RESEARCH ARTICLE

Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production

Natalia Trabal Fernández[1], José M. Mazón-Suástegui[1], Ricardo Vázquez-Juárez[1], Felipe Ascencio-Valle[1] & Jaime Romero[2]

[1]Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico; and [2]Laboratorio de Biotecnología, Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Macul, Santiago, Chile

Correspondence: Ricardo Vázquez-Juárez, Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, Baja California Sur 23096, Mexico.
Tel.: +52 612 123 8484;
fax: +52 612 125 3625;
e-mail: rvazquez04@cibnor.mx

Received 3 June 2013; revised 3 December 2013; accepted 5 December 2013. Final version published online 13 January 2014.

DOI: 10.1111/1574-6941.12270

Editor: Julian Marchesi

Keywords
bivalve molluscs; bacterial community; molecular-based techniques.

Abstract

The resident microbiota of three oyster species (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) was characterised using a high-throughput sequencing approach (pyrosequencing) that was based on the V3–V5 regions of the 16S rRNA gene. We analysed the changes in the bacterial community beginning with the postlarvae produced in a hatchery, which were later planted at two grow-out cultivation sites until they reached the adult stage. DNA samples from the oysters were amplified, and 31 008 sequences belonging to 13 phyla (including *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes*) and 243 genera were generated. Considering all life stages, *Proteobacteria* was the most abundant phylum, but it showed variations at the genus level between the postlarvae and the adult oysters. *Bacteroidetes* was the second most common phylum, but it was found in higher abundance in the postlarvae than in adults. The relative abundance showed that the microbiota that was associated with the postlarvae and adults differed substantially, and higher diversity and richness were evident in the postlarvae in comparison with adults of the same species. The site of rearing influenced the bacterial community composition of *C. corteziensis* and *C. sikamea* adults. The bacterial groups that were found in these oysters were complex and metabolically versatile, making it difficult to understand the host–bacteria symbiotic relationships; therefore, the physiological and ecological significances of the resident microbiota remain uncertain.

Introduction

Oysters are the most abundantly harvested and cultivated bivalve molluscs in the world. Over the past 20 years, oyster cultivation in Mexico has improved. Commercially important molluscs such as the Pacific oyster *Crassostrea gigas* (Thunberg, 1793), *Crassostrea sikamea* (Anemiya, 1928) and the regional species *Crassostrea corteziensis* (Hertlein, 1951) are cultured in Mexico (Castillo-Durán *et al.*, 2010; Mazón-Suástegui *et al.*, 2011). Oysters with high economic value have been studied from a technical point of view to establish the optimum conditions for their cultivation. The influence of environmental factors including salinity, temperature and culture density has been the focus of numerous studies (Gosling, 2003; Mazón-Suástegui *et al.*, 2008, 2011). However, the knowledge regarding the microbiota that are associated with these marine animals during commercial production is lacking. Successful oyster production depends on the appropriate transference of postlarvae to the field and the survival of the adults in the environment (Gosling, 2003; Mazón-Suástegui *et al.*, 2008, 2011). This survival may be related to the bacterial community that is associated with the oysters. 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 (Prieur *et al.*, 1990; Harris, 1993). One of the main problems in oyster

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aquaculture is the repetitive mortality episodes that are most often caused by bacterial pathologies and can dramatically reduce commercial production. These infectious outbreaks affect the larval and postlarval stages in hatcheries as well as juveniles and adults that are cultured in natural conditions (Moriarty, 1997; Romalde & Barja, 2010). The identification of the resident microbiota of an organism is important because the microbiota can be disturbed by environmental changes that subsequently allow transient microorganisms to gain an advantage and cause disease (Moriarty, 1990, 1997).

The association between bivalve molluscs and gut microorganisms is typically attributed to the ingestion of bacteria (Prieur *et al.*, 1990; Harris, 1993). Research on aquatic organisms has indicated that the resident microbiota are involved in a variety of beneficial roles, including the development of the host gastrointestinal tract, nutrition (providing vitamins, enzymes and essential fatty acids for the host), immune responses and disease resistance (Prieur *et al.*, 1990; Harris, 1993; Moriarty, 1997). An increased susceptibility to infections may be related to a lack of the barrier provided by the microbiota that normally competes with pathogenic microorganisms for nutrients and space in the intestinal tract or produces substances that inhibit pathogens (Gatesoupe, 1999; Gómez-Gill *et al.*, 2000; Prado *et al.*, 2010; Kesarcodi *et al.*, 2012).

Currently, methods to characterise microbial populations in oysters involve bacteriological cultivation, and *Vibrio* and *Pseudomonas* spp. are the organisms that are most frequently isolated (Kueh & Chan, 1985; Harris, 1993; Pujalte *et al.*, 1999; Najiah *et al.*, 2008). In general, the *Vibrio* species are frequently associated with diseased oysters and have been detected using selective culturing (Paillard *et al.*, 2004; Thompson *et al.*, 2005). However, bacterial identification from oyster homogenates using culture methods indicates that the number of colonies grown on agar was < 0.001% of the total bacteria that were present in the oyster (Romero & Espejo, 2001).

In recent years, culture-independent studies have identified the microbiota in the following hatchery-raised and wild oysters: *C. gigas* (Hernández-Zárate & Olmos-Soto, 2006; Fernández-Piquer *et al.*, 2012), *Crassostrea virginica* (LaValley *et al.*, 2009), *Saccostrea glomerata* (Green & Barnes, 2010), *Ostrea chilensis* (Romero *et al.*, 2002), *Crassostrea iredalei* (Najiah *et al.*, 2008) and *Chama pacífica* (Zurel *et al.*, 2011). However, these studies did not focus on the changes in the composition of the microbiota that may occur during oyster growth under commercial production conditions. With the exception of the study by Romero *et al.* (2002), the previous reports do not distinguish between resident and transient bacteria, which may lead to an overestimation of the bacterial diversity of the microbiota. During larval development, transient microbiota rapidly become residents of the oyster microbiota (Brown, 1973; Kueh & Chan, 1985; Kesarcodi *et al.*, 2012), although little is known about the dynamics and stability of the microbiota during the juvenile and adult growth stages. Colonisation of the oyster gastrointestinal tract by bacteria is particularly dependent upon the external environment because of the flow of water passing through the digestive tract, and the life stage and physiological state of the invertebrate marine organism can influence the composition of the gut microbiota (Harris, 1993; Gatesoupe, 1999; LaValley *et al.*, 2009). Recently, we showed that the gastrointestinal resident microbiota that was associated with *C. corteziensis* and *C. gigas* showed composition differences according to the cultivation sites and growth stages that were examined, that is the postlarval, juvenile and adult stages (Trabal *et al.*, 2012). The microbiota composition was determined by sequencing temperature-gradient gel electrophoresis bands, and the analysis revealed the presence of *Betaproteobacteria*, *Firmicutes* and *Spirochaetes*, but the overall bacterial diversity was low compared with that shown in other studies. High-throughput molecular-based techniques, such as 16S rRNA gene-based pyrosequencing, can be used for a more in-depth characterisation of complex bacterial communities, and samples can be obtained directly from their environment, thus eliminating the need to isolate and cultivate specific microorganisms (Roesch *et al.*, 2007; Sundquist *et al.*, 2007; Petrosino *et al.*, 2009). This study provides the first research where the 16S rRNA gene-based pyrosequencing analysis of the V3–V5 regions was used to determine the composition and diversity of the resident microbiota associated with *C. corteziensis*, *C. gigas* and *C. sikamea* postlarvae and to document the changes in the bacterial microbiota when those postlarvae were planted at two grow-out cultivation sites (Bahia Magdalena, BM; Bahía Topolobampo, BT), where they later reached the adult stage.

Materials and methods

Oyster collection

Crassostrea corteziensis, *C. sikamea* and *C. gigas* postlarvae were collected (n = 30 by each species) at the optimal size for planting from the hatchery; oysters at this stage are known as spats and have a shell height of 3–5 mm at 6 weeks of age after settling. In September 2009, a set of postlarvae was transferred (planted) to two different grow-out cultivation sites to reach the adult stage. These postlarvae were cultured exclusively at the bottom of the cages for improved stability and food supply. Two different grow-out cultivation locations were used: Punta

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Botella (PB; 25°18′1452″N, 112°04′35″W) at Bahía Magdalena and Bahía de Topolobampo (BT; 25°54′N, 108°33′ W); the detailed descriptions of the grow-out cultivation areas and environmental conditions can be found in Trabal *et al.* (2012). Thirty adult oysters of each species and each cultivation site were collected when they had reached a size of 6–12 cm in shell height and an age of 7–12 months after the postlarvae planting (from July to September 2010). The samples were transported on ice to the laboratory. Upon arrival, the surfaces of the shells were scrubbed with filtered, UV-sterilised seawater for epifauna elimination. Next, the shells were washed with 70% ethanol and were depurated for 72 h in filtered, UV-sterilised seawater with constant aeration according to the scheduled depuration process (FDA, 1992). The transient microbiota was removed from the oysters using methods previously reported by Son and Fleet (1980) and Lee *et al.* (2008). The external valves were thoroughly cleaned to remove surface contamination, and the oysters were then carefully opened, leaving the animal intact. The postlarvae and adult oysters were dissected in a sterile Petri dish using one sterile scalpel blade for each oyster. Postlarval oysters were vigorously rinsed in sterile seawater, the intervalve liquid and adductor muscles were eliminated, and the organisms were frozen in alcohol (70%) at −20 °C. The gastrointestinal tract tissues of the adult oysters were separated and were frozen at −20 °C. In all cases, the internal appearance of the oyster bodies was examined at the time of dissection, and only healthy organisms with no evidence of infection were used in the study.

DNA extraction, 16S rRNA gene library construction and pyrosequencing

The DNA was extracted from 270 individual samples that consisted of 30 postlarvae and 30 adults from each cultivation site (BM, BT) for each species of oyster. The DNA was obtained from the total tissue of the postlarvae and from the gastrointestinal tissues of the adults. The tissues were homogenised with the help of a disposable and sterile plastic pestle and by incubation with a lysis buffer containing Tris–EDTA–SDS [100 mM NaCl, 50 mM Tris (pH 8), 100 mM EDTA (pH 8)], sodium dodecyl sulphate (SDS 1%) and 100 μL of lysozyme (50 mg mL^{-1}) for 1 h at 37 °C. The homogenised tissue was then incubated for 12 h at 65 °C with 20 μL of proteinase K (20 mg mL^{-1}; Sigma, St. Louis, MO). Following lysis, 100 μL of 5 M NaCl was added, the mixture was stirred and 80 μL of a solution of CTAB/NaCl (10% CTAB in 0.7 M NaCl) was added and incubated at 65 °C for 10 min. The DNA was extracted using a QIAmp® DNA Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The concentration and quality of the DNA were determined at $A_{260\ nm}$ and $A_{280\ nm}$ using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and 0.5% agarose gel electrophoresis with GelRed™ staining (Biotium, Hayward, CA). The total DNA from each individual sample was diluted in nuclease-free water to obtain a concentration of 100 ng μL^{-1}, which was used as a template to amplify the 570-bp region of the 16S rRNA gene (*Escherichia coli* position 357–926) that contained the hypervariable regions V3–V5 of the 16S rRNA gene. The primers 341 forward (5′-CCTACGGGAGGCAGCAG -3′) (Muyzer *et al.*, 1993) and 939 reverse (5′-CTTGTGCG GGCCCCCGTCAATTC-3′) (Rudi *et al.*, 1997), which annealed at positions 341–358 and 917–939 on the *E. coli* 16S rRNA gene, respectively, were used for the amplification. The primers used in the domain *Bacteria*-specific amplifications were selected because of their high variability (Andersson *et al.*, 2008) and because while they aligned *in silico* using the SEQUENCE MATCH software with reference sequences (1 921 179 16S rRNA genes) from the Ribosomal Database Project (RDP; Cole *et al.*, 2009), they showed greater alignment with the bacteria domain sequences and had lack of alignment with *Achaea* domain sequences; and it was not cross-amplified with the oyster genome. To reduce PCR-driven bias, we reduced the number of PCR cycles (Acinas *et al.*, 2005). PCR was performed with a reaction mixture (100 μL) containing 0.2 mM of each deoxynucleoside triphosphate, 1 U mL^{-1} AccuPrime Pfx DNA polymerase (Invitrogen, San Diego, CA), 1× polymerase reaction buffer, 2 mM $MgCl_2$ and 0.25 pmol mL^{-1} of each primer. The reaction mixtures were incubated in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturation at 95 °C for 10 min followed by 12 cycles at 95 °C for 1 min 30 s, 55 °C for 1 min 30 s, 72 °C for 1 min 30 s and a final elongation at 72 °C for 6 min for the first phase of the nested PCR. Subsequently, 1 μL of the amplified DNA (unpurified) was re-amplified using a PCR mixture that was identical to the previously described mixture and with an initial denaturation at 94 °C for 10 min followed by 20 cycles at 97 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min 30 s and a final elongation at 72 °C for 6 min. The PCR products were analysed using 1% agarose gel electrophoresis and GelRed™ staining (Biotium). The PCR amplification of the V3–V5 regions of the 16S rRNA gene was used to detect the bacterial communities that were present during different oyster growth stages (postlarvae and adult) and at the two grow-out cultivation sites. Three PCR amplifications were performed for each individual sample. The amplicons of the 16S rRNA gene for each sample, with final concentrations of ≥ 100 ng μL^{-1}, were mixed in equal concentrations to generate a final concentration of 300 ng μL^{-1} for nine sets of pooled

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samples (each set = postlarvae, adults BM, adults BT for each oyster species). Purification was performed using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI). After successful amplification and purification, the purified products that were obtained from the different sets of reactions were pooled in equal mass (molar) ratios, and 10 μg of the PCR product (concentration ≥ 50 ng μL^{-1} and D260/280 ≥ 2.0) was used for pyrosequencing on the FLX-Junior Sequencer (454 Roche Life Sciences, Branford, CT). Three different pyrosequencing sets were performed in which the nine samples of interest were represented using their corresponding multiplex identifiers (MIDs).

Sequence processing

The 16S rRNA gene raw reads were masked using the SeqClean software pipeline to eliminate sequence regions that would cause errors in the analysis process. Targets for masking included the ends that were rich in Ns (undetermined bases) and areas of low complexity (the nucleotide sequence with a single type of repeated nucleotide). Then, the potentially chimeric sequences that were detected using the Bellerophon approach with mothur software (Schloss *et al.*, 2009) were removed. Finally, only the sequences that showed a size ≥ 400 bp were considered for analysis, and cyanobacterial and eukaryotic sequences (chloroplast and mitochondrial 16S rRNA gene from algal cells) were removed.

The collection and sequence information is available on GenBank within the Sequence Read Archive under accession number SRA074278 sub 192249.

Operational taxonomic unit determination

The sequences generated by pyrosequencing were analysed using the mothur software (Schloss *et al.*, 2009) for the alignment and identification of operational taxonomic units (OTUs). A distance matrix was calculated from the aligned sequences using the dist.seqs script, and operational taxonomical units (OTUs; 97% sequence similarity) were assigned using a cluster script and the furthest neighbour clustering algorithm.

Taxonomic assignment

The sequences for each sample were given taxonomic assignments at a bootstrap confidence range of 95% using the RDP's (Cole *et al.*, 2009) Naïve Bayesian Classifier tool (RDP classifier) (Wang *et al.*, 2007). Sequences with identity scores > 97% were resolved at the genus level, and sequences with identity scores > 80% were resolved at the phylum level. The relative abundances were calculated based on the 16S rRNA gene pyrosequencing results and were expressed as the percentage of 16S rRNA gene sequences that were assigned to a given genus or phylum.

Rarefaction and diversity analyses

Based on the alignment, a distance matrix with the Phylip format was constructed. These pairwise distances served as the input to mothur (Schloss *et al.*, 2009) for clustering the sequences into OTUs of defined sequence similarity. These clusters served as OTUs to generate rarefaction curves. Shannon's diversity, Simpson index (Magurran, 1998) and Chao1 estimators (Hughes *et al.*, 2001; Chao & Bunge, 2002) were calculated to determine the diversity and richness of the OTUs within each sample. Diversity analyses were performed using mothur (Schloss *et al.*, 2009).

We analysed the beta-diversity of the bacterial communities that were associated with *C. corteziensis*, *C. sikamea* and *C. gigas* using a principal component analysis (PCA) (Krzanowski, 2000) that compared the OTUs that were detected in the postlarvae to adults from the two grow-out cultivation sites. Only principal components with eigenvalues > 1.0 were considered statistically significant. The PCA was performed using correlation matrices that were generated from a binary matrix (presence/absence) of the OTUs of each sample that was expressed as a value of the Dice similarity coefficient (Fromin *et al.*, 2002) using past (Hammer *et al.*, 2001). The PCA was conducted using the Statistica 10.0 software package (StatSoft Inc., Tulsa, OK).

Results and discussion

In this study, the gastrointestinal resident microbiota of three oyster species (*C. corteziensis*, *C. gigas* and *C. sikamea*) were characterised using a high-throughput sequencing approach (pyrosequencing) that was based on the V3–V5 regions of the 16S rRNA gene. The postlarvae were all produced at the same hatchery, and the adults were grown at BM and BT.

OTU, sequence classification and diversity of oyster bacterial microbiota

Characterisation of bacterial communities using PCR amplification of 16S rRNA gene can be biased by a number of factors such as multiple copies of the 16S rRNA gene, differences in primer binding and elongation efficiency of the PCRs and variation in the efficiency of DNA extraction (Hughes *et al.*, 2001; Acinas *et al.*, 2005; Claesson *et al.*, 2010; Wu *et al.*, 2010; Soergel *et al.*, 2012). Despite these limitations in the use of 16S rRNA

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gene amplicons, it is a robust tool and is the most commonly used molecular marker for identifying prokaryotes in varied environments (Hughes *et al.*, 2001; Acinas *et al.*, 2005; Wu *et al.*, 2010). Whether the unit of measurement is defined and constant, abundances and diversity among sites or treatments can be compared (Hughes *et al.*, 2001; Amend *et al.*, 2010). In this study, the utilised methods were carefully considered to reduce bias caused by DNA extraction and driven by PCR. For example, a method with a high extraction efficiency was used, and this method includes the use of CTAB, which can precipitate genomic DNA by its ability to remove polysaccharides from bacteria, and lysozyme, which digests cell wall components of Gram-positive bacteria (Shahriar *et al.*, 2011). Furthermore, two approaches were used as follows: one was based on presence–absence information for bacterial taxa (PCA), and the other took into account their relative abundance, included calculated diversity indices and estimated OTU richness and compared sample diversity with rarefaction curves.

The pyrosequencing produced 37 802 raw reads with an estimated average size of 451 bp for all of the oyster samples (including postlarvae and adults), and three biological replicates were included for each sample. After the removal of 16.6% of the total raw reads that could cause errors in the analysis process (low complexity, low quality, chimeric, and chloroplast and mitochondrial 16S rRNA gene sequences), 31 524 valid reads with a length $\geq$ 400 bp were used for further analysis (Table 1). OTUs were identified using the RDP classifier (Cole *et al.*, 2009) at the phylum and genus levels using confidence thresholds of $\geq$ 80% and $\geq$ 97% sequence identity, respectively (Table 1). Overall, 98.4% of the valid reads were classified at least to the phylum level (Table 1). At most, 6% of the sequences (*C. corteziensis* adults, BT cultivation site) were unclassifiable at the phylum level (Table 1). In contrast, $\leq$ 70% of all reads could be assigned to a bacterial genus. These differences demonstrate the limited ability to use 16S rRNA gene amplicons to discriminate beyond the genus level, and this limitation is occasionally due to the incomplete reference sequences of the 16S rRNA gene database available for the selected region in the amplification (Sundquist *et al.*, 2007; Liu *et al.*, 2008). To avoid these technical limitations during pyrosequencing of the 16S rRNA gene amplicons, in subsequent studies may be used more than one set of primers because using different targeted amplification regions can provide additional information for classification and can allow for better resolution at the genus level (Soergel *et al.*, 2012). Nonetheless, high-quality reads of similar average sizes were obtained in this study, and we were able to compare the different developmental stages and sampling sites.

The microbial diversity thus reflects the number of OTUs in the microbiota and not necessarily the number of defined species. It is also possible to predict the total numbers of OTUs that are present in these samples using the Chao1 richness estimate, as shown in Table 1. *Crassostrea corteziensis*, *C. gigas* and *C. sikamea* had a great richness of OTUs during both stages that were examined; however, the postlarvae of the three oyster species showed the highest richness based on the observed OTUs and Chao1 estimate, with values of 368/481, 240/305 and 367/503, respectively (Table 1). The Shannon–Weaver (H') and Simpson indices indicated that the microbiota associated with the postlarvae were more diverse than those associated with the adults, regardless of the species of oyster (Table 1). These results could be explained by the life stage of the oysters. The maturity of the gastrointestinal tract can especially influence the composition of the microbiota in marine organisms (Harris, 1993; Gatesoupe, 1999; Paillard *et al.*, 2004; Kesarcodi *et al.*, 2012). For example, it has been reported that the density and diversity of the bacteria in the intestines of molluscs decrease as it progresses from the zoea to the postlarval

Table 1. Summary of the characteristics of the oyster samples, sequences analysed, diversity and richness indices

Oyster species	Life stage	Geographical sites	Reads	Reads classified*	OTUs†	Chao-1	Simpson ($1-'\lambda$)	Shannon diversity (H')
Crassotrea corteziensis	Postlarvae	Bahía La Paz-Hatchery	9916	9768 (98.5)	368	481	0.98	4.47
Crassotrea corteziensis	Adult	BM cultivation site	1161	1130 (97.3)	165	249	0.94	3.67
Crassotrea corteziensis	Adult	BT cultivation site	1126	1060 (94.1)	117	157	0.90	3.21
Crassostrea gigas	Postlarvae	Bahía La Paz-Hatchery	3867	3846 (99.5)	240	305	0.96	3.99
Crassostrea gigas	Adult	BM cultivation site	3277	3120 (95.2)	141	237	0.59	1.84
Crassostrea gigas	Adult	BT cultivation site	1201	1192 (99.3)	140	234	0.88	3.11
Crassostrea sikamea	Postlarvae	Bahía La Paz-Hatchery	8469	8381 (98.9)	367	503	0.98	4.51
Crassostrea sikamea	Adult	BM cultivation site	1247	1243 (99.7)	79	133	0.50	1.57
Crassostrea sikamea	Adult	BT cultivation site	1260	1255 (99.7)	125	187	0.90	3.23

BM, Bahia Magdalena; BT, Bahía Topolobampo; OTUs, operational taxonomic units; RDP, Ribosomal Database Project.
*Number (percentage in parentheses) of sequences assigned to a given phylum using the RDP's (Cole *et al.*, 2009) Naïve Bayesian Classifier tool (RDP classifier; Wang *et al.*, 2007).
†The OTUs were defined using a 16S rRNA gene sequence similarity cut-off of 97% with the RDP (Cole *et al.*, 2009).

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stage (Brown, 1973; Tinh *et al.*, 2008; Kesarcodi *et al.*, 2012). Moreover, observations in *Pecten maximus*, *Mercenaria mercenaria*, *Argopecten* sp. and *C. gigas* adults have shown that adults are more selective regarding food ingested compared with the larvae, and this can affect the composition of the associated microbiota (Prieur *et al.*, 1990; Ward & Shumway, 2004). This ability to select ingested microorganisms could partly explain the differences in the bacterial diversity of the microbiota that are associated with the postlarvae and adults. However, it is also possible that the differences in richness and diversity that were observed between the adults and the postlarvae could be explained by the way in which the samples were processed. Although all of the organisms were depurated, it was impossible to dissect the gastrointestinal tracts of the postlarvae (due to their small size); therefore, these organisms were processed as homogenate tissue. Other authors have reported that microorganisms are also associated with the gills and mantle in species of mussels and oysters (Hernández-Zárate & Olmos-Soto, 2006; Duperron *et al.*, 2008; Zurel *et al.*, 2011), and these microorganisms could result in an overestimation of the bacterial diversity and abundance at this growth stage.

Rarefaction analysis (at a 97% sequence identity level) was performed to determine whether all of the OTUs that were presented in the data sets had been sufficiently recovered in the study (Fig. 1). The individual rarefaction curves for the postlarvae (SK-pL, C-pL) showed similar patterns of reaching a plateau, but, similar to the curves for the remaining samples, they did not reach a saturation or asymptotic phase (Fig. 1). This result suggests that a large number of unseen OTUs remained in the original samples and that additional pyrosequencing may be required to detect the additional phylotypes.

Bacterial phyla composition of the resident microbiota associated with oysters

Phylogenetic analysis of the sequences using the RDP classifier identified 13 phyla from the oyster microbial community. The bacterial communities were dominated

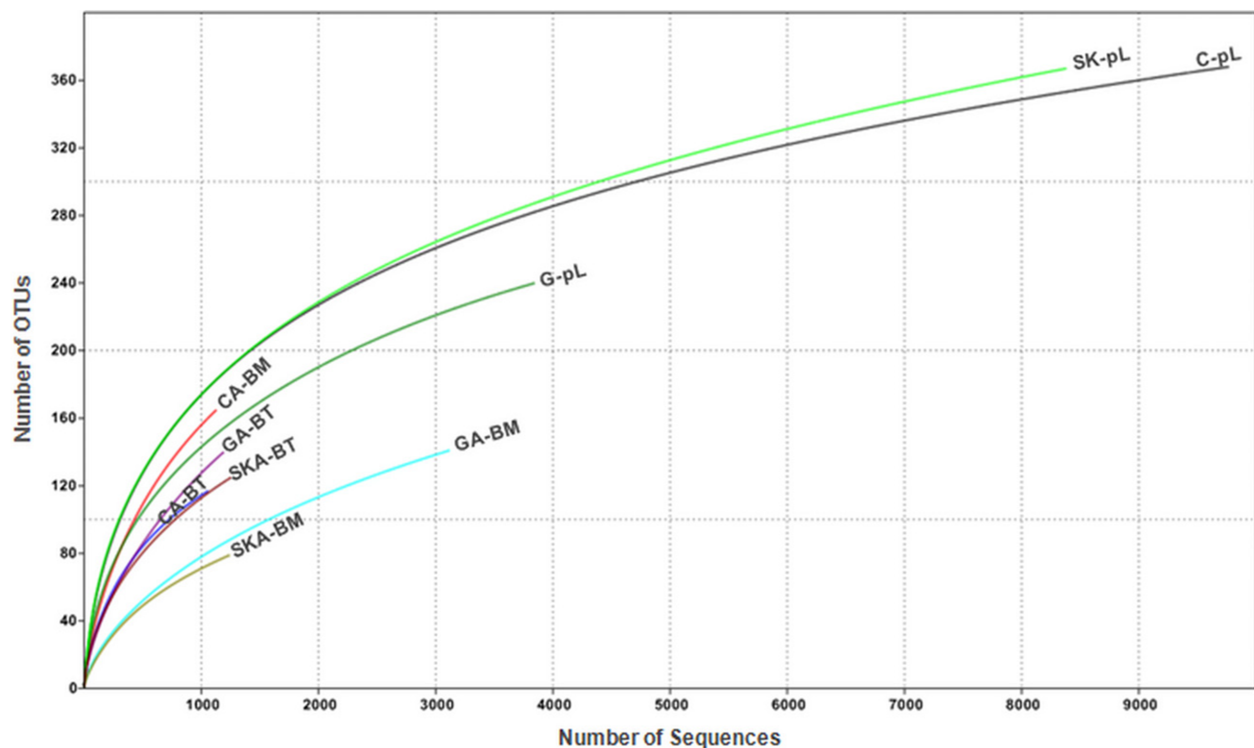

Fig. 1. Rarefaction curves for *Crassostrea corteziensis, Crassostrea gigas* and *Crassostrea sikamea* showing the number of OTUs (at 97% 16S rRNA gene sequence identity) as a function of the number of sequences analysed. SK-pL: Postlarvae of *C. sikamea*; C-pL: Postlarvae of *C. corteziensis*; G-pL: Postlarvae of *C. gigas*; SKA-BM: Adults of *C. sikamea* from the grow-out cultivation site at BM; CA-BM: Adults of *C. corteziensis* from the grow-out cultivation site at BM; GA-BM: Adults of *C. gigas* from the grow-out cultivation site at BM; SKA-BM: Adults of *C. sikamea* from the grow-out cultivation site at BT; CA-BM: Adults of *C. corteziensis* from the grow-out cultivation site at BT; and GA-BM: Adults of *C. gigas* from the grow-out cultivation site at BT.

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by the phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes*, in that order (Fig. 2a).

Proteobacteria, the largest and most phenotypically diverse phylum, was the most abundant phylum through all of the life stages and at the different cultivation sites. The prevalence of this phylum in oyster microbiota has been reported in previous studies (Vasconcelos & Lee, 1972; Pujalte *et al.*, 1999; Romero *et al.*, 2002; Hernández-Zárate & Olmos-Soto, 2006; Najiah *et al.*, 2008; Green & Barnes, 2010; Zurel *et al.*, 2011; Fernández-Piquer *et al.*, 2012). Two important roles have been recognised for *Proteobacteria* in marine invertebrates: first, they are able to degrade cellulose and agar, which are major components of the food that is consumed by these bivalve molluscs; and second, some marine bacterial species are capable of fixing nitrogen in the gastrointestinal tract of bivalves (Prieur *et al.*, 1990; Harris, 1993; Zehr *et al.*, 2003; Newell, 2004). In the postlarvae stages, the relative abundance of this phylum was between 58% and 72%. At the BM cultivation site, the microbiota of the *C. corteziensis*, *C. sikamea* and *C. gigas* adults consisted of 82%, 86% and 92% *Proteobacteria*, respectively. In contrast, at the BT cultivation site, the *C. corteziensis* and *C. sikamea* adult microbiota consisted of 87% and 51% *Proteobacteria*, respectively (Fig. 2a). For most of the samples, *Alpha*- and *Gammaproteobacteria* comprised most of the dominant classes (Fig. 2b), and both classes are known to be highly abundant in marine environments (Rappé *et al.*, 2000; Kersters *et al.*, 2006). However, massive sequencing allowed us to detect differences in the abundance and structure of these classes when comparing the bacterial communities at different growth stages and cultivation sites. In the postlarvae stage, the *Alpha*- and *Gammaproteobacteria* were most abundant (between 41% and 53%), while the adults showed variations in the abundance of each *Proteobacteria* classes that varied by species and site. In the adults, the most abundant class was Gamma*proteobacteria*, followed by *Beta*- and *Alpha-proteobacteria* (Fig. 2b). These results agree with those previously described for adult oysters (Table 2). *Proteobacteria* and *Bacteroidetes* usually dominate marine environments (Rappé *et al.*, 2000; Thomas *et al.*, 2011). In this study, *Bacteroidetes* was the second most abundant phylum in the oyster microbiota (Fig. 2a), similar to what has been reported by other authors (Hernández-Zárate & Olmos-Soto, 2006; Zurel *et al.*, 2011; Fernández-Piquer *et al.*, 2012). *Bacteroidetes* were more common in postlarvae (relative abundances were 26% for *C. gigas*, 32% for *C. corteziensis* and 37% for *C. sikamea*). However, this phylum had a low abundance (< 8%) in adults at both cultivation sites (Fig. 2a). The relative abundance of this phylum in the adults at the BT site was higher than at the BM cultivation site (Fig. 2a). *Bacteroidetes* are able to colonise various habitats, including the gastrointestinal tracts of several animals (Thomas *et al.*, 2011), including oysters such as *C. gigas*, *Chama pacific* and *Chama savignyi* (Table 2). *Bacteroidetes* are also found in the gut microbiota of mammals and are believed to play an

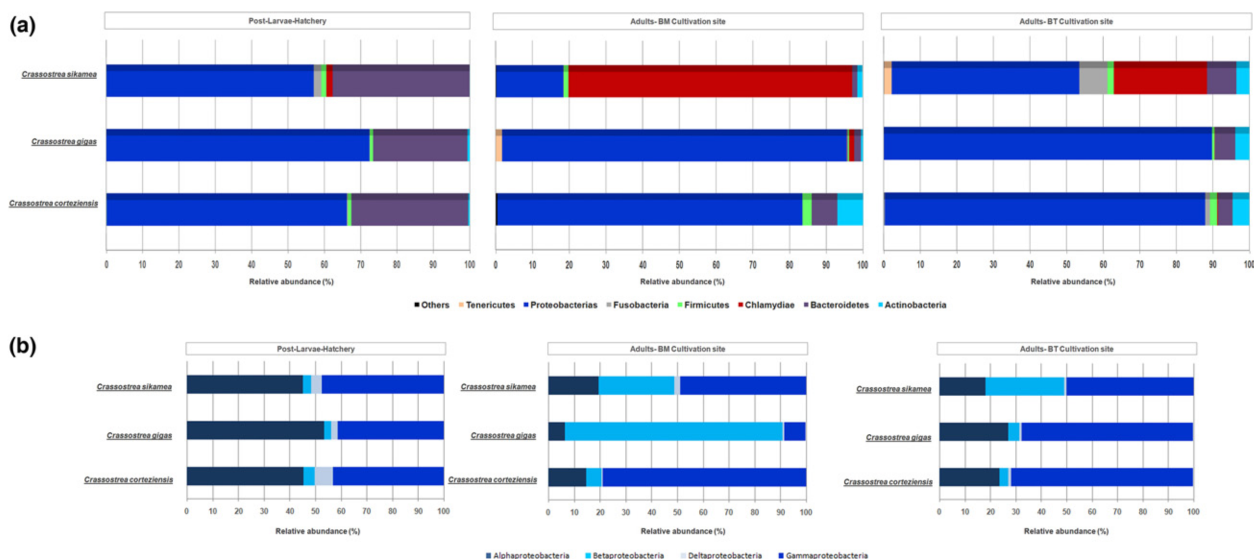

Fig. 2. Relative abundance of the bacterial phyla (a) and *Proteobacterial* classes (b) in the microbiota associated with oysters. The relative abundance was calculated based on the results of the 16S rRNA gene pyrosequencing and expressed as the percentage of 16S rRNA gene sequences that were assigned to a given phylum, not the total numbers of OTUs. Others: corresponds to the following phyla: *Acidobacteria*, *Chlorobi*, *Deinococcus-Thermus*, *Spirochaetes*, *Thermotogae* and *Verrucomicrobia*, with relative abundances ≤ 1%. BM: Grow-out cultivation site at Bahía Magdalena; BT: Grow-out cultivation site at Bahía Topolobampo.

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Table 2. Comparison of the microbiota composition (phylum level) in different oyster species obtained through the use of culture-independent techniques. The information was obtained from published data, and comparative data for each oyster are given with the appropriate author reference

Oyster species	Geographical location	*Proteobacteria*	*Bacteroidetes*	*Firmicutes*	*Fusobacteria*	*Actinobacteria*	*Spirochaetes*	*Chloroflexi*	Sample type	Technique used	References
Saccostrea glomerata	Australia	Alpha+++ Gamma+	ND	++	ND	+	+	+	Digestive gland Adult Not depurated	16S rRNA gene amplification, cloning and RFLP	Green & Barnes (2010)
Chama pacific	Israel	Gamma+++ Alpha++ Delta++	++	++	ND	+	ND	ND	Gills Adult Not depurated	ARISA and cloning 16S rRNA gene	Zurel *et al.* (2011)
Chama savignyi	Israel	Gamma+++ Beta++ Alpha, Delta+	++	++	ND	++	+	+	Gills Adult Not depurated	ARISA and cloning 16S rRNA gene	Zurel *et al.* (2011)
Crassostrea gigas	México	Gamma+++ Alpha, Beta+	+	+	ND	ND	ND	ND	Gills Adult Not depurated	16S rRNA gene amplification and FISH	Hernández-Zárate & Olmos-Soto (2006)
Crassostrea gigas	México	Gamma+++ Alpha, Beta+	ND	+	ND	ND	ND	ND	Digestive gland Adult Not depurated	16S rRNA gene amplification and FISH	Hernández-Zárate & Olmos-Soto (2006)
		Alpha+++ Beta, Gamma+ Delta+							Homogenate Adult Not depurated	T-RFLP and cloning 16S rRNA gene	Fernandez-Piquer *et al.* (2012)
Crassostrea gigas	Tasmania		++	+	++	ND	+	ND	Adult Depurated		
		Alpha+++ Gamma++ Beta, Delta+	+++	++	+	+	ND	±	Postlarvae Homogenate Depurated	Pyrosequencing 16S rRNA gene	This study
Crassostrea gigas	México	Beta+++ Gamma+++ Alpha++ Delta+	+	+	+	++	ND	±	Adult Gastrointestinal tract Depurated	Pyrosequencing 16S rRNA gene	This study
		Gamma+++ Alpha+++ Beta, Delta+	+++	++	+	+	±	±	Postlarvae Homogenate Depurated	Pyrosequencing 16S rRNA gene	This study
Crassostrea corteziensis	México	Gamma+++ Beta++ Alpha++ Delta+	+	+	+	+++	ND	±	Adult Gastrointestinal tract Depurated	Pyrosequencing 16S rRNA gene	This study
Crassostrea sikamea	México	Alpha+++ Gamma++ Beta, Delta+	+++	++	++	+	±	ND	Postlarvae Homogenate Depurated	Pyrosequencing 16S rRNA gene	This study
		Gamma+++ Alpha++ Beta, Delta+	+	+	++	++	ND	ND	Adult Gastrointestinal tract Depurated	Pyrosequencing 16S rRNA gene	This study

+++: High abundance; ++: Abundance; +: Low abundance; ±: Very low abundance; ND: No data or not detected.

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important role in the degradation of plant cell wall components such as cellulose and pectin (Thomas *et al.*, 2011). Therefore, this phylum may play a similar role in oysters.

The phylum *Firmicutes*, a Gram-positive group with a low GC content, was another common component of the microbiota of the three oyster species that were observed in this study. Although this phylum occurred in all life stages, their relative abundance was lower than those of the two above-mentioned phyla (Fig. 2a). This group is highly relevant in aquatic environments and is found in the microbiota of different oyster species (Table 2).

Bacteria belonging to the phylum *Actinobacteria* (high G + C Gram-positive bacteria) were nearly exclusively detected in adult oysters and showed different relative abundances at each cultivation site (BM and BT; Fig. 2a). This phylum has also been detected in adults of other oyster species (Table 2).

The following phyla were detected at low abundances: *Fusobacteria*, primarily detected in *C. sikamea* with relative abundances between 2% and 7.7%; and *Tenericutes*, identified in *C. gigas* and *C. sikamea* adults (Fig. 2a). *Acidobacteria*, *Chlorobi*, *Deinococcus-Thermus*, *Spirochaetes*, *Thermotogae* and *Verrucomicrobia* were detected in samples with relative abundance values < 1%. For these phyla, no relationship with the growth stage, oyster species or cultivation site could be identified. With the exception of *Acidobacteria*, which are typically associated with soil microorganisms (Kielak *et al.*, 2009), the phyla observed in this study have been reported at low abundances in other species of oysters (Table 2).

Furthermore, because of the description of crystalline style-associated bacteria (Tall & Nauman, 1981; Margulis *et al.*, 1991), we expected to find an abundance of *Spirochaetes* in the bacterial community; however, these bacteria were detected in very small quantities in the oyster samples that were analysed. Recently, Husmann *et al.* (2010) investigated the phylogeny of *Spirochaete* groups present in the crystalline styles of bivalves and found that *Spirochaetes* are not obligate symbionts for these bivalves.

Numerous studies have shown that pathogenic bacteria are not always efficiently eliminated during shellfish depuration (Rippey, 1994; Wittman & Flick, 1995; Romalde & Barja, 2010; Oliveira *et al.*, 2011), and this study was no exception. Although the organisms that were included in this study were purified and did not exhibit evidence of infection, we identified a dominance of *Chlamydia*-like organisms in *C. sikamea*. These bacteria were detected at a low abundance in the postlarvae (relative abundance of 1.7%), but their numbers were increased significantly in the gastrointestinal tracts of the adults at the two cultivation sites (relative abundance of 77.2% at BM and 25.5% at BT; Fig. 2a). This result highlights the importance of identifying the microbiota that are associated with the postlarvae because, once incorporated into the culture site for grow-out, the associated microbiota can be disturbed due to environmental changes, thus allowing transient microorganisms, in this case *Chlamydia*, to gain a temporary advantage. *Chlamydia*-like microorganisms have been reported by several authors to be parasites of a diverse group of bivalve molluscs; however, although injuries have been reported at the epithelial level has been reported little mortality due to these bacteria in cultivated adult bivalves (Renault & Cochennec, 1995; Paillard *et al.*, 2004; Romalde & Barja, 2010).

Composition of bacterial genera associated with oysters

Analysis of the obtained sequences identified 243 bacterial genera (> 97% similarity to the RDP reference), but only 77 of these genera had relative abundances ≥ 1% and are discussed in this section. Fifty-two genera were identified as belonging to *Proteobacteria*, and 30 of these genera are shown in Fig. 3. Genera with relative abundances ≤ 2% were included as other genera. The postlarvae stage had a greater bacterial diversity and a similar microbiota composition between oyster species. However, the adults had a lower diversity and showed differences in the bacterial community according to the cultivation site (Fig. 3). The pyrosequencing results showed that the predominant genus in the postlarvae was *Neptuniibacter*, followed by *Marinicella*, *Rhodovulum* and *Oceanicola* (Fig. 3), which are bacteria that are commonly found in the marine environment. The great bacterial diversity that was observed in this study has not been reported for these or other oyster species (Pujalte *et al.*, 1999; Najiah *et al.*, 2008; Green & Barnes, 2010; Zurel *et al.*, 2011; Fernández-Piquer *et al.*, 2012), and most are recently discovered. The adult and postlarvae stages showed different *Proteobacteria* community structures. At BM and BT, the dominant bacterial genera were *Burkholderia* and *Escherichia/Shigella* in the three oyster species, although *Umboniibacter* was also observed at BT (Fig. 3). It is important to note that *Vibrio* and *Pseudomonas* were not abundant components of the microbiota community that was associated with these oyster species, in contrast to previous reports (Kueh & Chan, 1985; Harris, 1993; Pujalte *et al.*, 1999; Najiah *et al.*, 2008). However, these results confirm the results obtained by us previously (Trabal *et al.*, 2012).

Sixteen *Bacteroidetes* genera were observed in the sequences identified (relative abundances ≥ 1%). The highest abundance and diversity of *Bacteroidetes* were found in the postlarvae stage, and the composition was similar between the different oyster species (Fig. 4). The most representative genera belonging to *Bacteroidetes* were

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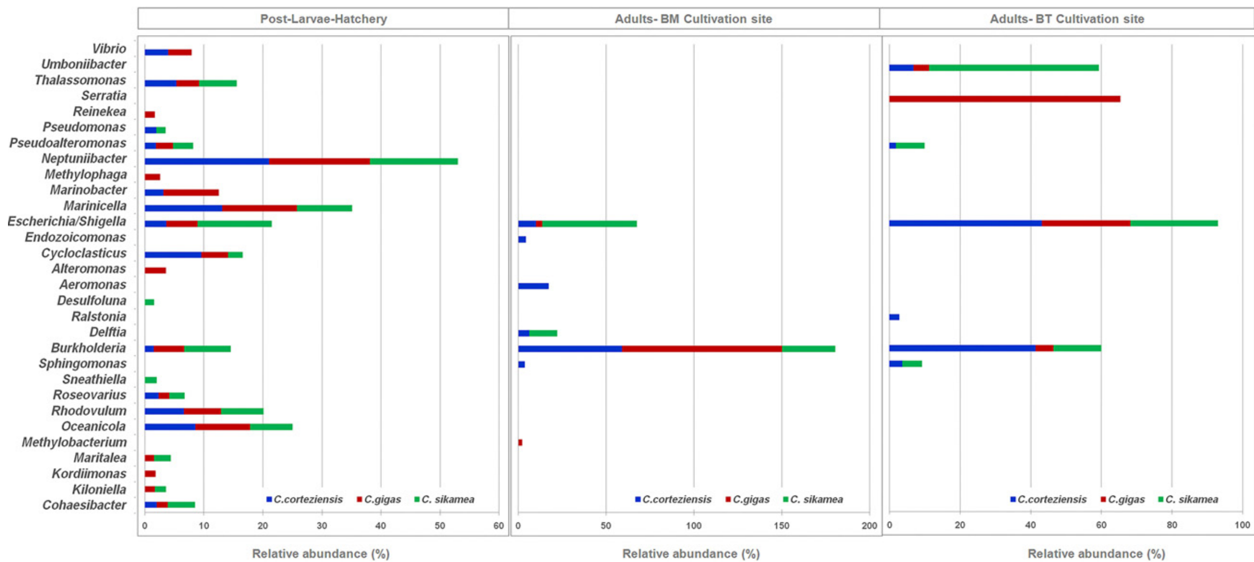

Fig. 3. Relative abundance of *Proteobacteria* genera identified as components of the microbiota associated with oysters. The relative abundance was calculated based on the pyrosequencing results of the 16S rRNA gene and expressed as the percentage of the 16S rRNA gene sequences that were assigned to a given genus, not the total numbers of OTUs. Others: indicates the bacterial genera that had relative abundances ≤ 2%. BM: Grow-out cultivation site at Bahía Magdalena; BT: Grow-out cultivation site at Bahía Topolobampo.

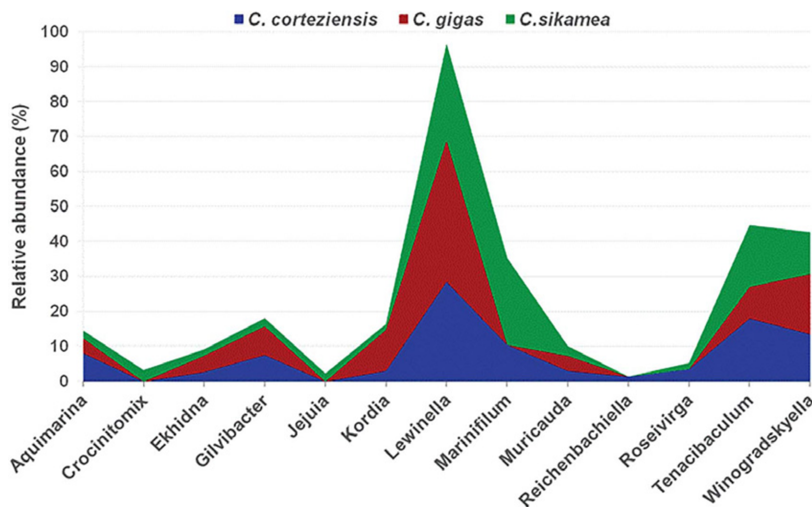

Fig. 4. Relative abundance of *Bacteroidetes* genera identified as components of the microbiota associated with postlarvae oysters. The relative abundance was calculated based on the pyrosequencing results of the 16S rRNA gene and expressed as the percentage of 16S rRNA gene sequences that were assigned to a given genus, not the total numbers of OTUs. Only values of relative abundance ≥ 1% are presented.

Lewinella, *Tenacibaculum*, *Winogradskyella* and *Gilvibacter* (Fig. 4). In contrast, only the *Tenacibaculum*, *Robiginitalea*, *Salinimicrobium*, *Sediminibacterium* and *Wautersiella* genera were detected at low abundances in the *C. gigas* and *C. sikamea* genera (data not shown). Most of the *Bacteroidetes* genera that were identified in this study belonged to the class *Flavobacteria*, and these results were comparable with results obtained for *C. gigas* adults by Fernández-Piquer *et al.* (2012). However, excluding *Winogradskyella* and *Gilvibacter*, the remaining genera of *Bacteroidetes* that were identified in this study have not been reported.

Representatives of the phylum *Actinobacteria* were detected in the postlarvae and adults; although in general, the highest abundance was observed in adults. *Propionibacterium* was the dominant genus because it was found at a high abundance in the adults of all three oyster species at both cultivation sites. *Propionibacterium* has been previously reported to be associated with *S. glomerata* (Green & Barnes, 2010), but its probable function was not determined. Presumably, *Propionibacterium* may be a contaminant that can come from the hands of people working in the rearing facilities.

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Although we identified sequences belonging to the *Firmicutes*, *Fusobacterium* and *Spirochaetes*, in this study (Fig. 2a), few genera belonging to these phyla could be identified.

We detected a large number of genera that reflected highly complex bacterial communities that were associated with oysters. Future studies designed to identify and localise any permanent symbionts and to further elucidate the specific metabolic role that the bacteria may be providing are needed.

Differences and similarities between the resident microbiota that were associated with oysters during cultivation

The variation in microbial composition between oyster species and sites is shown in Fig. 5. The observed postlarval cluster of the three oyster species was explained by two principal components (PC1 = 74.39%, $\lambda = 6.7$; PC2 = 13.71%, $\lambda = 3.6$) with an 88.1% cumulative variance. These results are in agreement with the similarities that were found in the relative abundances and compositions of the genera that were described above. These results suggest that, during the postlarval stage, the microbiota had a uniform bacterial composition that was independent of the host, possibly because these postlarvae were produced in identical conditions at the same hatchery, as explained in Trabal *et al.*, 2012;. Significant correlations were evident between the bacterial communities of *C. corteziensis* and *C. sikamea* when these oyster species were fattened at same grow-out cultivation site, which indicated that the environmental differences between the two collection sites played a key role in determining the bacterial communities (Fig. 5). Colonisation by bacteria in the oyster gastrointestinal tract has a particular dependence with the external environment because of the flow of water that passes through the digestive tract during feeding (Prieur *et al.*, 1990; Harris, 1993; Gatesoupe, 1999). We showed that the microbiota of *C. sikamea* and *C. corteziensis* were influenced by the conditions at the cultivation site, and we confirmed the results that were previously reported by Trabal *et al.* (2012) for *C. corteziensis*. The microbiota of *C. gigas* did not show the same behaviour as those of the other two species that were influenced by the cultivation site (Fig. 5), possibly because *C. gigas* show intraspecific differences in its microbiota composition. We had previously reported this phenomenon (Trabal *et al.*, 2012). Overall, in this study, we observed differences between the composition of the bacterial community that were associated with oysters at different stages of life or cultivated in different environments, but we did not detect variations in the bacteria that were present in oyster species that were grown under the same conditions. These observations suggest that the composition of the microbiota might result from the effects of multiple interacting variables, including local environmental factors and diet, although it may also be affected by the life stage of the oyster (especially by the maturity of the gastrointestinal tract) and perhaps by genetic differences of individuals (Prieur *et al.*, 1990; Harris, 1993; Gatesoupe, 1999; Paillard *et al.*, 2004; LaValley *et al.*, 2009; Karasov *et al.*, 2011; Kesarcodi *et al.*, 2012; Mouchet *et al.*, 2012). The oysters had a common core microbiota in the same cultivation site (Fig. 5), which

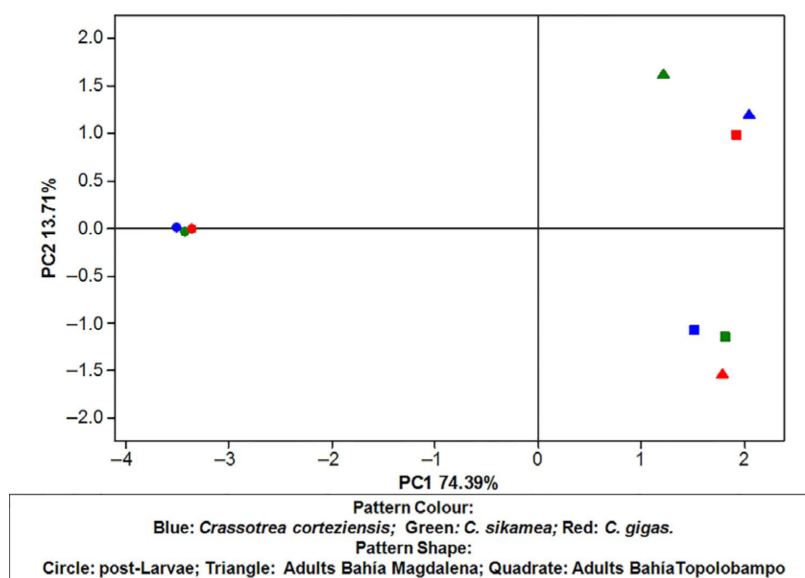

Fig. 5. Beta-diversity of the OTUs identified as components of the microbiota associated with oysters during the commercial production of oysters and determined by principal components analysis.

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could be explained because these animals had same diets, an analogous behaviour has been reported for other animals. Additional effort should be directed towards understanding the roles of environment variables (e.g. temperature, salinity, bacterioplankton and diet) that were not considered in this study but could influence the composition of the microbiota. Moreover, despite these differences, the phyla that were detected as components of the microbiota that were associated with oysters were the same as those reported by other authors (Table 2), and this may be explained by evolutionary adaptation because a subset of the microorganisms in the marine environment may have some benefit to the host (Giovannoni, 2004; Pommier *et al.*, 2007). Despite the differences in the diversity of the microbiota associated with oysters, our results show that some genera are strongly associated with the gastrointestinal tracts of *C. corteziensis*, *C. sikamea* and *C. gigas*. Presumably, certain attributes of these bacteria, such as adhesion to the gut wall, prevent expulsion from the intestine. *Burkholderia,* which were common to all samples, regardless of environmental conditions or life stage, may be symbiotically maintained by the oyster because of yet-unknown metabolic benefits that are provided by these bacteria. Members of the *Burkholderia* genus, which were previously identified as *Burkholderia cepacia* by Trabal *et al.* (2012), were acquired in the postlarval stage and remained associated with the gastrointestinal tracts of adult oysters at both cultivation sites (BM and BT). This bacterium was also detected in the Pacific oyster (Fernández-Piquer *et al.*, 2012) and was reported to be an endosymbiont of the sponge *Arenosclera brasiliensis* (Trindade-Silva *et al.*, 2012). Several studies have proved on the ability of the *Burkholderia cepacia* to produce a large number of secondary metabolites that inhibit a wide variety of pathogenic bacteria and degrade the organic acid capacity (Govan *et al.*, 1996; Mahenthiralingam *et al.*, 2008). Recently, this bacterium was shown to have an antagonistic effect on the pathogenic strains *Vibrio alginolyticus* and *Vibrio harveyi*, and this may be the primary function for the association of *Burkholderia* species with oysters (Campa-Córdova *et al.*, 2011).

The high-throughput sequencing approach revealed that *C. corteziensis*, *C. sikamea* and *C. gigas* harbour a diverse bacterial population that varies during the commercial production process. The microbial diversity suffered changes during growth, and these changes could be related to the site of grow-out, hatchery or cultivation. Digestive performance, in part, is dependent upon the distribution of the bacteria and the total population of resident microbiota. The bacterial groups that were found as part of the resident microbiota in these oyster species were complex and metabolically versatile; therefore, it is difficult to understand their roles as symbionts of these marine organisms. Many of the bacteria that were found in the bacterial populations associated with *C. gigas*, *C. corteziensis* and *C. sikamea* were described for the first time in our research, but the physiological and ecological significance of these populations remains unknown.

Acknowledgements

We would like to thank Acuícola Robles and Acuícola Cuate-Machado for providing the oysters used in this research. We also thank Hever Latisnere-Barragan of the Marine Biotechnology Laboratory (CIBNOR) and Raul Llera for technical support. Funding was provided by Consejo Nacional de Ciencia y Tecnología of Mexico (SEP-CONACYT grants 129025 and 106887). N. A. is a recipient of a CONACYT doctoral fellowship and an internship grant at the Instituto Nacional de Tecnología de los Alimentos (Universidad de Chile).

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