Microbiological quality and bacterial diversity of the tropical oyster *Crassostrea rhizophorae* in a monitored farming system and from natural stocks

M.T. Silva Neta[1], B.M. Maciel[2], A.T.S. Lopes[1], E.L.S. Marques[3], R.P. Rezende[2] and G. Boehs[2]

[1]Programa de Pós-Graduação em Ciência Animal,
Universidade Estadual de Santa Cruz, Ilhéus, BA, Brasil
[2]Departamento de Ciências Biológicas,
Universidade Estadual de Santa Cruz, Ilhéus, BA, Brasil
[3]Programa de Pós-Graduação em Genética e Biologia Molecular,
Universidade Estadual de Santa Cruz, Ilhéus, BA, Brasil

Corresponding author: B.M. Maciel
E-mail: caimbi@uol.com.br

Genet. Mol. Res. 14 (4): 15754-15768 (2015)
Received April 10, 2015
Accepted July 14, 2015
Published December 1, 2015
DOI http://dx.doi.org/10.4238/2015.December.1.27

ABSTRACT. Microbiological evaluation is one of the most important parameters for analyzing the viability of an oyster farming system, which addresses public health and ecological concerns. Here, the microbiological quality of the oyster *Crassostrea rhizophorae* cultivated in a monitored environment and from natural beds in Bahia, northeastern Brazil, was determined. Bacterial diversity in oysters was measured by polymerase chain reaction-denaturing gradient gel electrophoresis. Sequence analysis revealed that most bacterial species showed similarity with uncultured or unidentified bacteria from environmental samples, and were clustered into the phylum Proteobacteria. Diverse bacteria from cultivated (monitored) oyster samples were grouped in the same cluster with a high similarity

index (above 79%). Microbiological analyses revealed that these oysters did not contain pathogens. These results reflect the natural balance of the microbial communities essential to the maintenance of health and in inhibiting pathogen colonization in the oyster. On the other hand, bacterial diversity of samples from native stocks in extractive areas displayed a similarity index varying between 55 and 77%, and all samples were clustered separately from each other and from the cluster of samples derived from the cultivation area. Microbiological analyses showed that oysters from the extractive area were not fit for human consumption. This reflected a different composition of the microbial community in this area, probably resulting from anthropic impact. Our study also demonstrated that low temperatures and high rainfall limits the bacterial concentration in tropical oysters. This is the first study analyzing the total bacterial community profiles of the oyster *C. rhizophorae*.

Key words: Oyster cultivation; Quality control; Microbiological evaluation; Culture-independent methods; PCR-DGGE

INTRODUCTION

The Brazilian coast extends for approximately 8500 km, with an Exclusive Economic Zone corresponding to 4.3 km^2, housing a large diversity of coastal ecosystems (Castello, 2010). This large coastal extension includes several bays and estuaries containing mangroves that favor the cultivation of marine organisms. Oyster farming has gradually increased in these areas; oyster production by ostreiculture and extractive activity has reached approximately 2.5 and 1.2 t, respectively (MPA, 2011). In northeast Brazil, oyster farming has developed gradually with the use of native species *Crassostrea rhizophorae.*

According to Lenz and Boehs (2011), *C. rhizophorae* reproduces continually throughout the year in the southern State of Bahia. Santos (2011) demonstrated the zootechnical viability of oyster farming in this region; on the other hand, Brandão et al. (2013) made an inventory of the pathogens associated with this species and stated that its health was not seriously threatened, thereby recommending further cultivation and sale. Ostreiculture in small farm system has been developed in the Camamu Bay since 2006, with constant assistance of Universidade Estadual de Santa Cruz (UESC). However, ostreiculture is not monitored in the city of Ilhéus in Bahia, and the supply of oysters is derived exclusively from extractive activity. In this region, bivalves are appreciated by the local population and by tourists who commonly enjoy oyster consumption *in natura*. The consumption of this mollusk without prior cooking is a pre-disposing factor for outbreaks of foodborne disease.

Microbiological evaluation is one of the most important parameters of an oyster farming system. Oysters, which are filter feeders, are potential biomarkers of environmental pollution in marine and estuary areas. The filtration capacity of *C. rhizophorae* (approximately 5 L of water per hour) allows for the retention of 75% of environmental bacteria resistant to enzymatic degradation that survive the digestive process (Barros et al., 2005). Therefore, the microbiological quality of oysters is closely related to the sanitary conditions of water, and can be seriously affected by untreated effluents from urban or rural areas (Souza et al., 2012). The anthropic impact and water

pollution destabilize the microorganism population (La Valley et al., 2009), which is essential for the maintenance of health by inhibiting the pathogen colonization in oysters (Trabal et al., 2012). The misbalance of the microbial communities can negatively influence the aquaculture activities, leading to a possible increase in human pathogens (Forrest et al., 2009).

The study of microbial communities by culture-independent methodologies allows for the detection of microorganisms that cannot be cultured, because of the difficulty of developing media (for cultivation) that accurately resemble the specific environmental conditions (Ercolini, 2004). Su et al. (2012) have reported that the culture-independent fingerprinting technique based on polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) is successful in providing a profile representative of the bacterial community in different samples. In oysters, studies of the bacterial diversity have been conducted in *Crassostrea virginica* (La Valley et al., 2009), *Crassostrea gigas* (Trabal et al., 2012; Wang et al., 2014), and *Crassostrea corteziensis* (Trabal et al., 2012). Total bacterial community profiles of *C. rhizophorae* have not yet been studied by the culture-independent DGGE method.

Due to the growing economic and ecological importance of *C. rhizophorae* in Brazil, the objective of this study was to evaluate the microbiological quality of these oysters with regard to public health. For this purpose, oysters collected from a cultivation area and from native stocks located at the southern coast of Bahia, Brazil, were monitored.

MATERIAL AND METHODS

Study localities

The study was conducted in the coverage areas of the locality of Ilhéus (estuary of the Cachoeira River) and Camamu (Porto do Campo at Camamu Bay), both located on the southern coast of Bahia, northeastern Brazil (Figure 1). The climate can be characterized as tropical humid, with rainfall exceeding 60 mm during the driest months (annually approximately 1400 mm). Abiotic factors of these regions (temperature, rainfall, and salinity) are summarized in Figure 1. Mangroves are prominent in this region. In Ilhéus, specimens of *C. rhizophorae* were collected from the native stocks. In Camamu Bay, the oysters were obtained from a long-line farming system.

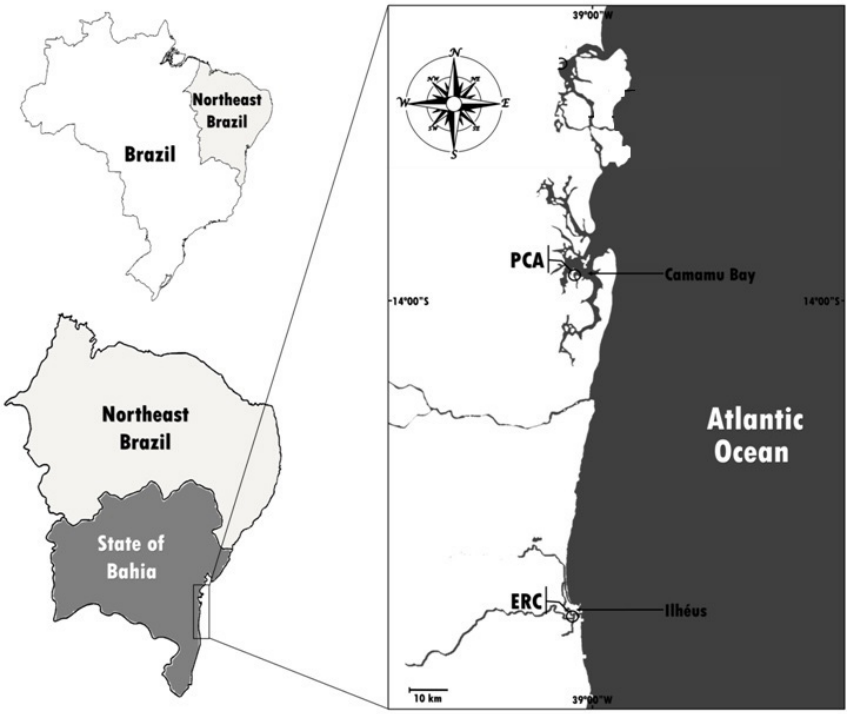
Sampling

Thirty oysters were collected at each time point (during April, July, and October, 2012) from two study areas, totaling 180 exemplars. Samples from the native stocks of Ilhéus were designated as I1, I2, and I3, and those from the farming system of Camamu Bay were marked as C1, C2, and C3, with reference to those collected during April, July, and October, respectively. The collected oysters were stored in isothermal boxes until their arrival at the Microbiology Laboratory at the Veterinary Hospital of UESC, Ilhéus, and were immediately analyzed.

Sample preparation and dilutions

The meat and the intervalvar fluid of 30 oysters were aseptically transferred to a sterile Becker, and homogenized for 5 min in a tissue mixer (Novatecnica, Brazil). The homogenate (25 g) was then mixed with 225 mL of the 0.1% peptone water (Acumedia). This mixture was homogenized

again and serially diluted (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) in duplicate. The samples were plated to determine the coliform and *Escherichia coli* counts at 35° and 45°C, to enumerate *Staphylococcus aureus*, detect *Salmonella* sp, the most probable number of *Vibrio parahaemolyticus*, and to obtain the total aerobic mesophilic plate count and total psychrotrophic plate count, in order to certify the microbiological quality of the oysters. Analyses were performed according to the Brazilian legislation described in the Normative Instruction No. 62 of August 26, 2003, of the Ministry of Agriculture, Livestock and Supply that addressed the Official Analytical Methods for Microbiological Analysis of Products of Animal Origin and of Water (Brasil, 2003).



	Ilhéus				Camamu[a]			
Abiotic factors	April	July	October	Average (± stand. dev.)	April	July	October	Average (± stand. dev.)
rainfall (mm)	58.5[b]	94.4[b]	134.7[b]	95.9 (± 38.12)	82.2	86.4	73.0	80.5 (±6.9)
environmental temperature (°C)	24.3[b] (± 0.8)	21.5[b] (± 0.6)	23.0[b] (± 0.9)	22.9 (± 1.4)	29.7 (± 1.7)	28.7 (± 3.2)	28.5 (± 1.9)	29 (± 0.6)
temperature of water (°C)				24.0[c] (± 1.2)	28.1 (± 1.9)	26.0 (± 1.4)	27.2 (± 1.1)	27.1 (± 1.0)
salinity (ppt)				28.0[c] (± 4.0)	24.9 (± 3.6)	26.2 (± 1.1)	30.3 (± 1.4)	27.1 (±2.8)

[a] Personal data obtained from Boehs's Research Project "Development and Technology Transfer in Shellfish Mariculture in Quilombolas Remnants Community of Porto Campo, Camamú, Bahia".
[b] Data from Ceplac/Cepec/Seram-Climatologia (Ilhéus, Brazil)
[c] Ourives et al., 2011

Figure 1. Map of study area on the south coast of Bahia, northeast Brazil, and abiotic parameters during the months of the study.

Enumeration of coliforms and *E. coli*

The total coliform and *E. coli* counts were determined by plating the samples on solid medium. Aliquots (1 mL) of each dilution were cultured on violet red bile agar (Himedia) and the plates were incubated at 35°C for 18-24 h. Five presumptive colonies were picked and each was transferred to a tube containing brilliant green lactose broth (Himedia), and incubated at 35°C. The tubes were examined at 24 and 48 h for gas production and to determine the coliform count at 35°C. One aliquot of each gas-positive tube was cultured in EC broth (Himedia) and incubated at 45°C. The tubes were also examined at 24 h for gas production and to determine the coliform count at 45°C. One aliquot of each gas-positive tube was cultured in eosin methylene blue agar (Himedia) and incubated at 45°C for 24 h. The suspect colonies were counted and tested by specific biochemical analysis (indole, methyl red, Voges-Proskauer, and Simon citrate test) to confirm the presence of *E. coli*.

Enumeration of *Staphylococcus aureus*

One milliliter of each dilution was divided on the surface of three Baird-Parker (BP, Acumedia) agar plates. The plates were incubated at 35°C for 48 h and five presumptive colonies were selected for catalase, coagulase, and thermostable DNase tests.

Detection of *Salmonella* spp

For detection of *Salmonella* spp, 25 g of the sample was mixed with 225 mL buffered peptone water and incubated at 37°C. After 24 h, 1 mL was transferred from each tube to 9 mL selenite-cystine (SC; Merck) broth and Rappaport-Vassiliadis (Merck) broth and incubated at 43°C for 24 h. A sample (1 mL) from each broth was plated onto xylose-lysine deoxycholate (Acumedia), Hektoen enteric (Himedia), and *Salmonella-Shigella* (Merck) agars. The plates were incubated overnight at 37°C. Typical colonies were submitted to biochemical screening on triple sugar iron agar (TSI; Himedia), lysine iron agar (Himedia), and urea agar (Merck).

The presence of *Salmonella* spp was confirmed by testing presumptive colonies using two sets of primers to amplify a conserved region for *Salmonella* genus ST11 (5'-AGCCAACCATT GCTAAATTGGCGCA-3') and ST15 (5'-TTTGCGACTATCAGGTTACCGTGG-3') (Aabo et al., 1993). The 25-µL PCR mixture contained 1X PCR buffer (Invitrogen), 1.25 mM $MgCl_2$, 200 µM of each deoxyribonucleoside triphosphate (Invitrogen), 10 pmol sense and anti-sense primers (Invitrogen), 1.25 U Taq DNA polymerase (Invitrogen), and one suspected *Salmonella* colony. The volume of the reaction mixture was made up with ultrapure water. The amplification cycle consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were visualized by loading 5 µL suspension onto 1% agarose gel, staining with SYBR® Safe (Invitrogen), and examining the same under UV light.

Most probable number of *V. parahaemolyticus*

V. parahaemolyticus content in the samples was detected and enumerated by homogenizing 50 g tritrated sample with 450 mL 0.1% peptone water (Acumedia) supplemented

with 3% NaCl. Decimal dilutions up to 10^{-3} were prepared from this homogenate, and 1 mL of each dilution was used to inoculate a series of three tubes containing Glucose Salt Teepol Broth (Himedia) supplemented with 3% NaCl. The tubes were incubated at 35°C for 24 h. Cultures showing growth were streaked on thiosulfate-citrate-bile salts-sucrose agar (Acumedia) plates and incubated at 35°C for 24 h. Presumptive colonies were submitted to Gram staining, motility testing, and biochemical screening on TSI media supplemented with 3% NaCl.

Total aerobic mesophylic, psychrotrophic, and Enterobacteriaceae plate counts

Aliquots (1 mL) of each dilution were transferred to plate count agar (PCA, Himedia) by pour plate method, and incubated at 35°C for 18-24 h to enumerate the total aerobic mesophylic content, and at 7°C for 7 days to enumerate the total microorganism content. The Enterobacteriaceae content was enumerated by inoculating 1 mL aliquots of each dilution onto Mac-Conkey agar (Merck) by the pour plate method, and incubating the plates at 35°C for 18-24 h. The colonies were then submitted to oxidase test in order to enumerate the oxidase-negative bacteria.

Bacterial diversity analysis by PCR-DGGE

The bacterial community in the oysters was analyzed by PCR-DGGE. A 100 mg aliquot of the macerated oysters was used to extract DNA, using the Easy-DNA® kit (Invitrogen). DNA samples were standardized to 100 ng, and mixed in equal quantities for use as templates in the PCR analysis. The mixed DNA samples were amplified using primers for the variable V3 region of the 16S rDNA. A region of approximately 200 bp was amplified using the primers F357 (5'-CGCCCGC CGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGTACGGGAGGCAGCAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3'). The underlined sequence in F357 indicates the GC clamp required for DGGE analysis (Muyzer et al., 1993). Each 50 µL PCR mixture contained 10 ng DNA sample, 1X PCR buffer (Invitrogen), 200 mM of each deoxyribonucleoside triphosphate (Invitrogen), 0.2 mM sense and antisense primers (Invitrogen), 3.0 mM $MgCl_2$ (Invitrogen), and 1.25 U Platinum® Taq DNA polymerase (Invitrogen). The amplification cycle consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 60°C, and extension for 1 min at 72°C, and a final extension for 30 min at 72°C. A 50-µL nested-PCR was performed using the PCR product from the first reaction under the same conditions. The nested-PCR products were purified using the PureLink® PCR Purification kit (Invitrogen), and submitted to DGGE analysis.

The DGGE analysis was performed using the DCode™ Universal Mutation System (Bio-Rad). A 20-mL sample of the PCR products of the rDNA sequences was analyzed on an 8% (w/v) polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) consisting of a 30-70% denaturing concentrate [100% denaturing agent corresponded to 7 M urea and 40% (v/v) deionized formamide]. The gels were run in 0.5X TAE buffer (20 mM Tris acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM disodium ethylene diaminetetraacetic acid; Merck, Darmstadt, Germany) at a constant voltage of 70 V (100 mA) for 16 h at 60°C. The bands were visualized by staining with silver nitrate (Merck), and the images were acquired using the Image Master™ 2D Platinum software (GE Healthcare) at a spatial resolution of 300 dpi. Each band was considered to be one ribotype, and the bands with identical migration positions were considered to be a part of the same ribotype.

Cloning procedures

The DGGE bands were excised and eluted in 30 µL ultrapure water incubated overnight at 4°C to allow for DNA re-dissolution. About 3 µL of this DNA solution was used for re-amplification as described above; this was then linked into a pCR 2.1-TOPO vector (Invitrogen) and transformed into electro-competent cells (*E. coli* TOP10; Invitrogen), as per the protocols provided by the manufacturer in the TOPO® TA Cloning® Kit (Invitrogen). The transformed cells were plated onto Luria-Bertani plates containing 50 µg/mL ampicillin, 40 µg/mL 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, and 100 µg/mL isopropyl-b-D-thiogalactopyranoside, and incubated overnight at 37°C. Blue and white colonies were screened to ensure the absence and presence of inserts, respectively, by performing colony PCR with vector-specific M13 primers (forward 5'-GTAAAACGACGGCCAG-3' and reverse 5'-CAGGAAACAGCTATGAC-3'). White colonies were selected to construct the clone libraries. Plasmid DNA was prepared using a Qiagen Plasmid Kit (Qiagen) and sent to be sequenced with M13 primers at the Ludwig Biotec Institute (Alvorada, RS, Brazil).

Phylogenetic analysis

The DGGE band-obtained DNA sequences were trimmed to remove the vector sequences and then subjected to the basic local alignment search tool (BLAST, Altschul et al., 1990) and SeqMatch in the Ribosomal Database Project II (RDP II; Cole et al., 2007), and aligned with relative 16S rDNA sequences using the ClustalW program (v.2; Larkin et al., 2007) and manually corrected. The phylogenetic analysis was conducted in the MEGA software (Tamura et al., 2011), and was inferred using the neighbor-joining method (Saitou and Nei, 1987). The distances were computed using the number of differences method (Nei and Kumar, 2000), and are in the units of the number of base differences per sequence. The stability of branches was assessed after bootstrapping with 500 replicates.

Statistical analyses

Quantitative microbiological results are reported as $\log_{10}$ microorganisms per gram. Mean and standard deviations of the quantities of bacteria were calculated and subjected to analysis of variance (ANOVA) and the Tukey test, in order to compare the variation between seasons. P values ≤ 0.05 were considered to be significant. Results of the Fisher exact test (F) are expressed in the text. Data was analyzed using the GraphPad Prism Software v.6.

Diversity results were analyzed by three multivariate analysis methods: cluster analysis, cluster analysis preceded by principal component analysis (PCA), and Venn diagrams. The cluster analysis was based on a binary matrix representing the presence/absence of bands in a sample. The similarity matrix was obtained according to the Dice similarity coefficient (S_D). Clusters were determined by sequentially comparing the patterns and constructing a relatedness dendrogram reflecting the relative similarities. The S_D calculation, and cluster and PCA analyzes were performed using the PAST software (Hammer et al., 2001), and Venn diagrams were constructed manually, taking into account the intersections of the obtained PCR-DGGE bands.

RESULTS

Bacteriological analysis

The total coliform count at 35°C was not significantly different among the oysters from both areas of study. In contrast, the coliform counts at 45°C and that of *E. coli* in samples obtained from the natural stocks of Ilhéus were approximately 4 log CFU/g in April (I1) and October (I3), while samples from Camamu showed negative values during all periods of sampling (Table 1). None of the 180 analyzed samples were contaminated with *S. aureus* or *V. parahaemolyticus.* The only positive sample for *Salmonella* was observed in the July sample obtained from the native stocks of Ilhéus (I2) (Table 1).

Table 1. Bacteriological analysis[a] (Log_{10} microorganisms/g) of *Crassostrea rhizophorae* obtained from the southern coast of Bahia, Brazil (N = 180).

Bacteriological analysis	Samples							
	Ilhéus (native stocks)				Camamu (farming oysters)			
	I1	I2	I3	Average	C1	C2	C3	Average
Coliform count at 35°C	4.2	4.0	3.9	4.0	4.4	4.0	3.2	3.8
Coliform and *Escherichia coli* counts at 45°C	4.2	-	3.9	2.7	-	-	-	
Staphylococcus aureus count	-	-	-		-	-	-	
Detection of *Salmonella* spp	-	+	-		-	-	-	
MPN of *Vibrio parahaemolyticus*	-	-	-		-	-	-	
Enterobacteriaceae plate count	5.4[b]	4.8	4.3	4.8[c]	4.1	3.4	3.1	3.5
Total aerobic mesophylic plate count	4.7[b]	4.1	4.1	4.3[c]	3.2	2.8	3.4	3.1
Psychrotrophic plate count	5.2[b]	3.9	3.9	4.3[c]	3.8	1.7	3.3	2.9

[a]Mean of experimental duplicates in five dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}). [b]Significant difference ($P < 0.0001$) by the Tukey test between places and sampling period. [c]Significant difference ($P < 0.0001$) by the Tukey test between the averages in each locality. (-) Negative result. (+) Positive result.

Compared to the samples obtained during the other time-points, and from the cultivation area of Camamu Bay, a significant increase was found in the counts of Enterobacteriacea (5.4 log CFU/g; $P < 0.0001$; $F = 2.56$), aerobic mesophilic ($10^{4.7}$ CFU/g; $P < 0.0001$; $F = 4.357$), and psychrotrophic micro-organisms (5.2 log CFU/g, $P < 0.0001$; $F = 6.606$) in samples obtained from Ilhéus in April (I1) (Table 1).

In general, oysters from the extractive area of Ilhéus showed higher levels of contamination than those from the cultivation area of Camamu, showing a significant increase in the average number of micro-organisms indicative of fecal and environmental contamination (thermotolerant coliforms and enterobacteria), and counts of total mesophilic and psychrotrophic micro-organisms (Table 1). On the other hand, samples from Camamu showed satisfactory results, exhibiting bacterial growth below of the limits established by the National Agency for Sanitary Surveillance of Brazil (Brasil, 2001).

Bacterial Diversity

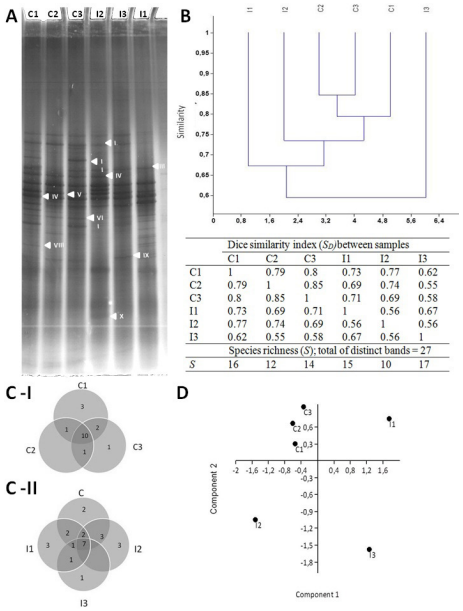
Analysis of the PCR-DGGE banding pattern of total bacteria obtained from oysters identified 27 distinct ribotypes upon combining all samples (Figures 2A and B). S_D was determined and a dendrogram of the bacterial profiles from the PCR-DGGE fingerprints was constructed (Figure 2B). The analysis of oysters from Camamu revealed high S_D values, above 79% (Figure 2B). Approximately 56% of the ribotypes at this site of study were common (10 common bands/18 total

bands); three ribotypes were distinct in C1 and one band was distinct in C3 (Figure 2C-I). Because of this high similarity, the Venn diagram grouped all samples from this area together (Figure 2C-II), to allow for a comparison with samples from Ilhéus. In the PCA score plot (PC1 *vs* PC2), samples from Camamu were also grouped together, with positive scores observed in PC2 (Figure 2D).

The bacterial diversity of samples from Ilhéus resulted in varying S_D values (between 55 and 77%) compared to other samples. The sample I3 showed the lowest mean S_D (59.6%) and was clustered as an external group (Figure 2B). The Venn diagram showed that 26% of the ribotypes were common to all samples (7 common bands/27 total bands); three different ribotypes were distinct in I1 and I2, and one band was distinct in I3 (Figure 2C-II). All samples from Ilhéus were plotted separately from each other in the PCA score plot and from the cluster of samples from Camamu (Figure 2D).

Ten ribotypes could be identified based on their sequences and most of them were related closest to microorganisms from the environmental samples (Table 2). Four ribotypes showed greater similarity to cultivable microorganisms, *E. coli* (ribotypes II and III), with similarities of 73 and 90%, respectively, and *Rhizobium* (ribotypes VI and X), with a similarity of 92%. It is important to emphasize that band III was similar to the pathogenic enteroaggregative *E. coli* O104:H4. The other six ribotypes showed greater similarity to uncultured or unidentified bacteria, and two of these (ribotypes I and V) were similar to uncultured bacteria from the live Pacific oyster *C. gigas* (Table 2).

The phylogenetic analysis revealed that most ribotypes were closer to uncultured or unidentified bacteria of the phylum Proteobacteria. Nevertheless, band V did not belong to the same clade, and was clustered into the phylum Spirochaetes in 100% of the bootstrap replications (Figure 3).



Dice similarity index (S_D) between samples

	C1	C2	C3	I1	I2	I3
C1	1	0.79	0.8	0.73	0.77	0.62
C2	0.79	1	0.85	0.69	0.74	0.55
C3	0.8	0.85	1	0.71	0.69	0.58
I1	0.73	0.69	0.71	1	0.56	0.67
I2	0.77	0.74	0.69	0.56	1	0.56
I3	0.62	0.55	0.58	0.67	0.56	1
Species richness (S); total of distinct bands = 27						
S	16	12	14	15	10	17

Figure 2. Denaturing gradient gel electrophoresis (DGGE) results. **A.** Banding patterns of samples from Camamu (Porto Campo at Camamu Bay) obtained during April (C1), July (C2), and October (C3); samples from Ilhéus (estuary of the Cachoeira River) obtained in April (I1), July (I2), and October (I3). Arrows and numbers indicate the excised bands that were subject to sequencing and phylogenetic analysis. **B.** Dendrogram representing the analysis of Dice similarity (also shown in the table) by paired groups of PCR-DGGE banding pattern between samples. **C.** Venn diagram showing the richness of bands detected by DGGE profiles between samples from Camamu (C-I) and between samples from Camamu and Ilhéus (C-II). **D.** Score plot of PCA analysis of PCR-DGGE profile.

Table 2. Nearest microorganism related to the denaturing gradient gel electrophoresis (DGGE) ribotype.

DGGE ribotype No.	Band presence						Nearest microorganism[a]	% sequence identity, accession No. of nearest relative	Origin of the nearest microorganism	References
	C1	C2	C3	I1	I2	I3				
I	X	X	X		X	X	Uncultured bacterium, clone 40H7 16S ribosomal RNA gene, partial sequence	73; JF827374.1	Live Pacific oyster (*Crassostrea gigas*)	Fernandez Piquer et al. (2012)
II	X						*Escherichia coli* C321. deltaA	73; CP006698	Genetic study	Lajoie et al. (2013)
III	X		X	X	X	X	*Escherichia coli* O104:H4 str. 2009EL-2071, complete genome.	90; CP003301	Clinical isolate from human with bloody diarrhea	Ahmed et al. (2012)
IV	X	X	X		X		Unidentified bacterium partial 16S rRNA gene, clone Mul1P3-95.	98; AJ518182	Sediments from reservoirs of different trophic state	Wobus et al. (2003)
V	X	X	X	X	X	X	Uncultured bacterium clone 40H7 16S ribosomal RNA gene, partial sequence	99; JF827374	Live Pacific oyster (*Crassostrea gigas*)	Fernandez Piquer et al. (2012)
VI	X	X	X	X	X	X	*Rhizobium* sp. JEYF16 gene for 16S rRNA, partial sequence.	92; AB069650	Isolated from yam bean (*Pachyrhizus erosus* L. Urban)	Unpublished
VII	X	X	X	X	X		Unidentified bacterium, partial 16S rRNA gene, clone Mul1-87	98; AJ518163	Sediments from reservoirs of different trophic state	Wobus et al. (2003)
VIII	X			X			Uncultured bacterium, gene for 16S rRNA, partial sequences, clone	96; AB099935	Environmental sample (deep-sea hydrothermal systems)	Unpublished
IX	X	X	X	X		X	Unidentified	ND		
X					X		*Rhizobium* sp. JEYF16 gene for 16S rRNA, partial sequence	92; AB069650	Isolated from yam bean (*Pachyrhizus erosus* L. Urban)	Unpublished

[a]Derived from the results of the molecular analysis based on the 16S ribosomal RNA gene. ND = not defined.

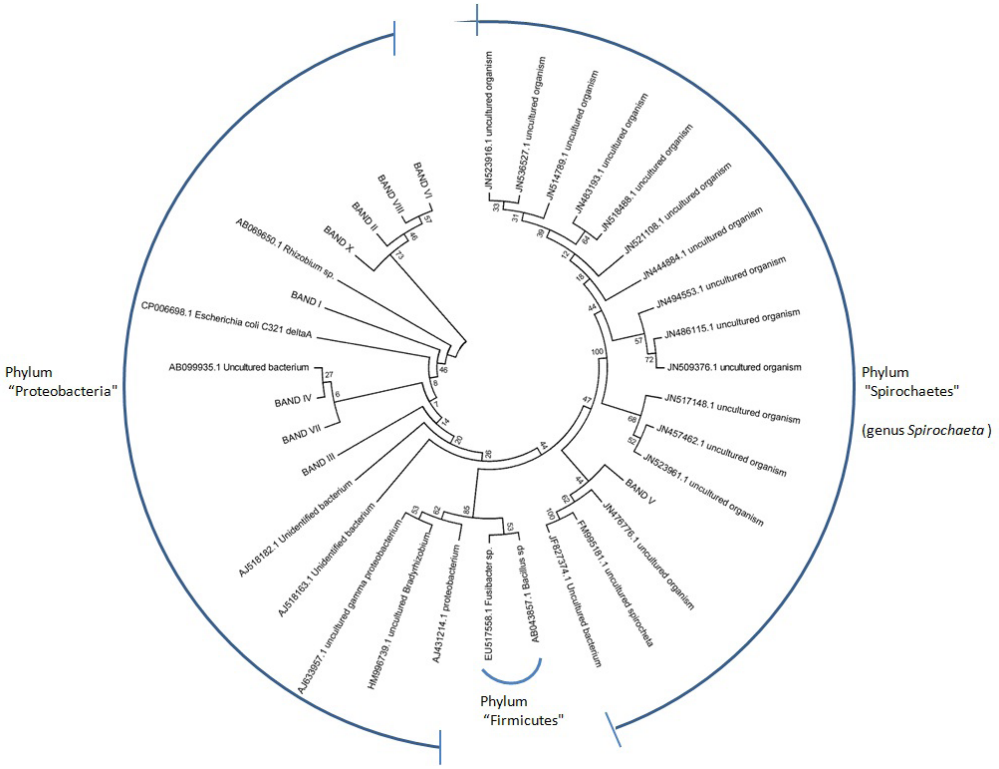


Figure 3. Phylogenetic relationship of DGGE bands inferred from 16S rDNA nucleotide sequences. Nucleotide sequences were aligned with their close relatives, and the phylogenetic tree was constructed by the neighbor-joining method after bootstrapping with 500 repetitions. The evolutionary distances were computed using the number of differences method, and are in the units of the number of base differences per sequence. The analysis involved 35 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1188 positions in the final dataset.

DISCUSSION

The microbiological results showed that the oysters from Camamu were fit for human consumption, as they showed negative results for thermotolerant coliforms, *S. aureus, Salmonella*, and *V. parahaemolyticus*, and a low number (<4 log CFU/g) of microorganisms indicative of quality, such as mesophylic and psychrotrophic counts (Table 1). Oysters are bio-indicators of environmental pollution; therefore, these results suggest that this area suffers minor anthropogenic impact, explainable by the very low density of human population (around 300 inhabitants). Therefore, oyster farming can be recommended in Porto Campo, based on the microbiological quality and food health standards.

On the other hand, oysters from the native stocks of Ilhéus were not appropriate for human consumption in almost all parameters analyzed, showing high counts (>4 log CFU/g) of total coliforms, Enterobacteriacea, and mesophylic and psychrotrophic microorganisms. In addition, 2.7 log CFU/g (average) thermotolerant coliforms and *E. coli* were found in these samples; the sample from July (I2) was found to be positive for *Salmonella* (Table 1). Samples from April (I1)

showed a significant increase in microorganism counts, compared to the other time-points. This could be attributed to the low rainfall observed during this period (58.5 mm, Figure 1), resulting in increased titers of microorganisms in the estuary and, consequently, increased accumulation of the microorganisms filtered by native oysters.

In a previous study at the Cachoeira River estuary, Sande at al. (2010) isolated and identified 68 microorganisms from bivalve mollusks (*C. rhizophorae* and *Tagelus plebeius*), including *Salmonella enterica* serovar Typhi, *E. coli*, and *Shigella* sp. However, the thermotolerant coliform count was below the safety limit established by legislation. Therefore, the authors warned of the risk of consuming raw bivalves even when the product was in accordance with the pertinent regulations. Therefore, the results of both studies (Sande et al., 2010 and our study) show that this part of the Cachoeira River estuary is seriously compromised with respect to bivalve harvesting, probably because of the dumping of untreated effluents, leading to the conclusion that the consumption of raw oysters from this region poses a serious risk to public health.

In a similar study conducted in the State of Bahia, Brazil, Pereira et al. (2007) showed that oysters from Maragogipe Bay, at the south-central region of the State, served in restaurants presented 22 to 13,000 MPN/g of total coliforms and 1.8 to 4600 MPN/g of thermotolerant coliforms. The authors also detected *Salmonella* spp in 8.4% of the samples analyzed. In oysters obtained from the Graciosa River estuary cultivation area, located at the south coast of Bahia, Silveira (2012) observed <1.8 to 54,000 MPN/g of thermotolerant coliforms in approximately 25% of the samples, with 12% of the water analyzed containing *Salmonella* spp. These studies showed that a part of the samples were not fit for consumption, and highlighted the need for a purification process before commercialization to diminish the microbial contamination.

The absence of microbiological quality in marine bivalves intended for consumption may pose a serious risk for human health. Microbial contamination can stem from the environmental itself, or may result from improper handling during transport and commercialization of the product. Therefore, the cultivation of bivalves should be accompanied by previous temporal studies conducted during different periods, wherein a thorough microbiological analysis must be systematically applied with an objective of protecting the human health and monitoring environmental contamination. Our findings showed that the monitoring of oyster farming in the Camamu Bay have been helpful in improving sustainable development in this region, as the consumption of uncontaminated oysters was encouraged.

Samples from the native stocks of Ilhéus and the farmed samples from Camamu displayed an average bacterial diversity of 14 species (*S*) (Figure 2). Although the average water temperature during the different sampling periods was almost constant, differences in the species richness were observed. The lowest abundance was observed in July (winter), when water and air temperature were lower, with values of 12 *S* in I2 and 10 *S* in C2 (Figure 2). This is in accordance with the results of previous studies (DePaola et al., 2003; Parveen et al., 2008), which reported higher bacterial quantities in environments with a higher water temperature.

However, temperature is not the unique parameter that can be evaluated to explain the species richness. A higher species richness was observed during months with lower rainfall, such as April in Ilhéus (58.5 mm) and October in Camamu (73 mm), with values of 16 *S* in I1 and 17 *S* in C3 (Figure 2). Therefore, we inferred that dry periods may allow for an increase in concentration and diversity of bacteria in aquatic environments. In a study of seasonal bacterial dynamics in oysters, Wang et al. (2014) concluded the rainy season in China to be the main reason for a limited concentration and diversity of bacteria in aquatic farming, since the highest bacterial diversity was

observed in fall (low rainfall), and not in the summer (high rainfall). In our study, both low temperature and high rainfall were found to be reasons limiting the bacterial concentration in oysters. Based on this, the best period of bivalve harvest in a tropical environment was concluded to be the winter season (low temperature and high rainfall); however, consumer demand is highest during the summer (high temperature and low rainfall). This observation highlights the greater responsibility of the producers in planning their cultivation to offer a greater quantity of the product in a season that favors microbial proliferation. Therefore, a rigorous quality control program that emphasizes microbiological analysis becomes imperative for successful production and commercialization.

Multivariate analyses showed that the bacterial diversity of samples from the farming area of Camamu had a high similarity index (>79%, Figure 2B); therefore, these were clustered together (Figure 2D). In contrast, samples from native stocks of Ilhéus showed different bacterial profiles during the study period. As bacterial diversity in oysters is associated with the local aquatic environment (La Valley et al., 2009; King et al., 2012) the high similarity index in samples from Camamu reflects the natural stability of the area. This was also inferred by the abiotic factors, which showed a low standard deviation between the average of different factors, such as rainfall, environmental temperature, water temperature, and salinity (Figure 1). These data favor the choice of this locality to be used for oyster cultivation.

The image of the oyster bacterial community provided by the culture-independent method (based on DGGE) allows the differentiation of bacterial species based on the differences in base pair composition (Ercolini, 2004), and provides a view of the population structure (mainly, the relative abundance of the main bacterial community) (La Valley et al., 2009). It is not valid in culture-based methods, because the use of enrichment and selective media favors the growth of specific microorganism groups and does not mimic the natural environmental conditions; therefore, many populations cannot be cultured. In this study, 10 different 16S rDNA sequences were recovered, cloned, and sequenced, and the majority was clustered into the phylum Proteobacteria, presenting homology with uncultured or unidentified bacteria from environmental samples (Figure 3; Table 2). The band V displayed a high similarity with an uncultured bacterium from the live Pacific oyster *Crassostrea gigas* (JF827374; Fernandez Piquer et al., 2012) and was clustered into the phylum Spirochaetes (Table 2; Figure 3). On the other hand, four sequences from the DGGE band showed greater similarity to cultured bacteria, such as *Rhizobium* (ribotypes VI and X, with 92% identity) and the pathogenic enteroaggregative *E. coli* O104:H4 (band III, with 90% identity). The genera *Rhizobium* is a nitrogen-fixing bacterium that can colonize in both terrestrial and marine environments of mangroves, and has been isolated from the sediment, rhizosphere, and root surfaces of various mangrove species (Sahoo and Dhal, 2009). The enteroaggregative *E. coli* O104:H4 strain was associated with a high prevalence of hemolytic uremic syndrome cases during a large outbreak of bloody diarrhea in Europe in May, 2011 (Ahmed et al., 2012). The identity percentages are, however, not sufficient to confirm the presence of these microorganisms; nevertheless, it can be used as an alert, because there is a possibility that these microorganisms can be filtered by marine bivalves. Therefore, the use of filtrating organisms, such as oysters, may increase the sensitivity of molecular methods that allow for the study of microbial diversity in aquatic environments, since it has been estimated that 90 to 99.9% of the microbes on the planet are not cultivable (Amann et al., 1995).

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors thank CAPES for funding the study and for the master's scholarship awarded to the first author. Research supported by FAPESB (grant #TSC0010/2011 and #RED0003/2012). We would also like to thank Dr. George Rêgo Albuquerque (UESC) and Dr. Martin Brendel (UESC) for their helpful comments on the manuscript, Tharcilla Braz Alves Pessoa (UESC) for assistance in the cloning procedure, and Maxwell Ribeiro (UESC) for the map design.

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