RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2016) 19:93-99
doi:10.2436/20.1501.01.267 ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Characterization of the microbiota associated to *Pecten maximus* gonads using 454-pyrosequencing

Aide Lasa, [1]Alex Mira,[2] Anny Camelo-Castillo,[2] Pedro Belda-Ferre,[2] Jesús L. Romalde[1]*

[1]Department of Microbiology and Parasitology, CIBUS, Universidade de Santiago de Compostela, Santiago de Compostela, Spain. [2]Department of Genomics and Health, Foundation FISABIO, High Research Center of Public Health, Valencia, Spain

Received 9 March 2016 · Accepted 3 June

Summary. A next-generation sequencing (NGS) approach was used to study the microbiota associated to *Pecten maximus* broodstock, applying pyrosequencing of PCR-amplified V1-V4 16S rRNA gene regions. We analysed the resident bacterial communities in female and male scallop gonads before and after spawning. DNA samples were amplified and quality-filtered reads were assigned to family and genus taxonomic levels using the Ribosomal Database Project classifier. A total of 18,520 sequences were detected, belonging to 13 phyla, including Proteobacteria (55%), Bacteroidetes (11,7%), Firmicutes (3%), Actinobacteria (2%) and Spirochaetes (1,2%), and 110 genera. The major fraction of the sequences detected corresponded to Proteobacteria, Beta- and Gammaprotebacteria being the most abundant classes. The microbiota of *P. maximus* gonad harbour a wide diversity, however differences on male and female samples were observed, where female gonad samples show a larger number of genera and families. The dominant bacterial genera appeared to be *Delftia*, *Acinetobacter*, *Hydrotalea, Aquabacterium, Bacillus, Sediminibacterium, Sphingomonas,* and *Pseudomonas* that were present among the four analysed samples. This next generation sequencing technique, applied for the first time in *P. maximus* (great scallop) gonads was useful for the study of the bacterial communities in this mollusc, unravelling the great bacterial diversity in its microbiota. [**Int Microbiol** 19(2): 93-99(2016)]

Keywords: *Pecten maximus* · gonads microbiota · next-generation sequencing (NGS) · molluscs pathogens · aquaculture

Introduction

Great scallop (*Pecten maximus*) is a bivalve mollusc species of great value in aquaculture due to its high market price. The main producers of this bivalve mollusc are France and United Kingdom [7]. However, the production of cultured scallops is still low since scallops at early life stages are susceptible to high mortalities. Due to their filter-feeding mechanism, bivalve molluscs have an abundant associated microbiota that can play an important role in their nutrition [5,6,25,29]. It has been proposed that the microbiota of shellfish is associated with the aquatic habitat and varies with factors such as salinity, bacterial load in the water, temperature, diet and rearing conditions [12,21]. Considering that scallops live in an environment with high concentration of microorganisms, opportunistic pathogens might be present [13,19], although their virulence would depend on the environment and bivalve conditions.

*Correspondence: J.L. Romalde
E-mail: jesus.romalde@usc.es

Most studies of bacterial communities associated with bivalves have been based on cultured strains, and in a study made in Chile with *P. maximus,* the main isolated bacteria were specific pathogens or probionts [24]. However, recently molecular methods have been applied to bacterial communities associated to molluscs. Sandaa et al. studied the bacterial communities associated with great scallop larvae using denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing [30]. They found that more than 50% of the 16S rRNA gene sequences among the different samples (algal cultures, hatchery systems and scallop larvae) belonged to Gammaproteobacteria class. Sequences from DGGE bands were assigned to uncultured Cytophaga-Flexibacter group, *Pseudoalteromonas* sp., *Vibrio* sp. and *Alteromonas/Pseudoalteromonas* group. In a study focused on the microbiota associated to oysters (*C. gigas* and *C. corteziensis*) in three different stages, temperature gradient gel electrophoresis (TGGE) bands were sequenced to determine the bacterial community revealing low bacterial diversity, with the main groups belonging to the Proteobacteria and Firmicutes phyla [33].

In the last few years, high-throughput pyrosequencing has been developed and applied to 16S rRNA gene analysis to enhance the knowledge about bacterial communities present on different natural and host-associated samples. The study of bacterial diversity associated to three oyster species [34], analysing V3-V5 regions of the 16S rRNA gene, generated sequences belonging to 13 phyla. Proteobacteria was the most abundant phylum, and within this phylum Alpha- and Gammaproteobacteria comprised the dominant classes.

The results presented here, to the best of our knowledge, mean the first data on the analysis of the microbiota associated to the gonads of cultured great scallop, before and after spawning, applying pyrosequencing of the 16S rRNA gene. The process of the spawning induction represents an important stressful factor, which may cause considerable changes in the resident microbiota of the scallop, increasing the risk of disease. Within the main routes of bacterial contamination to larval culture, such as incoming seawater, broodstock, and microalgal food, breeders could be one important route of pathogens input. Additionally, there are some evidences on other oyster species of bacterial transfer from parents to larval stages after the spawning [23], which may contain potential pathogens to larvae. In this sense, the current work aims to determine the effect of the spawning induction on the microbiota associated to scallop gonads. Our results will lead to a better understanding of the interactions between the host and its associated microbiota. In addition, it might constitute the optimization of a suitable model for future in-depth studies of the overall bacterial composition associated to the great scallop.

Material and methods

Sample collection. Four gonad samples of scallop broodstock were collected in a Norwegian hatchery located at Bergen (60º 30′ 53.77″ N, 4º 54′ 14.75″ W) on January 2011 and used for the analysis. Four samples, including two female (F) and two male (M) gonad samples were collected before (bS) and after (aS) spawning. The spawning was induced by thermal shock by the method described by Gruffyd and Beamont [10]. Before and after spawning, the external surface was washed by scrubbing under running water, washed with 70% ethanol and allowed to dry. The scallops were then opened aseptically by cutting the adductor muscle with a sterile scalpel. One gram of the gonad tissue was homogenized in 1 ml of artificial sterile seawater (ASW).The samples were stored at –20ºC until DNA extraction.

DNA extraction. DNA was extracted using the MasterPure Complete DNA and RNA Purification kit (Epicentre Biotechnologies) following the manufacturer´s instructions with a previous step of lysozyme (SIGMA) treatment (1 mg/ml, 37 ºC for 30 min). The DNA concentration and quality was determined by agarose gel electrophoresis (1% wt/vol agarose in Tris-acetate-EDTA buffer) and using NanoDrop ND-1000 spectrophotometer (Thermo Scientific). DNA extracted was stored at –20 ºC until use for PCR amplification.

PCR and pyrosequencing. A fragment of the 16S rRNA gene was amplified using the universal primers 27F and 785R with annealing temperature of 52 ºC and 20 cycles to minimize PCR biases [32]. The number of cycles was increased with equal conditions when the DNA concentration was insufficient. This approach has been used to reduce the frequency of nonspecific amplification [20]. The 27F universal primer was modified to contain an 8-bp "tag sequence" specific to each sample [16]. Barcodes were different in at least two nucleotides from each other to minimize mistakes in sample assignments. PCR products were purified using Nucleofast plates (Macherey-Nagel) following the manufacturer's instructions. The final concentration of DNA per sample was measured by picogreen fluorescence in a Modulus 9200 fluorimeter from Turner Biosystems. The pyrosequencing was performed unidirectionally from the forward primer at the Center for Advanced Research in Public Health (CSISP; Valencia, Spain) using 454-FLX sequencer (Roche) with Titanium Plus chemistry.

Quality assessment. Sequence end-trimming was performed to remove 10-bp windows with average quality values <20, as well as sequences of <250 bp and sequences with more than one ambiguous base call were removed using Ribosomal Database Project pyrosequencing pipeline (RDP) [35]. The program was used to separate samples according to the barcode sequences tagged to each forward primer. The possible chimeric sequences were detected with the UCHIME program [6], and an average of 2% of sequences for each sample were removed as possible chimeras.

Taxonomic analyses of sequence reads. The taxonomic assignment of the sequences was made using the RDP multiclassifier [35] using an 80% bootstrap confidence cutoff. For unidentified sequences a Blastn search was performed in order to lighten this issue. Many chloroplast sequences were detected (1550 sequences) that could be amplified with universal primers of the 16S rRNA gene [2], and removed before the

diversity analyses. For bacterial diversity estimation in the samples, the number of operational taxonomic units (OTUs) at 97% sequence identity was determined, and rarefaction analysis was carried out. Rarefaction curves were obtained by plotting the number of observed OTUs against the number of sequences. Equal number of sequences were used to minimize the biases caused by differences in the sequencing effort [31], using the minimum sample size. The diversity and richness of the samples were studied by calculating the Shannon and Chao1 indexes using the RDP Pipeline [35]. The overall composition of the microbial communities was compared using principal coordinate analysis (PCoA) performed by Fast Unifrac [11,15] using the weighted algorithm. This tool measures the similarity between bacterial communities based on phylogenetic distances. The number of shared OTUs between communities/samples was visualized using the Venn function in gplots [cran.r-project.org/package=gplots].

Significance tests based on the phylogenetic UniFrac distances [11] were performed. The P-values reported for multiple comparisons were adjusted by Bonferroni correction [25].

Results

The high-throughput sequencing approach was successfully applied to the microbiota associated to scallops. The number of reads filtered and the reads assigned at the genus level were higher on samples after spawning (Table 1). Despite the fact that differences on sequencing efficiency were observed, they being lower in sample M-bS, the numbers of genera and families were similar in the four samples. Evenness of samples appeared to be at the same level (Shannon–Wiener indexes) among samples, ranging from 5.56 to 6.20 values, indicating similar microbial diversity. However, Chao1 indexes showed differences among samples, and F-aS sample appeared to be the sample with highest richness (Table 1). The rarefaction analysis showed that the microbiota from female gonad samples was more diverse than the microbiota from male gonad samples (Fig. 1).

A total of 18,520 sequences were detected among the samples and sets of unclassified bacteria (data not shown), due to the 97% cutoff level of assignment, were manually assigned using a Blastn search, they having been classified as unculturable bacteria from other pyrosequencing studies.

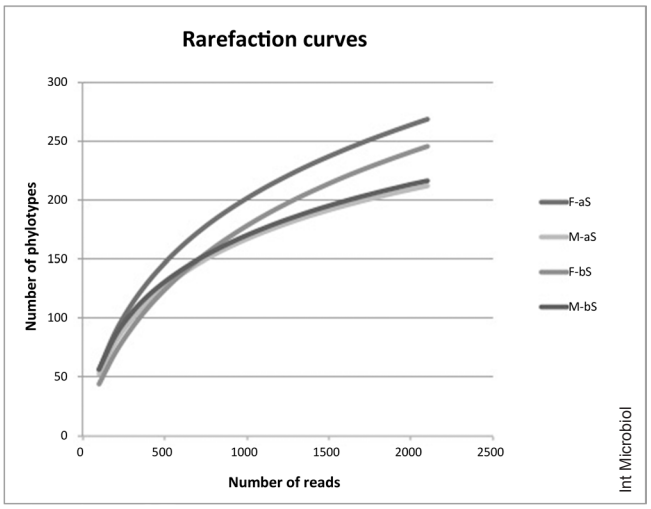

Fig. 1. Comparison of rarefaction curves among the four gonad samples, showing the number of OTUs (at 97% 16S rRNA gene sequence similarity) as a function of the number of sequences analysed. F-aS: female gonad sample after spawning; M-aS: male gonad sample after spawning; F-bS: female gonad sample before spawning; M-bS: male gonad sample before spawning.

However, the taxonomic assignment of the sequences pointed out a great diversity, with a total of 13 phyla (including Bacteroidetes, Proteobacteria, Spirochaetes, Actinobacteria and Firmicutes) and showed that the main bacterial classes were Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, Sphingobacteria, Actinobacteria and Bacilli. These groups represented more than 80% of the bacterial classes in samples with the exception of M-aS sample (Fig. 2A). Dominant families were the same among the four samples, namely Chitinophagaceae, Moraxellaceae, Comamonadaceae, Pseudomonadaceae and Sphingomonadaceae, they showing differences in their relative frequencies. Note that some families were more abundant in some pair of samples, such as Xanthomonadaceae and Rhodocyclaceae with higher frequencies in female gonad samples compared to male gonad samples, or the Enterobacteriaceae family that was not present in samples before spawning.

Table 1. Summary of the characteristics of the scallop samples, sequences analysed and diversity/richness indexes. F-aS: female gonad sample after spawning; M-aS: male gonad sample after spawning; F-bS: female gonad sample before spawning; M-bS: male gonad sample before spawning

Samples	Reads filtered	OTUs	No of genera	No of families	Shannon–Wiener Index	Chao1 index
F-bS	4395	1399	67	43	5.85	777.73
M-bS	2240	1162	41	35	5.56	526.50
F-aS	6742	2990	66	44	6.20	1482.12
M-aS	5143	2859	45	32	5.67	622.59

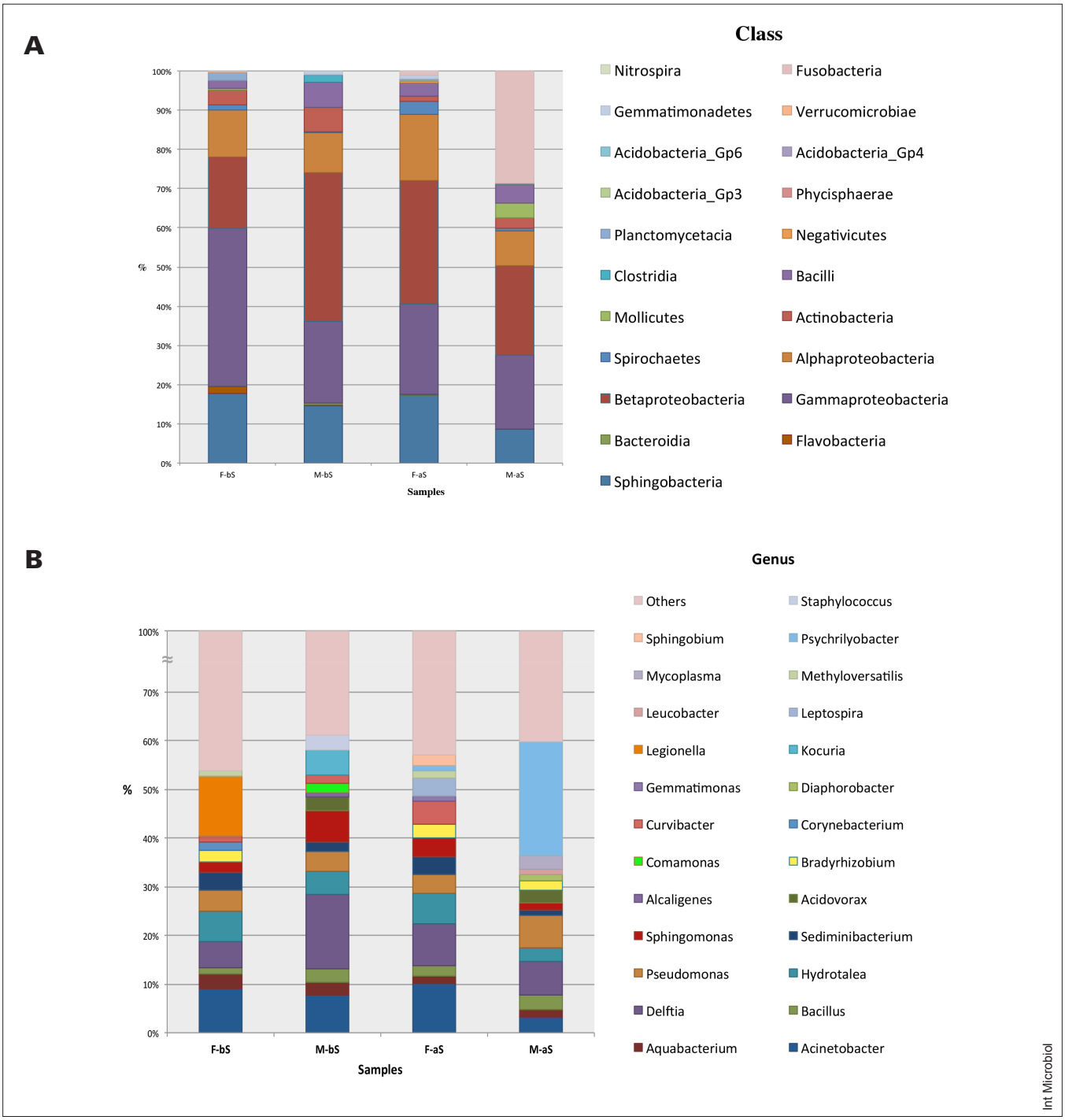

Fig. 2. Relative abundances of the bacterial classes (**A**) and genera (**B**) in the microbiota associated to scallop gonads. The graphs show the percentage (>1%) of 16S pyrosequencing reads assigned to different bacterial taxa. F-aS: female gonad sample after spawning; M-aS: male gonad sample after spawning; F-bS: female gonad sample before spawning; M-bS: male gonad sample before spawning.

Genera with the largest number of sequences detected, namely *Delftia*, *Acinetobacter*, *Hydrotalea, Aquabacterium, Bacillus, Sediminibacterium, Sphingomonas,* and *Pseudomonas*, appear in every sample with abundances over 1% of the total sequences assigned (Fig. 2B). The assignment at the genus level revealed the presence of more than 110 genera. However, approximately half of the sequences could not be assigned to any bacterial genus.

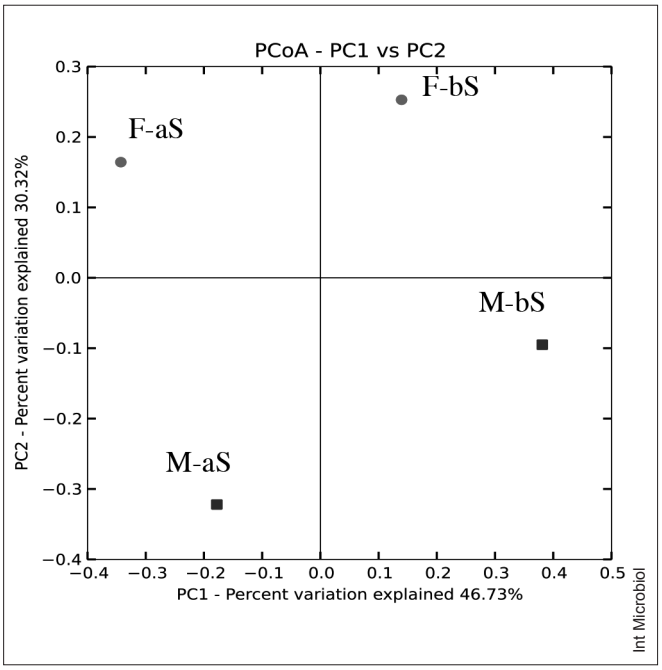

Fig. 3. Principal coordinates analysis (PCoA) from gonads samples according to microbiota composition. F-aS: female gonad sample after spawning; M-aS: male gonad sample after spawning; F-bS: female gonad sample before spawning; M-bS: male gonad sample before spawning.

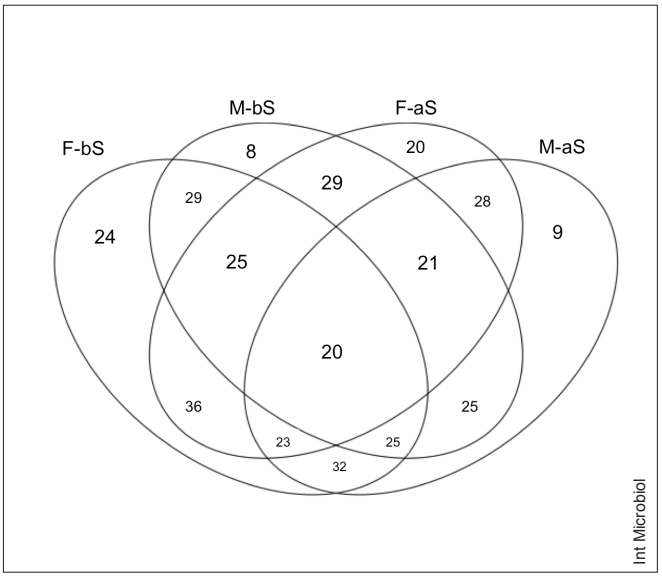

Fig. 4. Venn diagram showing the unique and shared genera among scallop samples.

The PCoA analysis showed that each sample could be differentiated by their microbial community composition (Fig. 3), where the two principal coordinates account for a 77.05% cumulative variance. The shared genera were shown via Venn diagram (Fig. 4) to compare differences among 4 gonad samples. Most genera (20–36) were shared between samples. Male gonad samples showed less unique genera (8–9), both before and after spawning. The second component of the analysis separated samples from male and female gonads and the principal component of the analysis separated gonads before spawning from samples after spawning. The microbial composition of the gonad samples differed significantly (*P*-test $\leq$ 1.0-e03) depending on sampling time (before and after spawning) and on the gonad part of the scallop (female or male).

Discussion

We found a large microbial diversity in the scallop gonad, where the variability of the bacterial communities among the samples was significant, as it could be seen in the PCoA analysis. Samples before and after spawning were selected to test whether the bacterial community changed or not. Thus, the method used to induce the spawning did not affect the composition of the main bacterial groups, which were conserved after the thermal shock. In fact, in all samples the main bacterial groups were the same, although with different abundances. Rarefaction curves and the total of sequences obtained were not completely homogeneous and more sequencing effort would be required to reach saturation.

Among the total of sequences detected, phylum Proteobacteria comprised the largest fraction. In fact, Betaproteobacteria and Gammaproteobacteria, which are known to be highly abundant in marine environments, were the most dominant classes [27]. It is known that most classes within Proteobacteria play important roles in bivalve molluscs, specifically they are able to degrade cellulose and agar, which are major components of the food consumed by these marine invertebrates, and some members of this phylum can fix nitrogen in the gastrointestinal tract of bivalves [18,21,36]. The 16S rRNA gene sequences from DGGE analysis obtained by Sandaa et al. [30] differed from our results, taking into account that they used algal cultures, hatchery systems and scallop larvae samples, where the main bacterial class was Alphaproteobacteria, except on larval samples, in which the predominant class was Gammaproteobacteria.Our results are in accordance with those of Trabal-Fernández et al., who have reported that Proteobacteria is the most abundant phylum in oysters [34]. However, they have observed differences in the abundances of Proteobacteria classes when comparing the bacterial communities at different growth stages and cultivation sites. In general, Alphaproteobacteria

and Gammaproteobacteria were the principal classes among the three oyster species, and only one sample showed Betaproteobacteria as the most abundant class**.** In this sense, studies about the bacterial communities of the North Atlantic deep-water [1] have provided similar results on the most abundant classes (Alphaproteobacteria) in the bathypelagic and subsurface waters, and only with depth, do the relative abundances of Gamma-, Delta- and Betaproteobacteria increase. In contrast to our results, Alphaproteobacteria appear to be one of most abundant classes both in oysters species and North Atlantic deep-water samples. However, we only analysed the microbial community of one organ and not the entire organism, which could explain these differences. Note that, for some of the main genera detected in this study, a small number of species has been described at the time of writing, namely *Hydrotalea* (2 species), *Delftia* (5 species), *Aquabacterium* (6 species) and *Sediminibacterium* (3 species) [14]. On average, these genera represented almost 20% of the whole bacterial community. By and large, these genera are inhabitants of different aquatic environments including marine freshwater and hot spring runoff habitats. The high abundances of these genera suggest that probably most species of these bacterial genera are still unknown. Note that no sequences belonging to *Vibrio* genus were detected and only a few sequences from *Pseudoalteromonas* genera were observed. These bacterial groups had been described as main components of the cultured microbial communities in several mollusc species, such as oysters [17,22], clams [28] and scallops [30]. However, our results agree with findings of Trabal-Fernández et al. [33,34], in which the presence of *Vibrio* species in different oysters species is low. The absence of *Vibrio* sequences may be related to the fact of having analysed only the gonad and not other organs nor the whole mollusc. The selection of the variable regions of the 16S rRNA gene and primer design might represent an important bottleneck on the detection of certain genera, such as *Vibrio* or *Pseudoalteromonas*, therefore it must be considered in future studies.

The next generation sequencing (NGS) approach revealed that scallop gonads harbour a diverse bacterial population before and after spawning. Despite some differences observed on the presence or abundances of certain genera, the dominant bacterial groups are the same among different samples. These results suggest that spawning should have minor effects on the bacterial composition of the gonads. However, the physiological and ecological importance of these bacterial communities it is still unknown. Further studies are needed to improve the knowledge about the microbiota present in these molluscs and their biological significance by increasing the number and type of samples, including more organs in the study, as well as other NGS techniques that will provide more accurate information. It is important to understand not only which bacterial groups are present but also what is the ecological relation between the host and the resident bacteria.

Acknowledgments. This work was supported in part by Project 245119 (REPROSEED) from KBBE-2009-1-2-11 Subprogram of the 7th Framework Programme (FP7), and grant AGL2013-42628-R from Ministerio de Economia y Competitividad (Spain). A.L. acknowledges the Ministerio de Ciencia e Innovación (Spain) for a research fellowship.

Competint interests. None declared.

References

1. Agogué H, Lamy D, Neal PR, Sogin ML, Herndl GJ (2011) Water mass-specificity of bacterial communities in the North Atlantic revealed by massively parallel sequencing. Mol Ecol 20:258-274
2. Benitez-Paez A, Álvarez A, Belda-Ferre P, Rubido S, Mira A, Tomás I (2013) Detection of transient bacteraemia following dental extractions by 16S rDNA pyrosequencing: a pilot study. PLoS One 8:e57782
3. Conza L, Pagani SC, Gaia V (2013) Presence of *Legionella* and free-living amoebae in composts and bioaerosols from composting facilities. PLoS One 8:e68244
4. Douillet P, Langdon CJ (1993) Effects of marine bacteria on the culture of axenic oyster *Crassostrea gigas* (Thunberg) larvae. Biol Bull 184:36-51
5. Douillet P, Langdon CJ (1994) Use of a probiotic for the culture of larvae of the pacific oyster *Crassostrea gigas* (Thunberg). Aquaculture 119:25-40
6. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194-2200
7. Food and Agriculture Organization of the United Nations (2014) *Pecten maximus* (Linnaeus, 1758). Species Fact Sheets. http://www.fao.org/fishery/species/3516/en
8. Garcia A, Goñi P, Cieloszyk J, Fernandez MT, Calvo-Beguería L, Rubio E, Fillat MF, Peleato ML, Clavel A (2013) Identification of free-living amoebae and amoeba-associated bacteria from reservoirs and water treatment plants by molecular techniques. Environ Sci Technol 47:3132-3140
9. Greub G, Raoult D (2004) Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 2:413-433
10. Gruffyd LD, Beamont AR (1970) Determination of the optimum concentration of eggs and spermatozoa for the production of normal larvae in *Pecten maximus* (Mollusca, Lamellibranchia). Helgoland Wiss Meer 20:486-497
11. Hamady M, Lozupone C, Knight R (2010) Fast UniFrac: Facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. ISME J 4:17-27
12. Harris JM (1993) The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. Microb Ecol 25:195-231
13. Lambert C, Nicolas JL, Cilia V, Corre S (1998) *Vibrio pectenicida* sp. nov., a pathogen of scallop (*Pecten maximus*) larvae. Int J Syst Bacteriol 48:481-487

14. List of Prokariotic Names with Standing in Nomenclature (2016) http://www.bacterio.net
15. Lozupone C, Hamady M, Knight R (2006) UniFrac-an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics 7:371
16. Mckenna P, Hoffman C, Minkah N, Aye PP, Lackner A, Liu Z, Lozupone CA, Hamady M, Knight R, Bushman FD (2008) The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. PLoS Pathog 4:e20
17. Najiah M, Nadirah M, Lee KL, Lee SW, Wendy W, Ruhil HH, Nurul FA (2008) Bacteria flora and heavy metals in cultivated oysters *Crassostrea iredalei* of Seitu Wetland, East Coast Peninsular Malaysia. Vet Res Commun 32:377-381
18. Newell RIE (2004) Ecosystem influences of natural and cultivated populations of suspension feeding Bivalve Mollusc: a review. J Shellfish Res 23:52-61
19. Nicolas JL, Corre S, Gauthier G, Robert R, Ansquer D (1996) Bacterial problems associated with scallop *Pecten maximus* larval culture. Dis Aquat Org 27:67-76
20. Patin NV, Kunin V, Lidström U, Ashby MN (2013) Effects of OTU clustering and PCR artifacts on microbial diversity estimates. Microb Ecol 65:709-719
21. Prieur MJ, Mvel G, Nicolas JL, Plusquellec A, Vigneulle M (1990) Interactions between bivalve molluscs and bacteria in the marine environment. Oceanogr Mar Biol Ann Rev 28:277-352
22. Pujalte MJ, Ortigosa M, Macián MC, Garay E (1999) Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. Int Microbiol 2:259-266
23 Riquelme CE, Chavez P, Morales Y, Hayashida G (1994) Evidence of parental bacterial transfer to larvae in *Argopecten purpuratus* (Lamarck, 1819). Biol Res 27:129-134
24. Riquelme CE, Hayashida G, Vergara N, Vasquez A, Morales Y, Chavez P (1995) Bacteriology of the scallop *Argopecten purpuratus* (Lamarck, 1819) cultured in Chile. Aquaculture 138:49-60
25. Riquelme CE, Jorquera MA, Rojas AI, Avendaño RE, Reyes N (2001) Addition of inhibitor-producing bacteria to mass cultures of *Argopecten purpuratus* larvae (Lamarck, 1819). Aquaculture 192:111-119
26. Roesch LF, Casella G, Simell O, Krischer J, Wasserfall CH, Schatz D, Atkinson MA, Neu J, Triplett EW (2009) Influence of fecal sample storage on bacterial community diversity. Open Microbiol J 3:40-46
27. Romalde JL, Diéguez AL, Doce A, Lasa A, Balboa S, López C, Beaz-Hidalgo R (2012) Advances in the knowledge of the microbiota associated with clams from natural beds. In: da Costa González F (ed) Clam fisheries and squaculture. Nova Science Publishers, New York, pp 163-190
28. Romalde JL, Diéguez AL, Lasa A, Balboa S (2014) New *Vibrio* species associated to molluscan microbiota: a review. Front Microbiol 4:413
29. Ruiz-Ponte C, Samain JF, Sanchez JL, Nicolas JL (1999) The benefit of a *Roseobacter* species on the survival of scallop larvae. Mar Biotechnol 1:52-59
30. Sandaa RA, Magnesen T, Torkildsen L, Bergh O (2003) Characterisation of the bacterial community associated with early stages of Great Scallop (*Pecten maximus*), using Denaturing Gradient Gel Electrophoresis (DGGE). Syst Appl Microbiol 26:302**e**311
31. Schloss PD, Gevers D, Wescott SL (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS One 6:e27310
32. Sipos R, Szekely AJ, Palatinszky M, Marialigeti K, Nikolausz M (2007) Efect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. FEMS Microbiol Ecol 60:341-350
33. Trabal-Fernández N, Mazón-Suastegui JM, Vázquez-Juárez R, Asencio-Valle F, Morales-Bojórquez E, Romero J (2012) Molecular analysis of bacterial microbiota associated with oysters (*Crassostrea gigas* and *Crassostrea corteziensis*) in different growth phases at two cultivation sites. Microb Ecol 64:555-569
34. Trabal-Fernández N, Mazón-Suástegui JM, Vázquez-Juárez R, Ascencio-Valle F, Romero J (2014) Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during comercial production. FEMS Microbiol Ecol 88:69-83
35. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261-5267
36. Zehr JP, Jenkins BD, Short SM (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ Microbiol 7:539-554