



# Study of the microbiota associated to *Ruditapes decussatus* and *Ruditapes philippinarum* clams by 16S rRNA metabarcoding, dilution to extinction, and culture-based techniques

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**Abstract** The study of the microbiota associated to clams is important not only to know their sanitary status but also to prevent pathobiology events. The use of different microbiological techniques can help to obtain a better picture of the bacterial diversity of clams as well as to isolate new bacterial taxa. In this study, two clam species, *Ruditapes decussatus* and *R. philippinarum*, were analyzed in two locations of

Galicia (northwest of Spain) in April and October, by combining classic culturing, dilution-to-extinction approach, and 16S rRNA gene target sequencing. 16S rRNA gene target sequencing revealed a great diversity within the clam samples, shedding light into the vast microbial communities associated to these bivalves. All samples were dominated by the same bacterial genera in the different periods, namely *Mycoplasma*, *Vibrio*, and *Cutibacterium*. The  $\alpha$ -diversity in the samples obtained during the month of October was lower and showed the dominance of rare bacterial taxa, such as *Methylobacterium* or *Psychrobacter*. Dilution-to-extinction technique demonstrated its usefulness to culture rare bacterial taxa that were not found in clams under the classic culturing techniques, including *Rahnella*, *Brachybacterium*, *Micrococcus*, *Jantirobacter*, and *Lelliottia*. Altogether, our study provides valuable information on the microbiota associated to *R. decussatus* and *R. philippinarum*, demonstrating the high complexity and dynamics of these microbial populations.

Diego Gerpe and Aide Lasa have contributed equally to this work.

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extinction · Metataxonomy

## Introduction

Bivalve production, including clam species *Ruditapes decussatus* (Linnaeus, 1758) (carpet shell clam) and

*R. philippinarum* (Adams & Reeve, 1850) (Manila clam), represents an important economic activity in the aquaculture of Galicia (NW Spain). Due to their filter-feeding habit, clams accumulate a rich and diverse bacterial microbiota (Balboa et al., 2016). The study of such microbiota is essential to know the sanitary status, as well as to determine the pathobiological bases of the periodic disease outbreaks affecting clam populations. On the other hand, the introduction of seeds and adult specimens from other countries, due to the overexploitation of natural beds, has increased the risk the introduction of foreign pathogens (Prado et al., 2005).

So far, culturable fraction of clams has been deeply studied (Paillard et al., 2004; Romanenko et al., 2008; Balboa et al., 2016) in which the predominant phylum was  $\alpha$ -Proteobacteria and the most abundant genus was *Vibrio*, representing approximately 60% of microbiota. Bacteria with aerobic metabolism constituted around 35% of the microbiota, being *Pseudoalteromonas* and *Shewanella* the predominant genera (52%, 8%, and 16%, respectively). Other genera as *Tenacibaculum*, *Psychrobacter*, *Alteromonas*, *Polaribacter*, *Luteimonas*, *Marinomonas*, *Lacinutrix*, or *Corbelia* were also found in less percentages (Balboa et al., 2016). Dilution-to-extinction technique has been previously used for the screening of underexplored bacteria in marine, plant biomass, or soil environments (Bartelme et al., 2020; Benitez et al., 2021; Diaz-Garcia et al., 2021) and retrieves bacterial isolates that otherwise are not culturable under normal lab conditions.

In recent years, bivalve–microbiota relationships have faced new challenges of crucial importance with the introduction on Next-Generation Sequencing (NGS) technologies. In this sense, symbiotic interactions and pathobiotic events have been extensively explored as well as the mollusc responses to environmental perturbations (Gootenberg & Turnbaugh, 2011; McFall-Ngai et al., 2013; Milan et al., 2018; Lasa et al., 2019). Culture-independent techniques have been introduced to describe microbial community populations associated with bivalves, including clams and oysters (Wegner et al., 2013; Lokmer et al., 2016; Milan et al., 2018; Vezzulli et al., 2018; King et al., 2020; Gerpe et al., 2021). The composition and establishment of the microbial association is influenced by several biotic and abiotic factors, including physiological, immune, and genetic characteristics

of the host, the environment, and the interactions among the microorganisms. NGS-based studies of the microbiota of different bivalve molluscs have shown the overestimation using classical techniques of certain bacterial groups or genera, such as *Vibrio* (Trabal et al., 2014; Roterman et al., 2015; Lokmer et al., 2015; Lasa et al., 2016; Lokmer et al., 2016; Vezzulli et al., 2018). Moreover, these studies have revealed the presence of uncultured species and also some bacterial genera never described before in studies with culture-based techniques.

In the current work, we combined the use of different culture-based techniques, classic culturing, and dilution to extinction, together with a metataxonomic approach, in order to obtain a complete picture of the microbial diversity associated to clams. Thus, the study of the microbiota associated to whole specimens was carried out using 16S rRNA amplicon target sequencing. We have also included classic culturing techniques on marine agar (MA) and Thiosulfate-Citrate-Bile-Sucrose (TCBS) plates for comparative purposes. Finally, the dilution-to-extinction approach, reducing nutrient stress for oligotrophic and facultative oligotrophic bacteria adapted to the marine environment, was employed to attempt the isolation of rare taxa that can be masked in the media used with conventional cultivation techniques. Besides, two different sampling periods and sites were selected to include temporal and spatial variability in the analysis.

## Material and methods

### Clam samples

In this study, specimens of two species of clams, *Ruditapes decussatus* (carpet shell clam) and *Ruditapes philippinarum* (Manila clam), were collected in Redondela (42° 17' 40.4" N 8° 36' 57.2" W) and Carril (42°36'50.4" N 8°46'39.1" W) (WGS84) during April and October of 2015. Water temperature was registered on site, ranging in Redondela from 13.7 °C (April) to 16.5 °C (October) and from 13 °C (April) to 15.2 °C (October) in Carril. Immediately after collection, clam samples were transported to the laboratory in a cool box (4 °C), approximately during 3 h, divided in different packs corresponding to each clam species.

Each sample comprised 25 individuals. After washing with distilled water in order to eliminate epibiotic bacteria from the outer shell, clams were aseptically opened and whole specimens were homogenized for bacterial isolation and DNA extraction for 16S rRNA metabarcoding analysis.

#### DNA extraction

Bacterial cells of each homogenate were separated from eukaryotic cells and concentrated by gradient centrifugation using OptiPrep™ gradient density medium as previously described (Gerpe et al., 2021). The final pellet was resuspended in 1 ml of PBS and stored at  $-20\text{ }^{\circ}\text{C}$  until DNA extraction.

DNA was extracted using the MasterPure Complete DNA and RNA Purification kit (Epicentre Biotechnologies) following the manufacturer's instructions. DNA concentration and quality were determined by agarose gel electrophoresis (1% wt/vol agarose in Tris–acetate-EDTA buffer) and using NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and QUBIT 3 fluorometer (Invitrogen). Extracted DNA was stored at  $-20\text{ }^{\circ}\text{C}$  until use for PCR amplification.

#### Metabarcoding sequencing and 16S rRNA analysis.

Genomic DNA from each sample was used for the amplification of 16S rRNA gene using primers targeting the hypervariable regions V3/V4 (Lee et al., 2012): 338F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTCCTACGGGAGGCAGCA-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3'). The 16S rRNA amplicons were verified by gel electrophoresis on a 2% agarose gel using GreenSafe DirectLoad (NZytech) for the staining. DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and QUBIT.

Amplicons were sequenced at Sistemas Genómicos (Valencia, Spain) using Illumina MiSeq platform, generating paired-end  $2\times 250$ -bp reads. Illumina reads were analyzed for quality control using FastQC software (Brabham Bioinformatics). Quality trimming of reads was performed based on quality scores ( $Q < 30$ ) and length trimming (200 base pairs bp), using Trimmomatic 0.32 (Bolger et al., 2014)

program, as well as chimera detection and removal. The filtered paired-end reads were then merged using the command fastq-join (Quast et al., 2013) and clustered at 97% level of similarity into OTUs. Chimera detection and removal were performed.

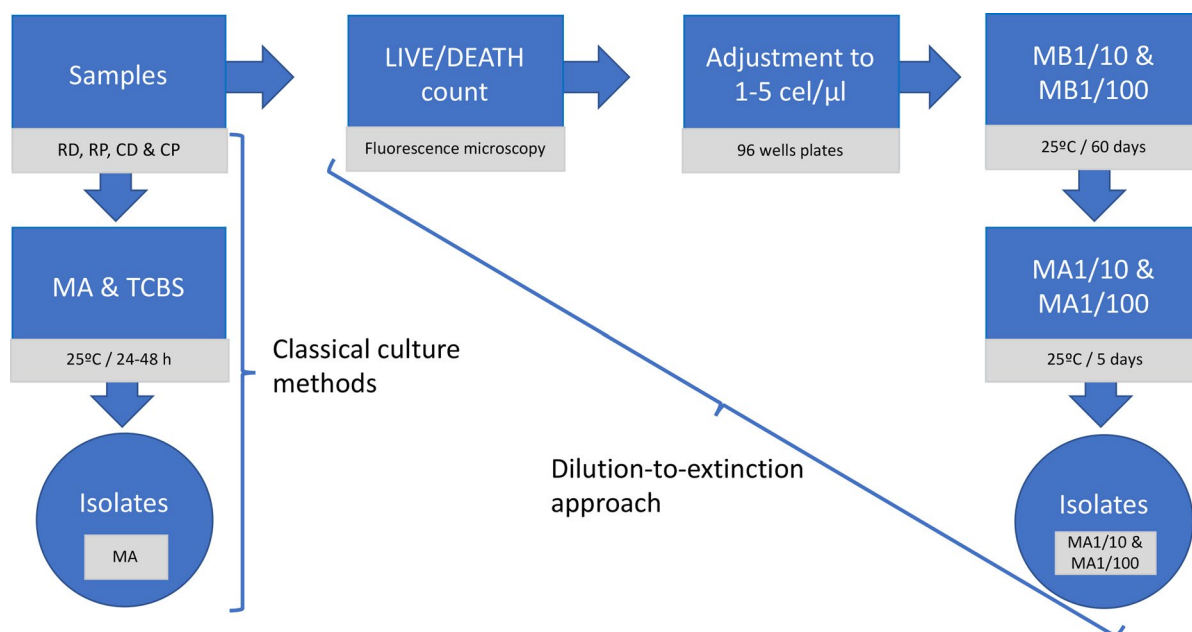
Ribosomal RNA gene reads were classified against the non-redundant version of the SILVA SSU reference taxonomy (release 123; <http://www.arb-silva.de>). For bacterial diversity estimation in the samples, the number of operational taxonomic units (OTUs) at 97% sequence identity was determined, and rarefaction analyses were carried out. Briefly, the reads were aligned against the 16S rRNA sequences of the SILVA database followed by a quality filtering, including length, ambiguity, and homopolymer checks. A de-replication step was performed to collapse identical reads into one single sequence and OTU's were clustered at 3% divergence threshold. The mitochondria, chloroplasts, and unassigned reads were deleted for the taxonomic analysis. Non-metric Multidimensional Scaling (NMDS) and Analysis of similarities (ANOSIM) were performed from the dissimilarity matrix using vegan package of R (Clarke 1993; Oksanen, 2017).

#### Bacterial isolation

Serial dilutions of the homogenized samples from the October sampling period were carried on with PBS and 100  $\mu\text{l}$  of each dilution was cultured in marine agar (MA, Difco) and Thiosulphate-Citrate-Bile-Sucrose (TCBS, Oxoid) at  $24\pm 1\text{ }^{\circ}\text{C}$  for 48 h. Isolated colonies were culture in marine agar and preserved at  $-80\text{ }^{\circ}\text{C}$  in marine broth (MB, Difco) supplemented with 15% of glycerol.

#### Dilution-to-extinction approach

For the experiment (Fig. 1), 20 clams were selected in October, 5 specimens of *R. decussatus* and 5 of *R. philippinarum* from each sampling site (Carril and Redondela). As before, clams were aseptically opened and the soft tissues of each clam were homogenized. The number of viable bacteria in the clam homogenates was determined using Live/Dead BacLight bacterial viability kit (Invitrogen) and were visualized and counted in a fluorescent microscope. Samples were diluted to 1–5 cells/well in 96-well microplates containing marine broth



**Fig. 1** Dilution-to-extinction and classic culture methodology. Sample codes: RD, *Ruditapes decussatus* from Redondela; RP, *R. philippinarum* from Redondela; CD, *R. decussatus* from Carril; CP, *R. philippinarum* from Carril

diluted 10 or 100 times (1–5 cells/well). These cultures were incubated at  $24 \pm 1$  °C in the absence of light for 60 days. Then, each well, containing bacterial growth, was streaked onto marine agar plates (MA, Difco) diluted 10 (MA1/10) and 100 times (MA1/100). Then, isolated colonies were cultured in marine agar 1/10 and 1/100 and stored at  $-80$  °C in marine broth (MB, Difco) diluted 1/10 and 1/100 supplemented with 15% of glycerol.

#### 16 s rRNA gene sequencing

For all the obtained isolates, DNA was extracted using the InstaGene Matrix (Bio-Rad) following the manufacturer's instructions. The 16S rRNA gene sequence was amplified and sequenced using the universal primer pair 27F (5' -AGAGTTTGATCC TGGCTCAG-3') and 1510R (5' -GGTTACCTT GTTACGACTT-3') as described by Hutson et al. (1993). Sequence data analyses were performed using DNASTAR Lasergene SEQMAN program and 16S rRNA sequence similarities were determined using the EzTaxon-e server ([www.eztaxon-e.ezbiocloud.net](http://www.eztaxon-e.ezbiocloud.net)) and the BLASTN program.

## Results

### 16S rRNA sequence metataxonomy

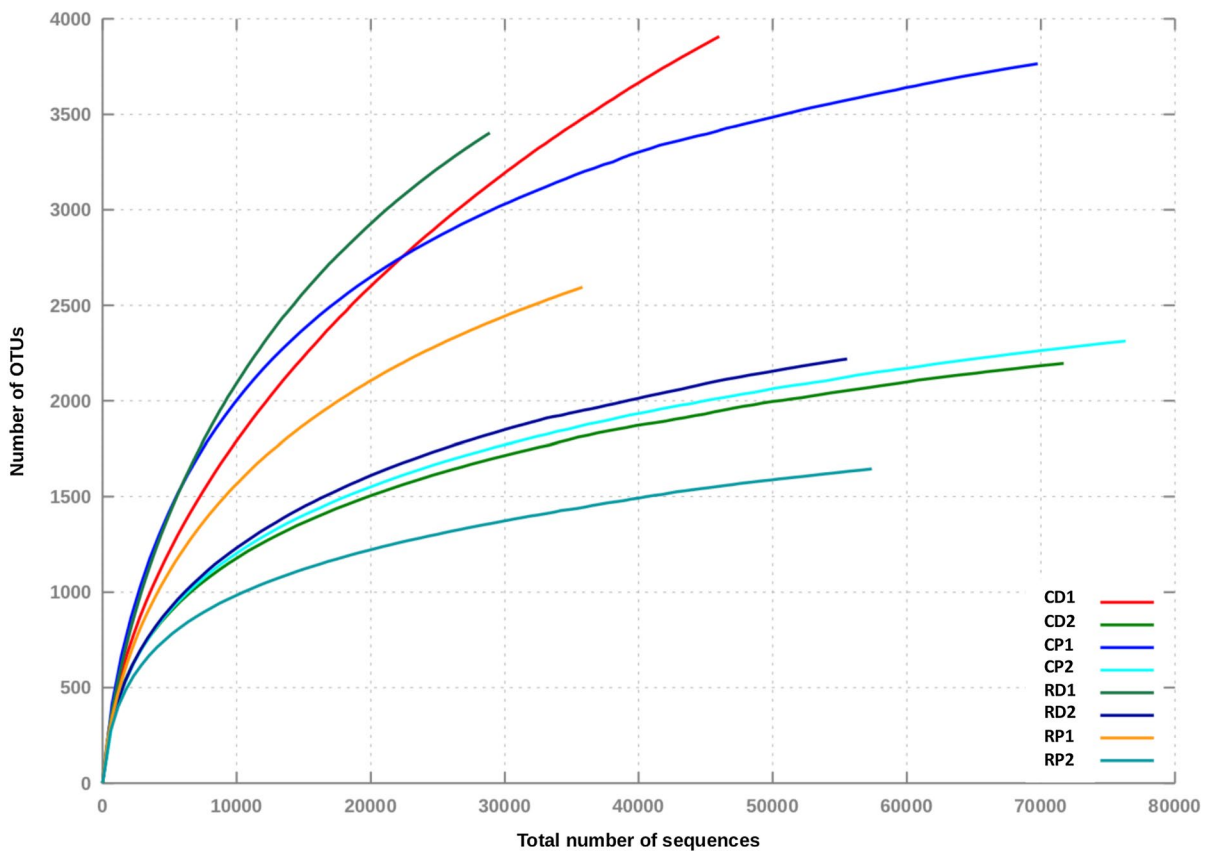
After filtering raw sequences obtained from V3/V4 region of 16S rRNA, a total of 441,672 reads were obtained from samples of *R. decussatus* and *R. philippinarum* with an average length ranging from 453 to 461 pb and a total of 22,044 OTUs were obtained (Table 1). Rarefaction analysis (at 97% sequence identity level) of *R. decussatus* and *R. philippinarum* reflected higher  $\alpha$ -diversity in April samples rather than October (Fig. 2).

Taxonomic assignment of the sequences using the non-redundant version of SILVA database identified *Proteobacteria* and *Actinobacteria* as the main phyla in all samples (Fig. 3). Other phyla with lower relative abundances included *Tenericutes*, *Firmicutes*, and *Bacteroidetes*. Samples taken in October showed an increase in the relative abundance of *Proteobacteria* group in both sites and clam species. On the other hand, *Actinobacteria* displayed a different pattern depending on the sampling site, with a reduction in Carril in October, while in Redondela the relative abundance was increased during this period.

**Table 1** Summary of the characteristics of all samples of *R. decussatus* and *R. philippinarum*, sequences analyzed, and diversity/richness indexes

Sample	N° sequences	Length	OTUs	% Classified	Sequences observed once	Sequences observed twice	Chao-1
CD1	46036	457	3908	99.02	1817	685	6313.01
CD2	71785	461	2196	92.94	615	254	2936.41
CP1	69785	460	3765	94.95	856	467	4546.92
CP2	76376	458	2313	99.41	618	266	3027.06
RD1	28904	460	3403	97.09	1336	623	4832.13
RD2	55553	453	2219	99.45	642	288	2930.98
RP1	35823	460	2595	94.16	863	413	3493.44
RP2	57410	455	1645	93.21	456	169	2255.24

C = Carril, R = Redondela;  
D = *R. decussatus*, P = *R. philippinarum*;  
1 = April and 2 = October

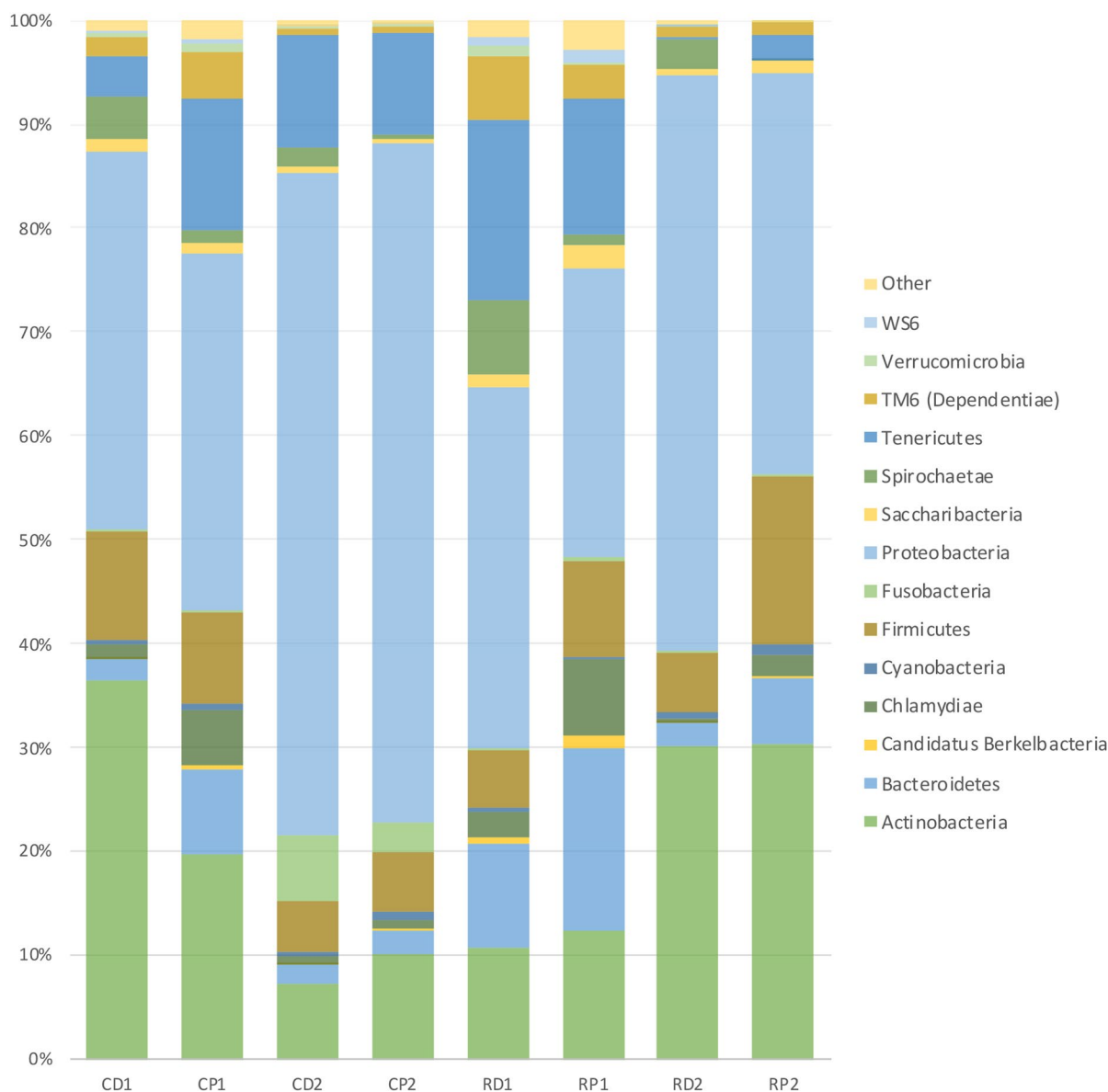


**Fig. 2** Rarefaction analysis of samples of *R. decussatus* and *R. philippinarum* showing the number of OTUs (at 97% 16S rRNA gene sequence identity) as a function of the number of sequences analyzed. Sample codes: RD, *Ruditapes decussatus*

from Redondela; RP, *R. philippinarum* from Redondela; CD, *R. decussatus* from Carril; CP, *R. philippinarum* from Carril. 1, April sampling; 2, October sampling

16S rRNA amplicon analysis at the genus level revealed a great bacterial diversity associated to *R. decussatus* and *R. philippinarum* species (Fig. 4).

*Mycoplasma* genus was detected in all samples showing more stability in Carril than in Redondela, which showed a drop in their relative abundance from 17.8%



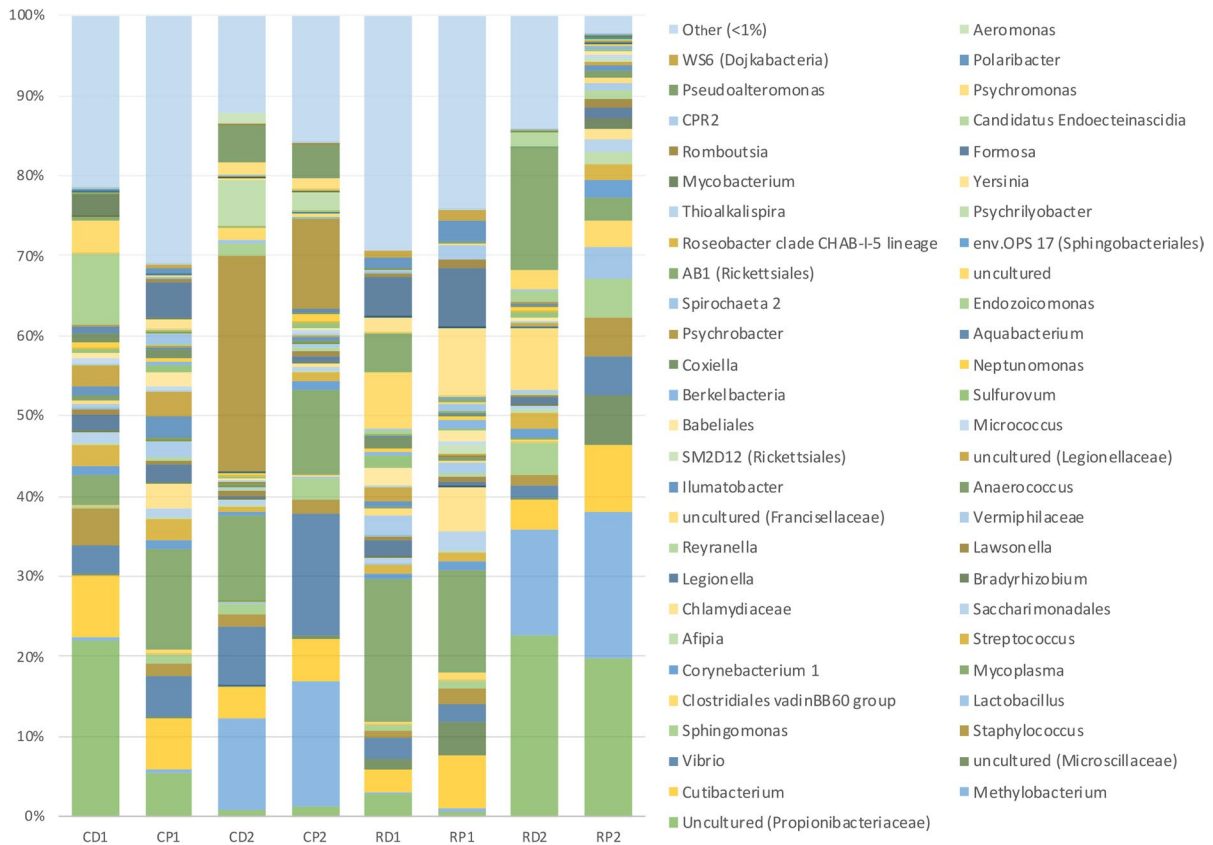
**Fig. 3** Relative abundances of bacterial phyla associated to *R. decussatus* and *R. philippinarum*. The graph shows the percentages (>1%) of the 16S rRNA reads assigned to different bacteria taxa. Sample codes: RD, *Ruditapes decussatus* from

Redondela; RP, *R. philippinarum* from Redondela; CD, *R. decussatus* from Carril; CP, *R. philippinarum* from Carril. 1, April sampling; 2, October sampling

in April (RD1) sample to 1% in October (RD2) sample. *Vibrio* genus was also present in all samples with higher relative abundances in Carril site and showing an increase from April to October sampling period. Other genera detected in all samples at relative abundances higher than 5% included *Staphylococcus*, *Streptococcus*, or *Cutibacterium*. Other genera, such

as *Psychrobacter*, were detected in all samples; however, at very low relative abundances except on samples gathered in Carril in October, both *R. decussatus* (CD2) and *R. philippinarum* (CP2), at high relative abundances (26.8% and 11.2%, respectively). Similarly, *Methylobacterium* genus was detected in all samples, although only samples obtained in October





**Fig. 4** Relative abundances of bacterial genera associated to *R. decussatus* and *R. philippinarum*. The graph shows the percentages (>1%) of the 16S rRNA reads assigned to different bacteria taxa. Sample codes: RD, *Ruditapes decussatus* from

Redondela; RP, *R. philippinarum* from Redondela; CD, *R. decussatus* from Carril; CP, *R. philippinarum* from Carril. 1, April sampling; 2, October sampling

at both locations were at high relative abundances (ranging from 11.5% to 18.2%). Interestingly, the NGS amplicon sequencing approach allowed the identification of several uncultured bacteria, such as *Propionibacteriaceae* or *Microscillaceae*, which in some cases reached relative abundances above 20%, as in *R. decussatus* samples from Carril in April (CD1) or from redondela in October (RD2).

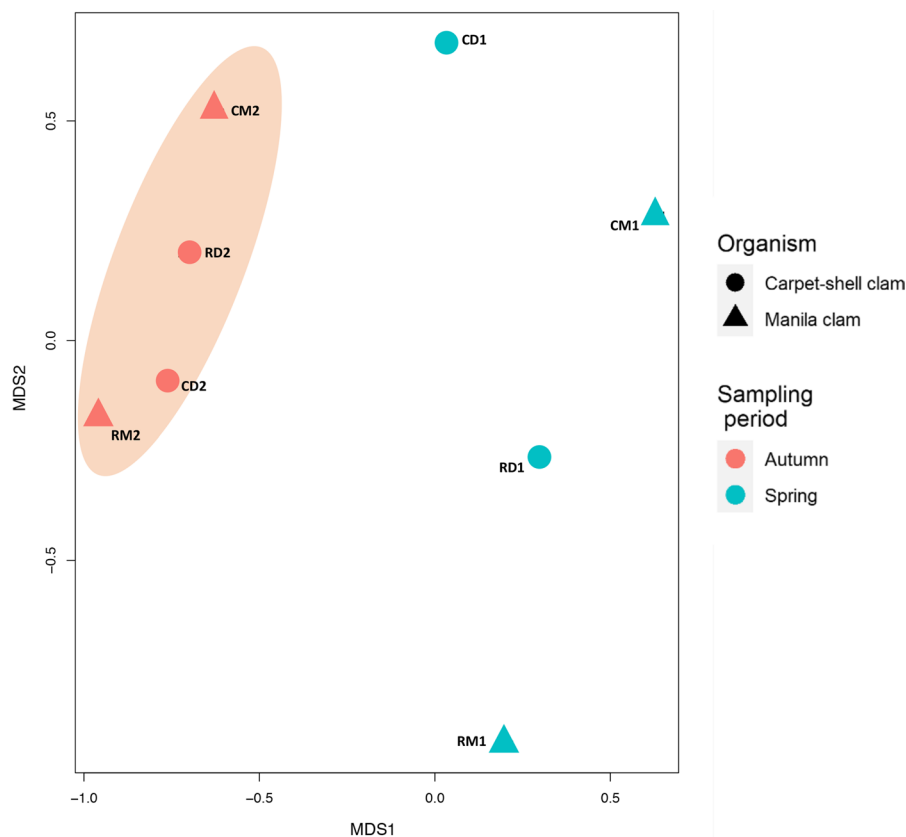
Microbial composition differences have been reflected when a Non-metric Multidimensional Scaling (NMDS), applied on ANOSIM distance matrix, analysis was performed. This analysis allowed to further investigate whether the clam species, sampling site, and sampling period affected the microbial composition of *R. decussatus* and *R. philippinarum* species (Fig. 5). This analysis demonstrated that samples from October clustered together, regardless their geographical origin or clam species. On the other hand,

samples from April were separated further apart one to each other.

#### Classical culture-based technique

Bacterial isolation from specimen homogenates resulted in a total of 94 isolates from MA and TCBS culture media (Supplementary Tables S1 and S2), which, in overall, consisted of 37 strains from *R. decussatus* and 13 from *R. philippinarum* in the localization of Redondela and another 21 belonged to *R. decussatus* and 23 *R. philippinarum* in the localization of Carril. In all samples, *Vibrio* was the most abundant genus with percentages from 42 to 57%, together with *Pseudoalteromonas* that was well represented in Carril (Fig. 6). Together with these two genera, *Shewanella* was one of the predominant genera in samples of *R. philippinarum* from Carril and

**Fig. 5** 2D representation of Non-metric Multidimensional Scaling (NMDS) plots applied on ANOSIM distance matrix. Ellipse indicates group of samples. Sample codes: RD, *Ruditapes decussatus* from Redondela; RP, *R. philippinarum* from Redondela; CD, *R. decussatus* from Carril; CP, *R. philippinarum* from Carril. 1, April sampling; 2, October sampling



*R. decussatus* from Redondela with percentages of 19.52% and 20.59%, respectively. Other strains isolated in lower proportions were identified as *Bacillus*, *Tenacibaculum*, *Photobacterium*, *Aliiroseovarius*, *Agarivorans*, *Cellulophaga*, *Endozoicomonas*, *Pseudomonas*, *Kiloniella*, *Acinetobacter*, and *Aliivibrio* (Fig. 6; Supplementary Tables S1 and S2).

#### Dilution-to-extinction isolates

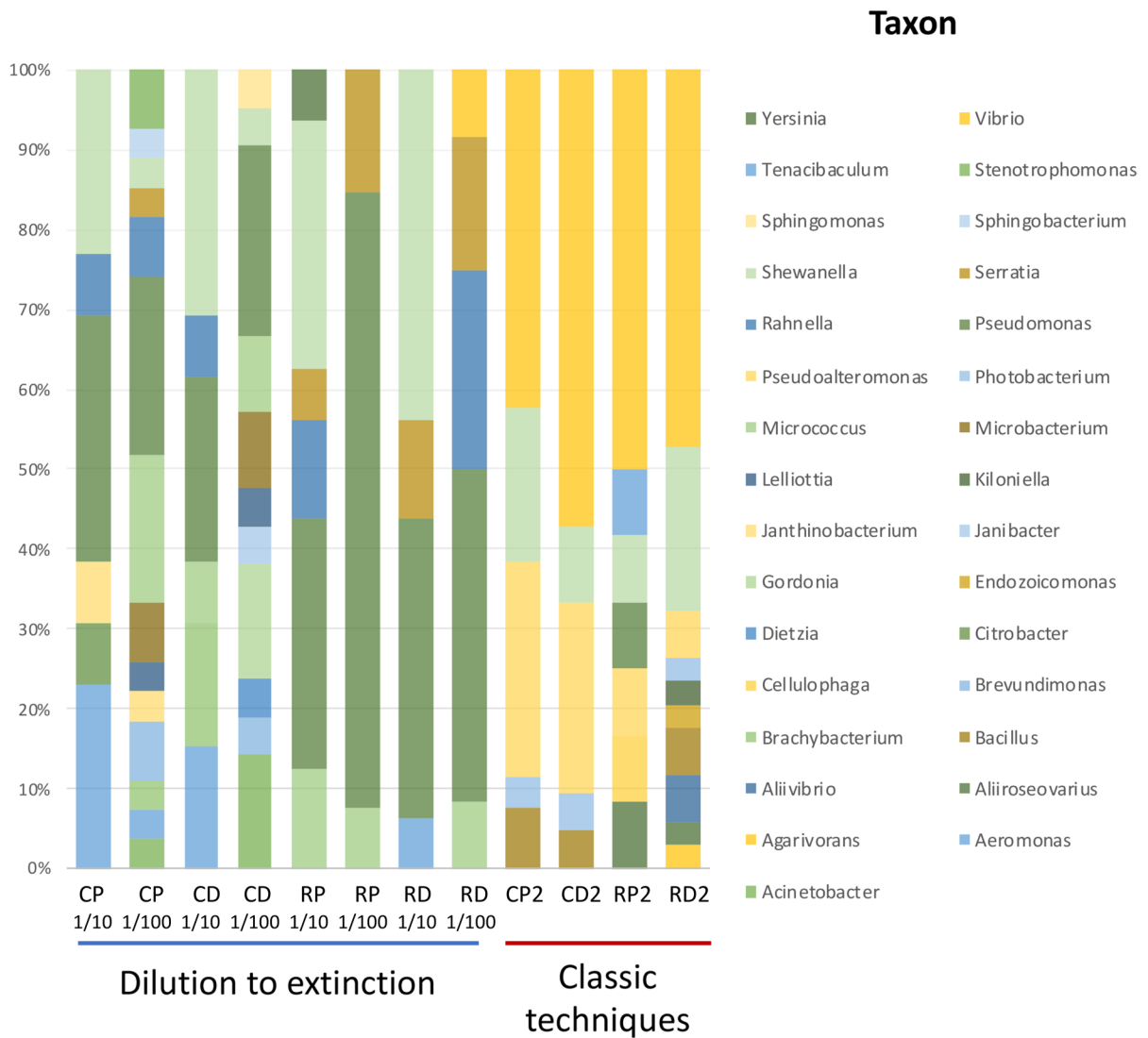
The dilution-to-extinction technique allowed the isolation of 131 isolates after 60 days of incubation with no light at 24 °C (Supplementary Tables S3 and S4). In Carril, 34 strains belonged to *R. decussatus* clam, 13 in MA 1/10 (CD1/10), and 21 in MA 1/100 (CD1/100). On the other hand, 40 strains were isolated from *R. philippinarum* in Carril site, of which 13 were obtained from marine agar diluted 10 times (CP1/10) and 27 from marine agar diluted 100 times (CP1/100). In Redondela, 27 isolates were obtained from *R. decussatus*, 15 from MA1/10 (RD1/10) and 12 from MA1/100 (RD1/100). From

*R. philippinarum* species 30 isolates were obtained, 16 isolates from MA 1/10 (RP1/10) and 14 from MA 1/100 (RP1/100).

16S rRNA gene sequence analysis of the isolated bacteria showed the predominance of *Gammaproteobacteria* group in all samples, followed by *Actinobacteria*, which was more abundant in samples from dilution-to-extinction approach than in samples cultivated on MA and TCBS, especially in Carril. At the genus level, the dominance of the genus *Pseudomonas* was observed in all samples of MA1/100, while in samples isolated from MA1/10 *Pseudomonas* and *Shewanella* were the most abundant genera (Fig. 6, Supplementary Tables S3 and S4). In general, samples from Carril showed higher number of different genera, especially samples obtained from MA1/100.

In CP1/10 *Pseudomonas*, *Shewanella*, and *Aeromonas* were the most abundant genera (30.77%, 23.08%, and 23.08%, respectively). Other three genera, *Janthinobacterium*, *Citrobacter*, and *Rahnella* were less abundant (7.69% each). *Pseudomonas*, *Shewanella*, *Aeromonas*, and *Brachybacterium* were





**Fig. 6** Relative abundances of culturable bacterial genera associated to *R. decussatus* and *R. philippinarum*, obtained by classic and dilution-to-extinction techniques in the sampling performed in October. The graph shows the percentages (> 1%) of the isolates assigned to different bacteria taxa according

to the 16S rRNA gene sequences. Sample codes: RD, *Ruditapes decussatus* from Redondela; RP, *R. philippinarum* from Redondela; CD, *R. decussatus* from Carril; CP, *R. philippinarum* from Carril

the most abundant genera in CD1/10 sample (23.07%, 30.77%, 15.38%, and 15.38%, respectively), while *Micrococcus* and *Rahnella* were less abundant, 7.79%.

Higher diversity was found in samples cultivated in MA1/100. *Pseudomonas* and *Micrococcus* constituted the 40% of the microbiota of CP1/100 sample. The other 60% of microbiota was constituted by several genera, including *Shewanella*, *Aeromonas*,

*Rahnella*, *Janthinobacterium*, *Lelliottia*, *Microbacterium*, *Brachybacterium*, *Brevundimonas*, *Acinetobacter*, *Vibrio*, *Stenotrophomonas*, *Sphingobacterium*, and *Serratia* (percentages from 3.57 to 7.14%). In CD1/100 samples, *Pseudomonas*, *Acinetobacter* and *Gordonia* represented more of the 50% of microbiota. *Micrococcus* and *Microbacterium* were found with percentages close to 10%. The remaining bacteria groups such as *Shewanella*, *Lelliottia*,

*Brevundimonas*, *Sphingomonas*, and *Janibacter* made up a total of 28%.

In Redondela samples, microbiota was represented by few genera. In samples cultivated in MA1/10, *Pseudomonas* and *Shewanella* were the most abundant genera that represented about 60% in RP1/10 and 80% in RD1/10 of the total of microbiota. *Micrococcus* and *Rahnella* represented 25% of the microbiota in RP1/10. In samples from this site cultivated in MA1/100 *Pseudomonas* was the most abundant bacteria accounting for 77% of total of microbiota in *R. philippinarum* and 42.67% in *R. decussatus*. *Serratia* genus presented percentages around 15–16% in both clam species and *Rahnella* genus was well represented in RD1/100 (25%).

## Discussion

The present study combined culture-dependent techniques together with a metataxonomic approach in order to study the microbiota associated to *R. decussatus* and *R. philippinarum* clam species. The use of different techniques provided a complete overview of the microbial population associated to these two clam species, from the already well-defined culturable fraction to the non-culturable vast microbial community. Indeed, the dilution-to-extinction experiment made possible the assessment of the microbial population, by successfully cultivating 131 new bacterial isolates.

Previous study of microbiota of *R. philippinarum* and *R. decussatus* reported the predominance of *Vibrio* and *Pseudoalteromonas* genera (Romalde et al., 2013; Balboa et al., 2016). These two genera represented more than 50% of the isolates on MA and TCBS media, followed by other bacteria frequently found in association with marine organisms or in the seawater, such as *Shewanella*, *Photobacterium*, *Aliivibrio*, or *Bacillus*. Furthermore, these two genera were not detected within the strains obtained by dilution-to-extinction culturing, in which *Pseudomonas* was the predominant genus in most samples. Some genera detected by dilution-to-extinction experiment were not detected with classic culturing technique in this study or in previous studies on *R. philippinarum* and *R. decussatus* clams (Balboa et al., 2016), including *Rahnella*, *Micrococcus*, and *Lelliottia*. These bacteria are frequently found in aquatic environments, but they have also been reported to cause bacteraemia

in humans, as for *R. aquatilis* (Ruimy et al., 2010). Besides, *Rahnella* and *Lelliottia* isolates were found during the experiment in several samples, both in diluted 1/10 and 1/100 MA plates, although they were not detected when using neither 16S rRNA gene amplicon sequencing nor classic culturing techniques. Other genera, such as *Janthinobacterium*, *Gordonia*, *Brachybacterium*, or *Microbacterium* isolated in dilution-to-extinction experiment, were also found using 16S rRNA gene amplicon sequencing but at very low relative abundances (<0.5%). As expected the restrictive selected culture conditions applied in the dilution-to-extinction effort, the number of isolates obtained was low in comparison to the number of OTUs detected with NGS technique, barely 0.6% of the total diversity. Different factors might be considered in future studies in order to culture a higher number of rare taxa, including quantification errors, culture media selection, nutrient availability, temperature, or time of incubation.

In general, seasonal and spatial variations were observed, pointing out the high complexity of these microbial populations. It is well known that the interaction between the host and their microbes provides them beneficial effects (Shapira, 2017; Torda et al., 2017; Offret et al., 2019), although, in bivalves, the characteristics and functionality of the associated microbiota are poorly understood (Desriac et al., 2014; Offret et al., 2019). Our work provides a new insight into the microbiota associated to *R. decussatus* and *R. philippinarum*, which, ultimately, demonstrates the complexity of such multicellular consortia. The results demonstrated that, although variability is observed, clam microbiota was dominated by few bacterial groups that are present in all samples in the different conditions, including *Mycoplasma*, *Vibrio*, and *Cutibacterium*, suggesting they might be considered as autochthonous bivalve bacteria.

*Vibrio* and bivalve mollusc interactions have been widely studied (Balboa et al., 2016; Destoumieux-Garzón et al., 2020) as pathogens or opportunistic pathogens but also as commensals or by establishing neutral associations. Vibrios are among the most common and widespread prokaryotes in temperate marine environments, representing the major culturable fraction of the marine microbial community (Ceccarelli et al., 2019); however, the abundance of *Vibrio* populations in molluscs can reach concentrations close to 100-fold higher than

those in seawater (Shen et al., 2009). In clams, they represent the main taxa in the culturable fraction as presented above; however, when a NGS approach is applied their real abundance compared to the whole community is clearly reduced.

*Mycoplasma* genus has been found associated with healthy oysters (Lasa et al., 2019) and was also dominant in clam hepatopancreas (Milan et al., 2018). It has been suggested that members of this genus might play a beneficial role in bivalve fitness and health status, although their role within the host is still largely unknown (Romero et al., 2002; King et al., 2012). Additionally, *Cutibacterium*, which was found at high relative abundances, is a novel genus (Scholz & Kilian, 2016), after splitting *Propionibacterium* into three different genera, and they have been mostly associated to humans, especially with acne (Platsidaki et al., 2018). However, our results indicate that the habitat of bacteria belonging to this genus may be larger than previously known and we also found several OTUs assigned to uncultured *Propionibacteriaceae*, indicating that these bacteria might be frequently found in marine environments.

The analysis of  $\alpha$ -diversity revealed lower microbial richness in *R. decussatus* and *R. philippinarum* in October samples, while NMDS analysis showed that microbial communities of samples obtained in autumn were more similar. Other studies revealed similar trends in oysters, where bacterial load and diversity were lower in winter (Pujalte et al., 1999; Zurel et al., 2011). Indeed, those samples showed the dominance of few bacterial taxa, besides *Mycoplasma*, *Vibrio*, and *Cutibacterium*, such as *Methylobacterium* and *Sphingomonas* in samples from Carril and Redondela in October or *Psychrobacter* in samples from Carril during October sampling period. These genera increased their relative abundances considerably respected to spring, when their concentration was residual. These changes in the microbiota composition may be related to a limited microbial diversity in the surrounding environment but also as a consequence of physiological changes that occur in clams during late spring/summer related to a great energy demand to complete gamete maturation and reproduction (Meneghetti et al., 2004). During this period, clams increase their filtration rates and increase the phytoplankton uptake, therefore the bacterial uptake is increased too.

## Conclusion

The present study explored into the high complexity of the microbial populations associated to *R. decussatus* and *R. philippinarum* clam species. Dilution-to-extinction technique revealed its usefulness for culturing rare bacterial species that, otherwise, would not be feasible. Overall, metataxonomic results demonstrated that the composition and dynamics of the associated microbiota are affected by seasonal periods, although few bacterial taxa appear to be autochthonous to these clam species.

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**Data availability** All data produced from this study are provided in this manuscript. Sequence files for all samples used in this study have been deposited at NCBI SRA with accession: PRJNA428215.

## Declarations

**Conflict of interest** The authors declare there is no conflict of interest.

**Ethical approval** The research complies with ethical standards.

**Consent for publication** Not applicable.

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