correlations between quantitative PCR and 16S rRNA gene sequencing for Pseudomonas were explained by the higher coverage of known Pseudomonas species by the designed genus-specific primers than the universal primers used in 16S rRNA gene sequencing. The designed genus-specific primers are proposed as rapid and cost-effective tools to evaluate the most abundant bacterial genera in fish-related or potentially other metagenomics samples.

KEYWORDS
16S rRNA gene, aquaculture site, abundant microbiota, eggs, genus-specific primers, larvae, qPCR

1 | INTRODUCTION

The influence of symbiotic and pathogenic interactions of bacterial microbiota on terrestrial and aquatic vertebrates is of high interest (Sharpton, 2018). The advent of cost-effective next-generation sequencing (NGS) has opened up new avenues of culture-free and high-throughput analysis of the entire microbiota in any ecosystem and has significantly modified the understanding of their role in animal health and disease (Cao et al., 2017). As the 16S rRNA gene is present in all bacteria, it is the most common reference gene for studies of bacterial phylogeny and taxonomy, and also for studies of the composition and the relative proportion of microorganisms in a
given habitat (Janda & Abbott, 2007; Simon & Daniel, 2011). The structure of the 16S rRNA gene explains its versatility for metagenomics since universal primers can be designed in regions that are highly conserved across species and the intervening hypervariable regions can be used to assign operational taxonomic units (OTUs) to the genus taxon (Baker et al., 2003; Wang & Qian, 2009). Furthermore, the hypervariable regions offer the opportunity for the development of genus and even species targeted quantitative PCR (qPCR).

Aquatic organisms are exposed to a ubiquitous and abundant microbiota and the intensification of aquaculture has increased interest in characterizing the microbiota of fish. Initially traditional microbiological approaches based on in vitro culture were used, but more recently metagenomics approaches have been deployed (Martínez-Porchas & Vargas-Albores, 2017). Aquaculture differs from terrestrial farming systems as there is a much larger number of species exploited and this is coupled to a wide variety of environmental conditions (e.g., temperature and salinity) and geographical locations (Linhart et al., 2021). The variability of fish microbiota has been linked to geography, species, and environmental conditions and indicates that fish microbiomes have a degree of farm site-specificity (Najafpour et al., 2021). Metagenomics studies targeting fish indicate that Vibrio and Pseudomonas are the dominant bacterial genera (reviewed by Egerton et al., 2018). However, the exclusive dependence on relative abundance data from NGS can lead to misinterpretation of microbial community structure (Jian et al., 2020). For this reason, it has been proposed that the use of genus-specific primers can provide complementary, quantitative data, to corroborate NGS results and contribute to better understanding microbial diversity and population structure (Zhou et al., 2014). A general literature review of microbiome studies in fish reveals that genus-specific primers for the most representative bacterial genera with high relative abundance are unavailable. Although genus-specific primers exist for the detection of pathogen-containing genera such as Aeromonas, Vibrio, Edwardsiella, and Streptococcus, their use has not been correlated with 16S rRNA metagenomic profiles in fish (D. Zhang et al., 2014).

We previously generated 16S rRNA metagenomics datasets for Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) eggs from several commercial production sites in Europe and identified the profile of the main bacterial genera (Najafpour et al., 2021). The objective of the present study was to develop a quick, cost-effective, and practical approach for large-scale screening of the core microbiome during aquaculture production cycles. We report the design of genus-specific primers, exploiting the hypervariable characteristics of the 16S rRNA gene, for the dominant bacterial genera represented in our in-house metagenomic 16S rRNA gene datasets from eggs, larvae, live feed, and tank water samples from seabream and seabass aquaculture sites in Europe (Najafpour et al., 2021). The efficiency and specificity of the genus-specific primers were confirmed by qPCR and sequencing of the PCR amplicons, and coverage of each genus was validated by comparison of qPCR and metagenomics data (Najafpour et al., 2021).

## EXPERIMENTAL PROCEDURES

### 2.1 Selection of target genera

The summarized workflow for genus choice and primer design is presented in Figure 1. The most represented bacterial genera in seabream and seabass hatcheries in Europe were identified using in-house metagenomic datasets of 16S rRNA gene sequences obtained from eggs, larvae, live feed, and hatchery water (sequenced by Lifesequencing S.L.-ADM, Spain and Stab Vida, Lda, Portugal). The corresponding 16S rRNA gene sequences of target genera were obtained from the LPSN and Silva (SSU r138.1) databases (Parte et al., 2020; Quast et al., 2013).

### 2.2 Sequence alignment and primer design

Multiple sequence alignments of the retrieved 16S rRNA gene sequences from the LPSN and Silva databases were performed using the MUSCLE algorithm (Edgar, 2004) in the Aliview platform v 1.27 (Larsson, 2014). Genus-specific forward (Fw) and reverse (Rv) primers were designed manually to obtain primers that amplified the maximum number of species in each of the target bacterial genera. The size of the amplicon was set at between 85 and 250 bp. Criteria used for PCR primer selection included the melting temperature (Tm), percentage of GC content, GC clamp, secondary structure, and the tendency to form primer-dimers. Primers were analyzed and optimized using the oligonucleotide sequence calculator.

![Figure 1: The workflow followed for the design of the bacterial 16S rRNA gene-specific primers for the most abundant genera in the microbiome of fish larvae, fish eggs, zooplankton, phytoplankton, and water. The most abundant genus detected in each data set was ranked using an in-house 16S rRNA gene database. The egg microbiome profile obtained from 16S rRNA gene sequencing has previously been reported (Najafpour et al., 2021).]
OligoEvaluator™ (Sigma-Aldrich, http://www.oligoevaluator.com) and OligoAnalyzer™ Tool (https://www.idtdna.com/calc/analyzer). In general, the threshold for primer selection included ΔG < −9 to minimize the likelihood of self-dimers, hairpins, and heterodimers and 50%-55% GC content for both the Fw and Rv primers to favor specific annealing to the targeted templates (Table A1). Primers that did not comply with the selection criteria were rejected, apart from the primers for Enterovibrio for which it was not possible to meet all the criteria (Table A1).

To further confirm that the designed genus-specific primer pairs would anneal to the maximal number of species in a given genus, in silico PCR simulations using TestPrime 1.0, available in the SILVA platform, was used (Klindworth et al., 2013). The primer pairs with the maximum specificity for each taxonomic group and with the best match to the selection criteria were selected and synthesized (Table A2, Specanaliitica, Carcavelos, Portugal). On arrival, primers were resuspended in sterile, nuclease-free water to prepare 100 μM stocks (Table A2).

2.3 | Evaluation of primer specificity by PCR amplification

The performance of the genus-specific primers was initially evaluated by running conventional PCR (Bio-Rad, T100 Thermal Cycler). Genomic DNA was extracted from seabream and seabass eggs, whole larvae, and rotifers (feed) using a DNeasy Blood & Tissue Kit (Qiagen) as previously described (Najafpour et al., 2021). The PCR was carried out using 2 μl (approximately 40–80 ng) of genomic DNA, 2.5 μl of 10× Dream Taq Green Buffer containing 20 mM MgCl2 (Thermo Scientific), 0.5 μl of nucleoside triphosphate 10 μM stock, 1.25 μl of the Fw primer (10 μM), 1.25 μl of the Rv primer (10 μM), 0.2 μl of Dream Taq DNA polymerase (5 U/μl, Thermo Scientific), and 17.3 μl of sterile, nuclease-free water to give a final reaction volume of 25 μl. The PCR thermocycle consisted of 1 cycle of 95°C for 3 min followed by 34 cycles of 95°C for 10 s, a gradient of melting temperatures tested for each primer pair (57–64°C) for 10 s and 72°C for 10 s and a final cycle at 72°C for 5 min. The genus-specific primers were tested in PCR amplification of genomic DNA extracted from larval intestines, whole larvae, rotifers, and egg samples of seabream and seabass that had a high relative abundance of a given bacterial genus in 16S rRNA metagenomics.

The 16S rRNA gene PCR reaction products were run on a 2% agarose gel in tris-acetate-EDTA buffer and the specific amplicons generated by each primer pair were purified using an Illumra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) following the manufacturer's instructions. The purified PCR products were ligated into the pGEM-T Easy cloning vector (Promega) which permits colony transformation (Promega, T100 Thermal Cycler). The primer performance was initially evaluated using a Bio-Rad CFX96 qPCR Instrument (Bio-Rad Laboratories). The primer performance was initially evaluated using a gradient of melting temperatures (Tm) in a reaction volume of 10 μl containing 200 nM of each primer, 2 μl of DNA (80 ng DNA/2 μl) for the samples, or 2 μl of serial dilutions (corresponding to 107–106 template copies in the reaction) for the standard curve, 5 μl of 2× Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium) and 2.4 μl of sterile nuclease-free water. Thermocycling conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, the optimized melting temperature for each primer pair for 10 s (between 58 and 61°C) and 72°C for 10 s. A final melting curve was generated by increasing the temperature up to 95°C in increments of 0.5°C every 10 s to confirm single reaction products were obtained. Control reactions included substitution of genomic DNA by water to confirm the absence of contamination.

2.4 | Real-time qPCR optimization and primer efficiency

After confirming primer specificity by amplicon sequencing, qPCR reactions were optimized using a Bio-Rad CFX96 qPCR Instrument (Bio-Rad Laboratories). The primer performance was initially evaluated using a gradient of melting temperatures (Tm) in a reaction volume of 10 μl containing 200 nM of each primer, 2 μl of DNA (80 ng DNA/2 μl) for the samples, or 2 μl of serial dilutions (corresponding to 107–106 template copies in the reaction) for the standard curve, 5 μl of 2× Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium) and 2.4 μl of sterile nuclease-free water. Thermocycling conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, the optimized melting temperature for each primer pair for 10 s (between 58 and 61°C) and 72°C for 10 s. A final melting curve was generated by increasing the temperature up to 95°C in increments of 0.5°C every 10 s to confirm single reaction products were obtained. Control reactions included substitution of genomic DNA by water to confirm the absence of contamination.

2.5 | Bacterial genus quantification and correlation with 16S rRNA microbiome profiling

To compare qPCR and 16S rRNA gene abundance estimates, Spearman correlations were used together with scatter plots generated using the R package ggplot2 v 3.3.5 (Wickham, 2016). Colwellia, Oleispira, Phaeobacter, Pseudomonas, and Psychrobium were quantified in nine egg samples (Najafpour et al., 2021, Table A3). Massilia, Phaeobacter, and Pseudomonas were quantified in seabream (n = 9; three larvae samples in the age range of 5–15 days posthatch [dph] and six samples in the age range of 43–58 dph) and seabass (n = 15; seven larvae samples in the age range of 5–7 dph and eight samples in the age range of 42–46 dph) larvae (Table A3). In the case
of Pseudomonas where the correlation between 16S rRNA gene sequencing and copy number determined by qPCR was low, further in silico analysis was done to assess genus-specific primer performance in comparison to the universal primers used for 16S rRNA metagenomics studies. In silico PCR v 0.5.1 implemented in Ubuntu (20.04.2 LTS) and unique Pseudomonas 16S rRNA gene sequences from the Silva database with a minimum length of 900 bp were used.

3 | RESULTS

3.1 | Primer specificity

In total, 11 bacterial genera were targeted based on their high relative abundance in fish-related samples as determined by 16S rRNA gene sequencing (Table 1). The species for which the 16S rRNA genes were readily amplified by each primer pair are indicated in Table A4 and the supplementary figure at https://doi.org/10.5281/zenodo.6301068. Primer specificity was confirmed by the presence of a single band in agarose gel electrophoresis (Figure A1) and the sequence of the PCR amplicon. In the phylogenetic tree, most of the 16S rRNA amplicon sequences generated clustered with the corresponding taxa in the SILVA 16S rRNA gene database, confirming the specificity of the genus-specific primers (Figure 2). In the Pseudomonas clade, the sequence of the 16S rRNA gene amplicon from two samples clustered with several Pseudomonas species including Pseudomonas azotifigens (AB189452; Figure 2a). In the Massilia clade, the sequence of the 16S rRNA gene amplicons clustered with unidentified Massilia sp. (AB639693). In the Psychrobium clade, the sequence of the 16S rRNA gene amplicons from two samples clustered with Psychrobium species, including an uncultured Psychrobium sp. (KT318702). Psychrobium conchae (AB930131; Figure 2a). In the Phaeobacter clade, the sequenced 16S rRNA gene amplicons clustered with Phaeobacter species, including Phaeobacter inhibens (CP010668), and Sedimentitalea sp. (JN018499; Figure 2a). In the Colwellia clade, one of the 16S rRNA gene amplicons clustered with unidentified Colwellia spp. (L10950 and JX569143; Figure 2a) and the other amplicon clustered with Thalassotalea sp. (Figure 2b).

3.2 | Primer efficiency and correlation between qPCR and 16S RNA gene abundance

The efficiency of bacterial genus-specific primers was evaluated using qPCR with the optimized annealing temperature of each primer pair (Table 2). All the genus-specific primers had an acceptable efficiency within the range of 92%-105.5%.

The five most abundant bacterial genera in seabream and sebass eggs were quantified. In general, the relative abundance profiles of the 16S rRNA gene sequencing and qPCR amplification of different bacterial genera were matched with some exceptions (Figure 3). A significant positive correlation was obtained for the relative abundance (%) of bacterial genera detected by both methods (Figure 4). In egg samples (n = 9) highly significant positive correlations were found for Colwellia (r = 0.82), Oleispira (r = 0.86), and Psychrobium (r = 0.86, Figure 4). In seabream (n = 9) and sebass (n = 15) larvae, Massilia (r = 0.32) and Phaeobacter (r = 0.83) abundance also had significant positive correlations (Figure 4). For Pseudomonas, no correlation was found between

| Bacterial genus   | Maximum relative abundance of each bacterial genus in different samples (%)a |
|-------------------|---------------------------------------------------------------------------|
|                   | Fish egg | Fish larva | Fish intestine | Rotifer | Artemia | Algae | Water |
| Pseudomonas       | 3.8      | 5.2       | 5.4           | 2.1     | 14.8    | 1.3   | -     |
| Massilia          | -        | 36.1      | 1.2           | -       | -       | 2.2   | 89.6  |
| Psychrobium       | 31.3     | 8.6       | 3.7           | -       | -       | -     | 33.4  |
| Phaeobacter       | -        | 3.4       | -             | -       | -       | -     | -     |
| Marinomonas       | 1.4      | 9         | 2.7           | 24.2    | 6.4     | -     | 14.6  |
| Polaribacter      | 11.8     | 13.6      | 94.7          | -       | -       | 4.7   | 19.2  |
| Alkalimarinus     | 1.9      | 3.2       | 29.4          | -       | -       | -     | -     |
| Enterovibrio      | -        | -         | 94.4          | -       | -       | -     | -     |
| Photobacterium    | 1.5      | 3.2       | 21.4          | -       | -       | -     | 40.8  |
| Oleispira         | 6.6      | 2.2       | 1.7           | -       | 0.3     | -     | 8.3   |
| Colwellia         | 9.5      | 4.8       | 1             | -       | -       | -     | 20.6  |

*Identified in the 10 top bacterial genera in each sample type. (−) signifies not detected within the most abundant bacterial genera of the specific sample type.
the qPCR results for larvae ($r = -0.063$, $n = 24$) and egg ($r = -0.22$, $n = 9$) samples and the 16S rRNA gene sequencing (Figure 4). In silico PCR analysis comparing the Pseudomonas-specific primers and the universal primers generally used for metagenomics (Klindworth et al., 2013) identified 27,734 and 26,780 unique sequences of the Pseudomonas 16S rRNA gene (total number in Silva = 60,869, filtered sequences number with the minimum size 900 bp = 41,607), respectively.

**4 | DISCUSSION**

Genus-specific primers for rapid and cost-effective high-throughput monitoring of core microbial genera of seabream and seabass aquaculture were successfully developed. The potential functional importance of the abundant bacterial genera selected makes further studies about their turnover during larval fish aquaculture important due to their high potential impact on production.
Several studies have focused on *Pseudomonas* spp. due to their widespread distribution and the presence of species that are human, animal, and plant pathogens (Palleroni, 2015). For example, *P. bactica* is a pathogen of marine fish, and primers targeting the *gyrB* gene (c390–F1 and c390–R1) and *rpoD* gene have been developed for rapid diagnosis (López et al., 2016). A multiplex PCR based on *oprI* and *oprL* genes was developed for the detection of *Pseudomonas* strains from a bacterial collection isolated from water (Matthijs et al., 2013). The qPCR comparisons, in the present study, of the abundance of five bacterial genera (*Colwellia*, *Oleispira*, *Pseudomonas*, *Psychrobium*, *Phaeobacter*) in the egg samples revealed a higher than expected copy number of the *Pseudomonas* genus relative to the results of the 16S rRNA metagenomic sequencing. The *Pseudomonas* genus-specific primer pair designed in the present study were of broad scope and had the potential to anneal to 226 out of the 254 16S rRNA gene sequences represented in the LPSN and SILVA databases. Insight into why a higher than expected copy number of *Pseudomonas* was detected by qPCR came from in silico PCR comparisons of the *Pseudomonas* genus-specific and the 16S rRNA gene universal primers (Silva database, SSU r138.1) since it revealed the universal primers (Klindworth et al., 2013) had lower efficiency and annealed to fewer of the *Pseudomonas* sequences represented in the database. Furthermore, the relatively short amplification product generated by the *Pseudomonas* genus-specific primers favors high qPCR efficiencies compared with previous primers designed to amplify a larger DNA sequence encompassing the ITS1 region (Locatelli et al., 2002). Bergmark et al. (2012) reported *Pseudomonas* and *Burkholderia* genus-specific primers for their detection in soil and proposed the use of genus-specific primers for both qPCR and 16S rRNA gene sequencing approaches to overcome issues related to the resolution of the 16S rRNA gene databases, although the exponential increase in available bacterial 16S rRNA gene sequences has now ameliorated this problem (Glöckner, 2019).

A high relative abundance of *Oleispira* and *Colwellia* genera was observed in our in-house database of 16S rRNA gene sequences obtained for egg and water samples from aquaculture installations. The copy number of *Oleispira* and *Colwellia* in the same samples determined by qPCR using genus-specific primers and their relative abundance using 16S rRNA gene sequencing gave a strong positive correlation. However, amplicon sequencing of products generated by

| Bacterial genus     | Primer | Size (bp) | Tm (°C) | Eff. (%) | R²  |
|---------------------|--------|-----------|---------|----------|-----|
| *Pseudomonas*       | Pseu-F | 250       | 61      | 99.9     | 0.99|
|                     | Pseu-R |           |         |          |     |
| *Massilia*          | Mass-F | 142       | 61      | 96.4     | 1.0 |
|                     | Mass-R |           |         |          |     |
| *Psychrobium*       | Psyc-F | 128       | 61      | 97.7     | 0.99|
|                     | Psyc-R |           |         |          |     |
| *Phaeobacter*       | Phae-F | 149       | 61      | 97.6     | 0.99|
|                     | Phae-R |           |         |          |     |
| *Marinomonas*       | Mari-F | 140       | 58.6    | 95.8     | 0.99|
|                     | Mari-R |           |         |          |     |
| *Polaribacter*      | Pola-F | 85        |         | 98.1     | 1.0 |
|                     | Pola-R |           |         |          |     |
| *Alkalimarinus*     | Alka-F | 165       | 61      | 95.9     | 1.0 |
|                     | Alka-R |           |         |          |     |
| *Enterovibrio*      | Entv-F | 163       |         | 105.5    | 0.97|
|                     | Entv-R |           |         |          |     |
| *Photobacterium*    | Phot-F | 167       | 58      | 92       | 1   |
|                     | Phot-R |           |         |          |     |
| *Oleispira*         | Olei-F | 171       | 58.2    | 95.2     | 1.0 |
|                     | Olei-R |           |         |          |     |
| *Colwellia*         | Colw-F | 188       | 61      | 98.3     | 1.0 |
|                     | Colw-R |           |         |          |     |
the Colwellia primers yielded a Colwellia-specific amplicon and another amplicon that matched the Thalassotalea genera of the Colwelliaceae family. The similarity between the sequence of the Colwellia genus-specific primers and the 16S rRNA gene sequence of Thalassotalea (LPSN database) should be considered when using the primers. Oleispira and Colwellia are marine hydrocarbon-degrading bacteria (Mason et al., 2014) and obligate hydrocarbonoclastic bacteria (OHCB) and are usually present in the environment in very low numbers (Golyshin et al., 2010). Pollution or the addition of hydrocarbons to water induces a rapid bloom of OHCB (Kasai et al., 2002) and indicates that variations in Oleispira and Colwellia abundance may be a useful indicator of excess hydrocarbons in aquaculture systems due, for example, to addition of lipid enriched feeds.

In seabream and seabass eggs, Pseudophaeobacter was relatively more abundant than Phaeobacter, which was not detected by 16S rRNA gene sequencing in eight out of nine egg samples (Najafpour et al., 2021). In contrast, Phaeobacter was more common in 16S rRNA gene sequences of seabream and seabass larvae, and the Pseudophaeobacter genus was not detected (in-house database). Phaeobacter spp. are common in marine organisms and the environment (Martens et al., 2006; Yoon et al., 2007; D. C. Zhang et al., 2008) and have been proposed as a probiotic against pathogenic Vibrio spp. in seabass and cod, Gadus morhua (D’Alvise et al., 2013; Grotkjær et al., 2016), although in juvenile squid they were associated with mortality (Won, 2009). The abundance-dependent detection of Phaeobacter or Pseudophaeobacter in seabream and seabass samples highlights one of the challenges when designing primers for short amplicon targets of 16S rRNA genes that show high similarity across bacterial genera. The amplification of Phaeobacter or Pseudophaeobacter by the genus-specific qPCR primers is unsurprising considering the recent reclassification of the species in Leisingera-Phaeobacter to Sedimentatia and Pseudophaeobacter (Breider et al., 2014). Therefore, it is proposed that the Phaeobacter genus-specific primers designed in our study be designated group-specific primers.

The primers for Psychrobium were highly specific and this genus was present in all analyzed samples of eggs, larvae, and environmental water and a positive correlation existed between the results of qPCR and 16S rRNA gene sequencing. The lowest number of species were detected for the Psychrobium genus compared with the other targeted genera and it was easier to design primers specific for this genus. The high specificity of the Psychrobium primers was assigned to the high variability of the Psychrobium 16S rRNA gene compared with other bacteria.

Six of the bacterial genera, Massilia, Marinomonas, Polaribacter, Alkalimarinus, Enterovibrio, and Photobacterium, for which genus-specific primers were designed, had low relative abundance in the 16S rRNA metagenome of eggs samples (Najafpour et al., 2021).
Nonetheless, the identified genera were well represented in seabream and seabass larvae, rotifers, and tank water, and therefore genus-specific primers were validated in qPCR. The designed genus-specific primers revealed a high relative abundance of the *Massilia* genus in seabream and seabass larvae, *Marinomonas* in rotifer, and *Alkalimarinus, Polaribacter, Photobacterium, and Enterovibrio* in larval seabream intestine. These genera have previously been reported in a wide diversity of samples and experiments. For example, *Massilia* spp.
are widespread in soil (Y. Q. Zhang et al., 2006), drinking water (Gallego et al., 2006), and plants (Ofek et al., 2012) and was a dominant genus in the gastrointestinal microbiota of the herbivorous grass carp, Ctenopharyngodon idellus (Li et al., 2014). Marinomonas species are abundant in seawater (Ivanova et al., 2005; Yoon et al., 2005) and were highly abundant in the microbial community of rotifer cultures prepared as a feed for fish larvae (Rombaut et al., 2001). Polaribacter species have been isolated from Antarctic soil and in biofilms on stones from the North Sea (Choo et al., 2020; Kim et al., 2013), and the genus was detected in the intestine of marine organisms and algae (Hyun et al., 2014; Nедашковская et al., 2013; Wei et al., 2018). *Alkalimarinus sediminis* was isolated from marine sediment in Shandong Province, China (Zhao et al., 2015), and *Alkalimarinus* species were found in bone-eating worms (*Osedax mediterranea*) from the Mediterranean Sea (Hewitt et al., 2020). Healthy and diseased Dentex and Sparus aurata (bony fishes) cultured in Spanish Mediterranean aquaculture contained *Enterovibrio coralii* strains and possibly *Enterovibrio nigricans* (Pascual et al., 2009). *Photobacterium* has been isolated from seawater, mussel, eggs of spiny lobster and fish intestine and the bioluminescence and pathogenicity of some species (e.g., *Photobacterium damsela*) has made studies of them a priority (Egerton et al., 2018; Labella et al., 2017; Osorio et al., 1999).

## 5 | CONCLUSION

Genus-specific primers were developed for *Pseudomonas, Massilia, Psychrobium, Phaeobacter, Marinomonas, Polaribacter, Alkalimarinus, Enterovibrio, Photobacterium, Oleispira*, and *Colwellia*. Most of the designed primers were highly efficient in qPCR and were genus-specific as shown by amplicon sequencing and phylogeny. Difficulty was encountered in the design of genus-specific primers for *Phaeobacter* and *Pseudophaeobacter* since the short amplicons of the 16S rRNA gene encompassed regions with 93%–98% identity and so these primers were designated group-specific. The genus-specific primers designed in this study will be useful for rapid evaluation and quantification by qPCR of target bacterial genera in fish-related samples and potentially other metagenomic samples since the genus targeted are ubiquitous in a diversity of environments. An interesting observation was that the 16S rRNA gene universal primers used to profile microbiomes do not cover all identified species of some bacterial genera such as *Pseudomonas* and this has the potential to create bias in metagenomics studies and reinforces the value of complimentary qPCR studies. Overall, the designed genus-specific primers provided a rapid and cost-effective evaluation of abundant bacterial genera in samples and can therefore contribute to understanding the modulation of abundant microbiota in complex microbial communities with a potentially high impact on host biology.

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## CONFLICTS OF INTEREST

None declared.

## ETHICS STATEMENT

None required.

## AUTHOR CONTRIBUTIONS

Babak Najafpour: Formal analysis-lead, investigation-lead, methodology-lead, Writing–original draft-lead; Patricia Pinto: Formal analysis-supporting, methodology-supporting, writing–review & editing-supporting; Adelino V. M. Canario: Methodology-supporting, project administration-supporting, resources-supporting, supervision-supporting, writing–review & editing-supporting; Deborah M. Power: Conceptualization-lead, formal analysis-supporting, funding acquisition-lead, investigation-lead, methodology-supporting, project administration-lead, resources-equal, supervision-lead, writing–original draft-supporting, writing–review & editing-lead.

## DATA AVAILABILITY STATEMENT

All data are provided in full in this paper apart from amplicon sequences of 16 S rRNA genes which are available at www.ncbi.nlm.nih.gov under accession numbers OM685062-OM685080. The supplemental figure is available in Zenodo at https://doi.org/10.5281/zenodo.6301068 (The alignment and identity of 16S rRNA genes and genus-specific primer sequences for different bacterial species of each target bacterial genus).

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Figure A1: Analysis of PCR products (5 µl) on 2% agarose gel electrophoresis after staining with GreenSafe. The first lane (L) represents the DNA ladder and the size (base pair [bp]) of some markers is shown in red on the left-hand side. Each lane contains the amplicon generated using the genus-specific primer pairs (P) and sample type (S): P = Massilia (Mass), S = 49 days post hatch (dph) seabream larvae; P = Phaeobacter (Phae), S = 9 dph seabass larvae; P = Pseudomonas (Pseu), S = 46 dph seabass larvae; P = Psychrobium (Pyc), S = 39 dph seabream larvae; P = Alkalimarinus (Alka), S = intestine of seabream larvae; P = Polaribacter (Pola), S = intestine of seabass larvae; P = Photobacterium (Phot), S = intestine of seabream larvae; P = Marinomonas (Mari), S = rotifer; P = Oleispira (Olei), S = seabream eggs; P = Enterovibrio (Entv), S = intestine of seabream larvae; P = Colwellia (Colw), S = seabream eggs. The size of the PCR products amplified with each primer pair is presented in red (e.g., Mass, product size 142 bp). PCR, polymerase chain reaction.

Table A1: Evaluation of primer GC content, dimer and secondary structure formation, and length using the OligoAnalyzer™ Tool

| Bacterial genus primer pair | GC (%) | Self-dimer (ΔG kcal. mole⁻¹) | Hetero-dimer (ΔG kcal. mole⁻¹) | Hairpin (ΔG kcal. mole⁻¹) | Length (bp) |
|----------------------------|--------|-------------------------------|-------------------------------|----------------------------|-------------|
| Pseudomonas                |        |                               |                               |                            |             |
| Fw                         | 61.1   | −6.68, −0.96                  | −4.95, −1.34                  | −2.18, −1.97               | 18          |
| Rv                         | 52.4   | −6.69, −1.57                  | −6.35, −1.34                  | −3.24, −3.13               | 21          |
| Massilia                   |        |                               |                               |                            |             |
| Fw                         | 55     | −3.61, −0.96                  | −6.35, −1.34                  | 0.39, 1.24                 | 20          |
| Rv                         | 47.8   | −6.84, −0.96                  | −0.01, 0.28                   |                            | 23          |
| Psychrobium                |        |                               |                               |                            |             |
| Fw                         | 55     | −7.05, −1.47                  | −6.69, −1.34                  | −2.86, −1.87               | 20          |
| Rv                         | 52.4   | −3.61, −0.96                  | −1.17                         |                            | 21          |
| Phaeobacter                 |        |                               |                               |                            |             |
| Fw                         | 52.4   | −3.61, −0.96                  | −3.61, −1.34                  | −1.17                      | 21          |
| Rv                         | 66.7   | −5.02, −1.34                  | −0.97, −0.41                  |                            | 18          |
| Marinomonas                |        |                               |                               |                            |             |
| Fw                         | 57.9   | −9.75, −0.96                  | −6.44, −1.34                  | −2.04                      | 19          |
| Rv                         | 50     | −7.6, −1.57                   | −0.73                         |                            | 19          |
| Polaribacter                |        |                               |                               |                            |             |
| Fw                         | 45.5   | −7.82, −1.34                  | −3.9, −1.34                   | −2.42                      | 22          |
| Rv                         | 50     | −3.61, −1.47                  | −0.27, 0.54                   |                            | 18          |
| Alkalimarinus               |        |                               |                               |                            |             |
| Fw                         | 50     | −4.85, −0.96                  | −8.33, −0.96                  | −0.11, 0.18                | 22          |
| Rv                         | 57.9   | −3.61, −0.96                  | −0.52, −0.4                   |                            | 19          |
| Enterovibrio                |        |                               |                               |                            |             |
| Fw                         | 55     | −3.14, −0.96                  | −15.48, −1.34                 | −1.25                      | 20          |
| Rv                         | 52.4   | −6.47, −0.96                  | −3.04                         |                            | 21          |
| Photobacterium              |        |                               |                               |                            |             |
| Fw                         | 55.6   | −8.54, −0.96                  | −8.54, −1.46                  | 0.9, 1.57                  | 18          |
| Rv                         | 61.1   | −7.05, −1.47                  | −0.87, −0.09                  |                            | 18          |
| Oleispira                   |        |                               |                               |                            |             |
| Fw                         | 61.1   | −7.05, −1.47                  | −6.36, −1.34                  | −0.87, −0.09               | 18          |
| Rv                         | 52.4   | −3.65, −0.96                  | 1.37, 1.41                    |                            | 21          |
| Colwellia                   |        |                               |                               |                            |             |
| Fw                         | 61.1   | −7.05, −0.96                  | −6.75, −0.96                  | −0.55, 0.15                | 18          |
| Rv                         | 47.8   | −3.61, −0.96                  | 0.49, 1.46                    |                            | 23          |

Note: ΔG = free energy required to break the structure, OligoAnalyzer™ Tool (https://www.idtdna.com/calc/analyzer).
## Table A2 Details of the genus-specific primers and their preparation for qPCR

| Primer target | Amount received (nmol) | Primer Melting temp. (°C) | Optical density (260 nm) | Sterile water (µl) |
|---------------|------------------------|----------------------------|--------------------------|-------------------|
| Pseudomonas   |                        |                            |                          |                   |
| Fw            | 9.7                    | 57.2                       | 2                        | 97                |
| Rv            | 10.2                   | 65.1                       | 2                        | 102               |
| Massilia      |                        |                            |                          |                   |
| Fw            | 9.5                    | 55.4                       | 2                        | 95                |
| Rv            | 7.8                    | 55.5                       | 2                        | 78                |
| Psychrobium   |                        |                            |                          |                   |
| Fw            | 10.1                   | 56.2                       | 2                        | 101               |
| Rv            | 10.5                   | 55.6                       | 2                        | 105               |
| Phaeobacter   |                        |                            |                          |                   |
| Fw            | 9.7                    | 55.9                       | 2                        | 97                |
| Rv            | 12.8                   | 59.7                       | 2                        | 128               |
| Marinomonas   |                        |                            |                          |                   |
| Fw            | 11.1                   | 56.3                       | 2                        | 111               |
| Rv            | 9.9                    | 52.8                       | 2                        | 99                |
| Polaribacter  |                        |                            |                          |                   |
| Fw            | 9.5                    | 52.8                       | 2                        | 95                |
| Rv            | 11.9                   | 50.8                       | 2                        | 119               |
| Alkalimarinus |                        |                            |                          |                   |
| Fw            | 9.2                    | 56.6                       | 2                        | 92                |
| Rv            | 11.3                   | 55.9                       | 2                        | 113               |
| Enterovibrio  |                        |                            |                          |                   |
| Fw            | 10                     | 56.0                       | 2                        | 100               |
| Rv            | 10.5                   | 55.9                       | 2                        | 105               |
| Photobacterium|                        |                            |                          |                   |
| Fw            | 9.7                    | 53.7                       | 2                        | 97                |
| Rv            | 12.2                   | 57.2                       | 2                        | 122               |
| Oleispira     |                        |                            |                          |                   |
| Fw            | 11.6                   | 50.6                       | 2                        | 116               |
| Rv            | 11                     | 54.0                       | 2                        | 110               |
| Colwellia     |                        |                            |                          |                   |
| Fw            | 10.8                   | 58.4                       | 2                        | 108               |
| Rv            | 9.7                    | 55.5                       | 2                        | 97                |

Abbreviations: Fw, forward primer; qPCR, quantitative polymerase chain reaction; Rv, reverse primer.

*Microliter of sterile nuclease-free water added to the lyophilized primers to give a 100 µM stock.
### TABLE A3  List of samples used to correlate qPCR and 16S rRNA gene abundance estimates

| Sample | Sample type | Site  | Species  | Sample code | Sequenced reads |
|--------|-------------|-------|----------|-------------|-----------------|
| E1     | Egg         | Site 1| Seabream | S1.SA.E.AD3  | 370,906         |
| E2     | Egg         | Site 1| Seabream | S1.SA.E.AD4  | 513,514         |
| E3     | Egg         | Site 1| Seabream | S1.SA.E.BD3  | 409,804         |
| E4     | Egg         | Site 1| Seabream | S1.SA.E.BD4  | 312,384         |
| E5     | Egg         | Site 2| Seabream | S2.SA.E.AD1  | 417,468         |
| E6     | Egg         | Site 2| Seabream | S2.SA.E.BD1  | 587,762         |
| E7     | Egg         | Site 3| Seabass  | S3.DLE.BD2   | 628,184         |
| E8     | Egg         | Site 3| Seabass  | S3.SA.E.AD2  | 616,148         |
| E9     | Egg         | Site 3| Seabass  | S3.SA.E.BD2  | 522,126         |
| L1–L3  | Larvae, 5–15 dph | F, G, I | Seabream | SA.1.JM     | 150,859         |
| L4–L10 | Larvae, 5–7 dph | A, F, H, E, D, G | Seabass | DL.1.JM     | 72,736          |
| L11–L16| Larvae, 39–58 dph | A, B, C, F, G, H | Seabream | SA.3.JM     | 105,275         |
| L17–24 | Larvae, 42–46 dph | A, C, D, E, F, G, H | Seabass | DL.3.JM     | 55,062          |

Abbreviations: dph, days posthatch; qPCR, quantitative polymerase chain reaction.

*Nine egg samples (E1–E9) used for qPCR reactions of five bacterial genera (*Pseudomonas*, *Oleispira*, *Colwellia*, *Psychrobium*, and *Phaeobacter*), and the details of their 16S rRNA gene sequencing profiles are available in Najafpour et al. (2021). "Factors driving bacterial microbiota of eggs from commercial hatcheries of European seabass and gilthead seabream" and the metagenomics raw data were deposited at NCBI SRA (sequence read archive) under project number PRJNA727018. Twenty-four larvae samples (L1–L24) and their corresponding qPCR and 16S rRNA gene sequencing data (available as in-house datasets) were also used to correlate qPCR and 16S rRNA gene abundance estimates of three genera (*Massilia*, *Phaeobacter*, and *Pseudomonas*).

*For larvae samples, the average number of the sequenced reads is presented for each age range.*

### TABLE A4  The numbers of potentially amplified and unamplified bacterial species with each of the designed genus-specific primer set

| Primer | Total number of speciesa | Number of amplified species | Unamplified species                                      |
|--------|--------------------------|----------------------------|----------------------------------------------------------|
| Pseu   | 254                      | 226                        | *P. luteola* (AB681955), *P. kirkiae* (MK159379), *P. ianjinensis* (MF083697), *P. asensus* (AJ871471), *P. caeni* (EU620679), *P. oryzihabitans* (AB681726), *P. zeshui* (JN411093), *P. duriflava* (EU046271), *P. litoralis* (FN908483), *P. psychrotolerans* (AJ575816), *P. rhizophyta* (MK759856), M. brevitalea (EF546777) |
| Mass   | 49                       | 48                         | M. brevitalea (EF546777)                                  |
| Psych  | 1                        | 1                          | -                                                        |
| Phae   | 6                        | 5                          | *P. italicus* (AM904562)                                  |
| Mari   | 33                       | 31                         | M. ushuaiensis (AJ627909), M. algida (KR003453)          |
| Pola   | 26                       | 20                         | *P. marinivivus* (KM017972), *P. huanghechanensis* (MH791326), *P. aestuariivivens* (MH791326), *P. porphyrae* (AB695286), *P. lacunae* (KJ728849), *P. pacificus* (KU315476) |
| Alka   | 1                        | 1                          | -                                                        |
| Photo  | 38                       | 35                         | *P. galatheae* (FJ457476), *P. salinisoli* (KP0544474), *P. halotolerans* (AY551089) |
| Olei   | 2                        | 2                          | -                                                        |
| Colw   | 21                       | 19                         | C. demingiae (U85845), C. hornerae (JN175346)             |
| Entv   | 6                        | 5                          | *E. pacificus* (JN118548)                                 |

*a16S rRNA genes of bacterial species present in the LPSN db (linked to the Silva db); a full list of the sequences used in the alignments is presented in the supplemental figure at https://doi.org/10.5281/zenodo.6301068*