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Metagenomic analysis of the bacterial microbiota associated with cultured oysters (*Crassostrea* sp.) in estuarine environments

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Abstract: In this work, we identified the bacterial microbiota associated with farmed oystersin estuarine regions of four states in the north eastern region of Brazil. During the drought and rainy seasons, for eight months, twenty oysters were sampled seasonally from seven different marine farms. In the laboratory, DNA extraction, amplification, and sequencing of the 16S rRNA gene were performed to establish the taxonomic units. We identified 106 genera of bacteria belonging to 103 families, 70 orders, 39 classes, and 21 phyla. Out of the total, 40 of the genera represented bacteria potentially pathogenic to humans; of these, nine are known to cause foodborne diseases and six are potentially pathogenic to oysters. The most prevalent genera were *Mycoplasma*, *Propionigenium*, *Psychrilyobacter*, and *Arcobacter***.** The results indicate the need for more systematic monitoring of bacteria of the genus *Mycoplasma* in oyster farming operations in the Brazilian north eastern region. Currently, *Mycoplasma is* not one of the microorganisms analysed and monitored by order of Brazilian legislation during the oyster production and/or commercialization process, even though this genus was the most prevalent at all sampling points and presents pathogenic potential both for oysters and for consumers.

Key words: contamination, cultivation, oyster farming, pathogenicity.

INTRODUCTION

The close relationship between vital functions of bivalve molluscs and the environment in which they live makes these animals recognized as bioindicators for monitoring environmental quality, and their health can affect the food safety of consumers (Kim & Powell 2006).

Oysters, in addition to naturally inhabiting estuarine environments, are widely cultivated in these places where food is plentiful for its development (Dame 2012). Oyster feeding takes place through filtration and capture of suspended particles present in the water. The phytoplankton and microzooplankton organisms, dissolved organic and inorganic material, and microorganisms such as bacteria are retained in the gills (Kach & Ward 2008). Such a feeding mechanism causes the entire microbiota present in the oysters to be directly associated with the inhabited aquatic environment, and this microbiota varies according to environmental factors, such as salinity, bacterial load in water, temperature, feed, and anthropic activities and management during production (Prieur et al. 1990).

Oysters naturally harbour a diverse bacterial microbiota, often composed of pathogenic bacteria, mainly by the species belonging to

the genera *Vibrio*, *Pseudomonas*, *Alcaligenes*, *Aeromonas*, *Flavobacterium*, *Bacillus,* and *Micrococcus* (Paillard et al. 2004). However, these bacterial species may also include some pathogens that are naturally present in cultured water, such as *Vibrio parahaemolyticus* and *V. vulnificus*, while other species are generally associated with the presence of faecal contamination in waters such as *V. cholerae*, *Salmonella* sp., *Escherichia coli*, *Shigella* sp., *Campylobacterium fasci* and *Yersinia enterocolitica* (USA 2020).

In addition to representing risks to public health when oysters are consumed, these bacteria can also cause the death of farmed oysters, drastically affecting commercial scale enterprises (Fernandez et al. 2014). The main bacterial genera that is present in some species of oysters (mainly *Crassostrea*) and known to cause mortality are *Vibrio* (Le Roux et al. 2002); *Nocardia* (Friedman et al. 1991); *Mycoplasma* (Azevedo 1993); *Rickettsia* (Azevedo & Villalba 1991) and *Chlamydia* (Renault & Cochennec 1995).

One of the main problems in identifying bacteria is related to the need for bacterial culture in laboratory conditions. It is estimated that less than 0.1% of all known bacteria are cultured by traditional methods (Nocker et al. 2004). In this context, methods based on the sequencing of bacterial genes have favored studies of bacterial communities in organisms and in marine environments, since it allows the complete mapping of the bacteria present in a certain sample from direct sample analysis (Postollec et al. 2011), without the need to culture bacteria.

One of the most advanced analytical methods for this purpose is metagenomics, which can be used in the further characterization of complex bacterial communities. The method allows the identification of bacteria in samples obtained directly from the environment, thus eliminating the need for isolation and cultivation; the results are fast, selective and high sensitive since the methods are able to detect specific gene fragments (Petrosino et al. 2009).

This work aimed to identify and characterize the bacterial microbiota of oysters grown in estuarine regions of four states of north eastern Brazil using next-generation sequencing as an analytical tool.

MATERIALS AND METHODS

Study area

The study was carried out at seven oyster farms of four states of the Brazilian north eastern region. These properties were considered the most representative oyster farms in each state by the Brazilian Service of Support to Micro and Small Companies (SEBRAE), as they presented know-how, level of technology and volume of production in accordance with the average values from each state. All the sampling sites were identified by the city and state that they are located in (Figure 1): city of Macau (Macau-RN) and city Tibau do Sul (Tibau do Sul-RN), in Rio Grande do Norte state; city of Marcação (Marcação-PB), in Paraíba state; city of Passo de Camaragibe (Passo de Camaragibe-AL) and city of Barra de São Miguel (Barra de São Miguel-AL), in Alagoas state; and city of Brejo Grande (Brejo Grande-SE) and city of Indiaroba (Indiaroba-SE), in Sergipe state. Among the selected sampling sites, the adopted oyster farming model was based on three types of fixed suspended systems: 1) racks - plastic "pillows" positioned on a PVC "table" (Macau-RN, Tibau do Sul-RN, Marcação-PB, Passo de Camaragibe-AL, Barra de São Miguel-AL); 2) floating baskets - plastic baskets attached to a guide wire that was fixed to a set of stakes buried in the bottom (Brejo Grande-SE

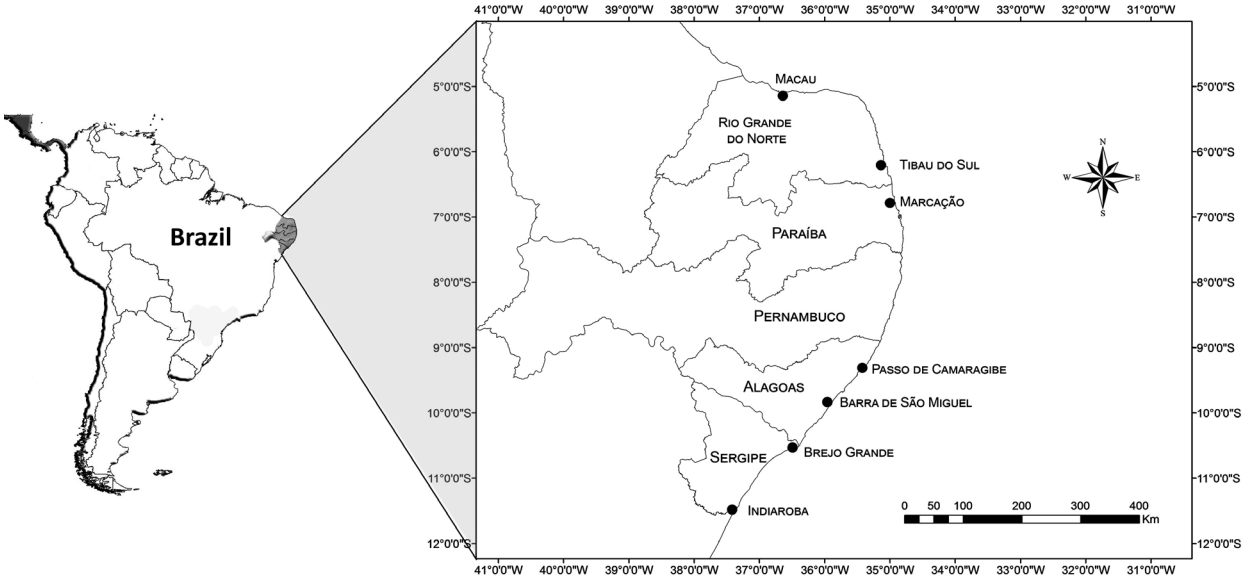

Figure 1. Location of the sampling sites of the farmed oyster used in the analysis of the bacterial microbiota.

and Indiaroba-SE); and 3) BST®- adjustable long-line oyster system (Indiaroba-SE).

Abiotic variables

The water temperature (°C), dissolved oxygen concentration (mg/L) (YSI® Pro 20, USA), salinity (g/L) (Instrutemp®, Brazil) and pH (Sensoglass® pH meter SP1400, Brazil) were measured along with the oyster sampling procedures. Precipitation data were obtained from the National Institute of Meteorology database (INMET 2016).

Sampling

Samples of *Crassostrea gasar* (Tibau do Sul-RN, Marcação-PB, Passo de Camaragibe-AL, Barra de São Miguel-AL, Brejo Grande-SE, and Indiaroba-SE) and *Crassostre arhizophorae* (Macau-RN) were collected during drought (Sep. and Oct. of 2015) and rainy (Mar. and Jun. of 2015) seasonal periods. Twenty oysters [height = 7.5 cm ± 1.8 (mean ± SD)] were collected at each sampling location during each sampling period. The sample size (n) was established through statistical calculation using the following formula:

$$n = \frac{N.Z^2.p.(1-p)}{Z^2.p.(1-p)+e^2.(N-1)}$$

Where N is the population of oysters, Z is the normalized standard variable associated with the level of confidence, *p* is the probability of the event, and *e* is the sampling error.

The collections were always carried out during low tides (ebb). At some sampling locations (Passo de Camaragibe-AL, Barra de São Miguel-AL, Brejo Grande-SE, and Indiaroba-SE), the oysters were occasionally submitted to air exposure. In others (Macau-RN, Tibau do Sul-RN, and Marcação-PB), they were always submerged during sampling.

The samples were sent by air to the Laboratory of Histology and Microbiology (LHM), belonging to the Integrated Group of Aquaculture and Environmental Studies, located at the Universidade Federal do Paraná (UFPR), in Curitiba, Paraná, Brazil. The transport period did not exceed 48 hours, as recommended by the

Codex Alimentarius Manual (Codex Alimentarius 1978) and the National Plan for Hygienic-Sanitary Control of Bivalve Mollusks (PNCMB) (Brasil 2012). For transportation, the oysters were placed in plastic bags and kept inside Styrofoam boxes (20 L) containing synthetic ice to ensure temperature maintenance (5.0 ± 1.5 °C). The temperature was monitored during the total period of transportation using portable temperature meters (Datalogger TagTemp Stick – Novus®, Brazil) that were set to measure temperature every five minutes. A cardboard foil was placed between the oysters and the synthetic ice to avoid direct contact between the samples and the ice.

Sample processing

Prior to DNA extraction, the oysters were externally cleaned with a sterile brush in running tap water. The valves were opened for the removal of the tissues and the intervalvar liquor. After homogenization of the sample in a stomacher (Marconi MA440, Brazil), 200 µL the homogenate was taken and stored in Eppendorf tubes for later extraction of total DNA using the Invitrogen kit (PureLink® Genomic DNA). The quality and quantity of the total DNA were evaluated by spectrophotometry at 260 nm and 280 nm of absorbance with a NanoDrop® 2000 spectrophotometer (ThermoScientific, USA).

The amplification and expression of the 16S rRNA gene were performed based on the methodology proposed by Caporaso et al. (2011), with adaptations described below. Briefly, the 16S rRNA gene amplification was performed by PCR analysis of the samples in a 10 µL system containing 5 µL of DNA (10 ng/L), 1 µL of universal primer mix (515F/806r), 1 µL of the first "adapter" and 5 µL of the Klentaq DV Readymix enzyme. Gene expression levels of the 16S gene were analysed in a thermocycler (Veriti 96 well Applied Biosystems, USA). The reactions were carried out at 94 °C for 3 minutes for initial denaturation of the DNA, followed by the amplification process (25 cycles at 94 °C for 45 seconds for final denaturation; 50 °C for 30 seconds for annealing; 68 °C for 1 minute for the initial extension and 10 minutes for 72 °C for the final extension) and kept in temperature-controlled conditions (0 - 4 °C).

For confirmation of the 16S gene amplification, the samples were tested on agarose gel (1%) in Tris / Borate / 1X EDTA buffer (0.09 M Tris-HCl, 0.09 M boric acid and 0.002 M EDTA). In the gel, 3 µL of the mixture with 2 µL of sample and 2 µL of FSUDS dye (bromophenol blue) were added to each well. After loading the gel, the samples were subjected to electrophoresis (one hour) at a constant electric voltage of 70 volts under 1X TBE buffer. Together with the samples, 3 µL of 1 Kb molecular weight marker was applied to the gel. The gel was stained with ethidium bromide (1%) (15 minutes), washed in water (10 minutes) and visualized in a UVP 3UV Transilluminator Imaging System.

The sequencing of the samples was performed on the next-generation Illumina MiSeq platform, which is capable of generating information on thousands of base pairs in a single run. The reagent kit used for sample sequencing was the MiSeq v2 500 cycle kit.

Data analysis

Sequence-generated 16S rRNA gene sequences were analysed according to the Quantitative Insights Into Microbial Ecology (QIIME®) protocol developed by Caporaso et al. (2010). Shannon-Winner diversity and dominance analyses were performed using the Past software, version 2.17, for the measurement of the diversity of species at each sampling site through the use of categorical data and for the estimation of

the predominance of bacterial species in each sample, respectively

The results obtained in the genetic analyses were also tabulated, ordered and classified according to their prevalence. It was established as a criterion of analysis that the prevalent genera would be those found in amounts equal to or greater than 5% in each sample analysed. The potential pathogenic role of the identified bacterial communities to humans and oysters was performed through scientific bibliographic review. A systematic search for articles was performed in scientific databases (i.e., Science Direct, Wiley, Springer Link, Google Scholar) using the IP of the UFPR.

After selection of the prevalent groups, the influence of the abiotic variables on the percentage of prevalent bacteria found in the samples was evaluated through multiple regression. Subsequently, Kruskal-Wallis test was performed to evaluate the occurrence of significant differences between the prevalent bacteria in each of the four sampling sites, between sampling sites and between seasons. All analyses were performed using Statistica 10.0 software (StatSoft®).

RESULTS

Bacterial microbiota

In the set of sample points evaluated, 106 genera of bacteria belonging to 103 families, 70 orders, 39 classes and 21 phyla were identified. The phyla with the highest prevalence were Tenericutes (21.7%), Spirochaetes (6.6%), Cyanobacteria (2.6%), and Fusobacteria (2.1%) (Table I). The prevalence of the other 17 phyla cumulatively reached 2.4% of the prevalence. Of all the genera found, 40 were of bacteria potentially pathogenic to humans, and only nine belonged to the group known to cause foodborne diseases (FD). Six genera [*Mycoplasma* (38.8%), *Photobacterium*

Table I The average percentage of phyla and genera of bacteria found in oysters cultivated in north eastern Brazil.

Phyla/Genera	% of bacteria
Tenericutes	21.7
Mycoplasma[a/b]	38.8
Acholeplasma[b]	0.1
Undefined	0.7
Spirochaetes	**6.6**
Borrelia[b]	0.5
Spirochaeta[b]	0.1
Treponema[b]	0.1
Undefined	9.5
Unclassified	0.1
Cyanobacteria	**2.6**
Synechococcus	0.7
Undefined	2.8
Fusobacteria	**2.1**
Propionigenium	4.5
Psychrilyobacter	3.8
Fusobacterium[b]	0.6
Cetobacterium[b]	0.4
Undefined	0.3
Bacteroidetes	**0.5**
Bacteroides[b]	0.6
Cloacibacterium[b]	0.4
Tenacibaculum	0.4
Fluviicola	0.2
Saprospira	0.2
Paludibacter	0.2
Lewinella	0.2
Roseivirga	0.2
Flammeovirga	0.1
Flavobacterium[c]	0.1
Crocinitomix	0.1
Aquimarina	0.1
Polaribacter	0.1
Maribacter	0.1
Winogradskyella	0.1
Chryseobacterium[b]	0.1
Owenweeksia	0.1
Rubricoccus	0.1
Fulvivirga	0.1

Table I. Continuation

Phyla/Genera	% of bacteria
Sediminibacterium	0.1
Kordia	0.1
Wautersiella[b]	0.1
Robiginitalea	0.1
Gramella	0.1
Unclassified	0.1
Proteobacteria	**0.4**
Arcobacter[c]	1.0
Shewanella[b]	0.8
Devosia	0.7
Francisella[b]	0.4
Psychrobacter[b]	0.3
Pseudoalteromonas[b]	0.3
Sulfurimonas	0.2
Thiomicrospira	0.2
Thiothrix	0.2
Phyllobacterium	0.2
Kaistobacter	0.2
Sphingomonas[c]	0.2
Photobacterium[a/b]	0.2
Vibrio[a/c]	0.2
Novispirillum	0.2
Marinomonas	0.2
Desulfococcus	0.1
Marinicella	0.1
Pseudomonas[a/c]	0.1
Phaeobacter	0.1
Oleibacter	0.1
Sulfurospirillum	0.1
Desulfovibrio[b]	0.1
Crenothrix	0.1
Novosphingobium[b]	0.1
Thalassospira	0.1
Hahella	0.1
Rhodospirillum	0.1
Bacteriovorax	0.1
Acinetobacter[b]	0.1
Desulfosarcina[b]	0.1
Candidatus Endobugua	0.1
Candidatus Portiera	0.1

Table I. Continuation

Phyla/Genera	% of bacteria
Janthinobacterium[b]	0.1
Oceanospirillum[a/b]	0.1
Reinekea	0.1
Octadecabacter	0.1
Alteromonas[a/c]	0.1
Erythrobacter	0.1
Sphingopyxis	0.1
Ferrimonas	0.1
Pedomicrobium	0.1
Thalassomonas	0.1
Glaciecola	0.1
Aminobacter	0.1
Desulfarculus	0.1
Agrobacterium[b]	0.1
Wolbachia	0.1
Methylomonas	0.1
Neptunomonas	0.1
Undefined	0.5
Fibrobacteres	**0.3**
Undefined	0.3
Chloroflexi	**0.3**
Undefined	0.3
Firmicutes	**0.2**
Leuconostoc[b]	0.7
Fusibacter	0.4
Staphylococcus[c]	0.2
Bacillus[c]	0.2
Acidaminococcus[b]	0.2
Streptococcus[b]	0.2
Guggenheimella	0.1
Clostridium[c]	0.1
Finegoldia[b]	0.1
Anaerococcus[b]	0.1
Alkaliphilus	0.1
Lactobacillus[b]	0.1
Undefined	0.1
Unclassified	0.3
Planctomycetes	**0.2**
Planctomyces[b]	0.2
Undefined	0.2

Table I. Continuation

Phyla/Genera	% of bacteria
Verrucomicrobia	**0.2**
Candidatus Xiphinematobacter	0.4
Coraliomargarita	0.2
Verrucomicrobium	0.2
Persicirhabdus	0.1
Rubritalea	0.1
Luteolibacter	0.1
Undefined	0.1
Crenarchaeota	**0.2**
Nitrosopumilus	0.2
Chlorobi	**0.1**
Undefined	0.1
Euryarchaeota	**0.1**
Undefined	0.1
Chlamydia	**0.1**
Candidatus Rhabdochlamydia [b]	0.1
Undefined	0.6
Gemmatimonadetes	**0.1**
Undefined	0.1
Lentisphaerae	**0.1**
Undefined	0.1
Actinobacteria	**0.1**
Undefined	0.1
Corynebacterium[b]	0.1
Undefined	0.1
Elusimicrobia	**0.1**
Undefined	0.1
Acidobacteria	**0.1**
Undefined	0.1
Caldithrix	**0.1**
Unclassified	0.1
Unclassified	**0.1**
Undefined	0.1
Unclassified	0.1
Undefined	**4.0**
Undefined	4.0

[a]Bacteria potentially pathogenic to oysters; [b]Bacteria potentially pathogenic to humans; [c]Bacteria that can be responsible for foodborne diseases in humans.

(0.2%), *Vibrio* (0.2%), *Pseudomonas* (0.1%), *Oceanospirillum* (0.1%), and *Alteromonas* (0.1%)] were identified as potentially pathogenic to oysters (Table I).

A large percentage of the 16S gene sequences (BD2-6, WH1-8, SC3-56, for example) were also observed for bacteria not yet specifically identified (named here as "unclassified"). Bacteria that were not adequately identified by the method were considered herein as "undefined" (Table I). After statistically comparing the data presented in Table I that presented a prevalence above 5%, no differences were detected among sampling sites ($p = 0.86$) or between drought and rainy seasons ($p = 0.24$).

When analysing the diversity index of Shannon-Winner for each sampling location during all sampling periods, it was observed that Tibau do Sul-RN presented higher bacterial diversity, followed by Marcação-PB and Macau-RN. On the other hand, the lowest diversity indexes were recorded in Indiaroba-SE, Camaragibe-AL, and Barra de São Miguel-AL. Indiaroba-SE presented greater genotype dominance due to the high percentage of *Mycoplasma* identified during the four sampling collections. For example, the prevalence of this genus was 71% in sample 2, while the other genera did not exceed 3.7% (Table II).

Of all the genera identified, those with the highest prevalence (equal to or greater than 5%) in the analysed samples were *Mycoplasma*, *Propionigenium*, *Psychrilyobacter* and *Arcobacter*. The last three bacterial genera occur naturally in marine environments, while *Mycoplasma* is part of the microbiota normally found in oysters (Table III).

Abiotic variables

There was great variation between the results of measurements of abiotic variables in the water

in all the collections, as would be expected in an estuarine environment. The oxygen concentration was below the limit considered tolerable by oysters (> 3 mg/L) (Mello 2007), during the first collection in Tibau do Sul-RN (1.61 mg/L) and the second in Brejo Grande-SE (1.08 mg/L). Some of the sampling time points at Brejo Grande-SE (collection 1, 2 and 4) and Indiaroba-SE (collection 1) presented temperatures above & range considered optimal (23-31 °C) (Ansa & Bashir 2007). Some of the sample points had lower salinity than those considered optimal for oysters (10-50 g/L) (Funo et al. 2015). For these cases, during the first sampling collection,

Table II. Dominance and Shannon-Winner diversity indexes obtained for bacteria present in oysters cultivated in north eastern Brazil.

Sampling site	Collection procedure	Number of genera	Dominance	Shannon Index
Macau-RN	1	18	0.44	1.27
	2	36	0.57	1.30
	3	16	0.83	0.51
	4	21	0.37	1.74
Tibau do Sul-RN	1	15	0.53	1.07
	2	22	0.30	1.51
	3	44	0.10	2.98
	4	25	0.71	0.78
Marcação-PB	2	35	0.33	1.93
	3	31	0.58	1.25
	4	23	0.57	1.09
Passo de Camaragibe-AL	1	9	0.85	0.38
	2	12	0.87	0.40
	3	22	0.52	1.40
	4	18	0.77	0.70
Barra de São Miguel-AL	1	18	0.58	1.05
	2	33	0.74	0.84
	3	21	0.85	0.49
	4	16	0.82	0.51
Brejo Grande-SE	1	19	0.39	1.34
	2	27	0.33	1.52
	3	18	0.32	1.39
	4	10	0.84	0.39
Indiaroba-SE	1	13	0.80	0.55
	2	11	0.88	0.29
	3	9	0.92	0.23
	4	17	0.79	0.56

Tibau do Sul-RN presented the lowest value (1 g/L), followed by Marcação-PB (5 g/L), Passo de Camaragibe-AL (7 g/L) and Indiaroba-SE (7 g/L). The pH, in turn, was within the tolerance limits of oysters (6.7-8.7) (Morales 1986) during all sampling collections (Table IV).

There were no multiple correlations (r^2 = 0.02, p = 0.23) between the prevalent bacteria (> 5%) in the samples and the abiotic variables measured. However, when the genera were analysed separately for abiotic variables, a negative correlation of pH with *Mycoplasma* was observed (r^2 = 0.25, p = 0.001).

DISCUSSION

With 96% sensitivity, the use of the molecular analysis tools adopted in the present work allowed the mapping of the bacterial microbiota of farmed oysters in the Brazilian north eastern region. This type of analysis has been carried out worldwide for several purposes, among which include the studies by King et al. (2012) to evaluate the bacteria found in the stomachs of farmed oysters (*C. virginica*) in Louisiana, USA; the studies by Chauhan et al. (2014), to describe the farmed oyster microbiota (*C. virginica*) in the Apalachicola Bay, USA; and the studies by Trabal et al. (2012) to differentiate the bacterial microbiota present in juvenile and adult oysters of *C. gigas* and *C. corteziensis* farmed on the coast of Mexico. In Brazil, the only work that used the same methodological analysis to assess the bacterial microbiota in oysters held in different storage conditions was recently published by Ostrensky et al. (2018).

Table III. Prevalent bacteria (≥5%) in the samples of oyster cultivated in north eastern Brazil.

Sampling site	Genera	Occurrence matrix	Prevalence(%)
Macau-RN	*Mycoplasma*	Oysters	22.4
	Propionigenium	Marine sediment	8.6
Tibau do Sul-RN	*Mycoplasma*	Oysters	42.3
	Propionigenium	Marine sediment	9.7
	Psychrilyobacter	Marine sediment	9.4
Marcação-PB	*Mycoplasma*	Oysters	35.3
	Arcobacter	Oysters/Contaminated water	6.7
Passo de Camaragibe-AL	*Mycoplasma*	Oysters	24.9
Barra de São Miguel-AL	*Mycoplasma*	Oysters	50.1
Brejo Grande-SE	*Mycoplasma*	Oysters	43.9
	Propionigenium	Marine sediment	12.1
	Psychrilyobacter	Marine sediment	14.8
Indiaroba-SE	*Mycoplasma*	Oysters	61.2

Table IV. Abiotic variables measured during the four sampling periods from oyster-farms in north eastern Brazil.

Sampling site	Collection procedure	Season	P (mm)	O (mg/L)	T (°C)	S (g/L)	pH
			Reference value				
			-	> 3[a]	23-31[b]	10-50[c]	6.7-8.7[d]
Macau/RN	1	Rainy	155.0	5.01	30.8	33	7.86
	2	Rainy	3.4	8.24	28.7	46	8.40
	3	Drought	0.0	6.99	28.7	49	8.32
	4	Drought	0.6	5.25	27.8	39	8.15
Tibau do Sul/RN	1	Rainy	317.0	1.61	29.8	1	6.71
	2	Rainy	301.2	4.85	28.4	27	8.02
	3	Drought	24.4	7.89	29.2	15	7.92
	4	Drought	10.8	4.57	27.8	24	7.56
Marcação/PB	1	Rainy	406.6	3.43	30.0	5	6.70
	2	Rainy	343.2	4.98	27.1	19	7.31
	3	Drought	56.9	3.74	28.3	20	7.33
	4	Drought	19.5	4.44	28.5	27	7.32
Passo de Camaragibe/AL	1	Rainy	150.6	5.02	31.0	7	7.13
	2	Rainy	287.1	4.93	27.2	12	7.71
	3	Drought	42.2	8.36	26.4	10	7.90
	4	Drought	35.8	5.91	28.5	27	7.84
Barra de São Miguel/AL	1	Rainy	58.6	6.90	31.0	30	8.30
	2	Rainy	347.9	4.28	28.0	16	7.59
	3	Drought	42.8	7.03	28.2	19	7.58
	4	Drought	53.4	4.58	28.5	25	7.54
Brejo Grande/SE	1	Rainy	40.5	4.88	31.7	28	7.13
	2	Rainy	152.0	1.08	32.2	26	8.05
	3	Drought	23.8	6.18	27.6	26	7.20
	4	Drought	20.6	10.1	32.1	28	7.88
Indiaroba/SE	1	Rainy	29.0	4.72	31.8	27	7.20
	2	Rainy	140.0	3.57	28.1	7	7.02
	3	Drought	28.0	6.46	27.2	22	7.18
	4	Drought	39.4	3.57	28.0	26	7.38

P: Precipitation; O: Dissolved oxygen; T: Temperature; S: Salinity. [a]Mello (2007) to *Crassostrea* sp. [b]Ansa & Bashir (2007) to *Crassostrea gasar.* [c]Funo et al. (2015) to *Crassostrea gasar*. [d]Morales (1986) to Crassostrea gigas.

The high percentage of bacteria of the taxa Tenericutes, Spirochaetes, and Proteobacteria in the oysters analysed corroborates the data found by Madigan et al. (2014) in oysters farmed in the Camden Haven, Australia. The authors verified that the microbiota of fresh *Crassostrea gigas* and *Saccostrea glomerata* comprised 53% Tenericutes, 27% Spirochaetes and 14% Proteobacteria, indicating a certain similarity in terms of the composition of the bacterial community of oysters grown in estuarine environments in regions as far away as north eastern Brazil and Australia.

Since the analysed oysters were cultivated for consumption, the presence of bacteria with pathogenic potential may represent a risk to the health of the consumer. Among the 40 bacterial genera identified, nine are responsible for foodborne diseases in humans. Of these nine genera, only the genus *Staphylococcus* (0.2%) is among the bacteria whose analysis is required by Brazilian legislation for commercialization and consumption [Resolution RDC N°12 of January 2001 of the Agência Nacional de Vigilância Sanitária (Brasil 2001) and Interministerial Normative Instruction MPA / MAPA Nº 07, of May 08, 2012 (Brasil 2012)].

The presence of *Staphylococcus* in oysters is usually associated with the manipulation of these animals, as this bacterium is found in the skin and mucosa of humans. This genus is divided into positive and negative coagulase staphylococci, and among the known species, the *S. aureus* bacterium is associated with the risk of human food poisoning, causing fever and vomiting in patients (Leroy et al. 2016). *Staphylococcus* can also cause bacteraemia, endocarditis and cutaneous infections (Tortora et al. 2012). However, as the method used here does not allow differentiation of negative coagulase *Staphylococcus*, it was not possible to draw conclusions about the pathogenicity of the bacteria of this group as identified in the analyses performed. Other bacteria genera that cause foodborne disease (*Arcobacter*, *Flavobacterium*, *Alteromonas*, *Bacillus*, *Clostridium*, *Sphingomonas*, *Vibrio*, and *Pseudomonas*) have also been registered; these bacteria are capable of causing diseases or physiological disturbances in humans, such as vomiting, fever, diarrhoea and abdominal muscle pains (Tauxe 2002). According to Kalyoussef & Feja (2014), when identified in foods, follow-up is necessary to determine preventive efforts, especially by including those bacteria genera in the mandatory reporting lists and thereby ensuring consumer food safety.

Of all the bacteria analysed that might present some pathogenicity to oysters (*Mycoplasma*, *Photobacterium*, *Vibrio*, *Pseudomonas*, *Oceanospirillum*, and *Alteromonas*), only *Mycoplasma* (ranging from 4.5 to 71% of the total number of the bacteria present in each sample) was dominant at all sampling locations, and the other genera did not exceed 0.3%. It is known that *Mycoplasma* proliferates and predominates in environments with high temperatures (King et al. 2012). According to Jaffe et al. (2004), these optimal growth temperatures range from 20-37 °C, depending on the species.

The genus *Mycoplasma* was first isolated in fish (*Tincatinca*) (Kirchhoff & Rosengarten 1984), although it has already been found by King et al. (2012) in abundance in the stomachs of *C. virginica* grown in Terrebonne Bay, Louisiana, USA, and in the gills of *C. gigas* as evaluated by Wegner et al. (2013) in the bay Sylt-Rømø-Bight and the bay Hörnum Deep in Germany. Paillard et al. (2004) reported that in bivalve mollusc larvae, the presence of *Mycoplasma* can cause infections, resulting in the consequent death of the animal. Azevedo (1993) associated the presence of *Mycoplasma* with the mortality of

cockles (*Cerastodermaedule*) in the estuarine region of Aveiro, Portugal.

In humans, however, *Mycoplasma* is also considered potentially pathogenic and may cause allergic inflammation, pneumonia, diabetes mellitus and multiple sclerosis (Razin 1996). In this study, no correlation was found between the environmental variables analysed and the bacteria present in oysters. However, it should be stressed that water samples and analyses were performed only at the time of oyster harvesting, with the objective of being used as an indicator of environmental quality. In extremely variable environments such as those that characterize the estuarine regions (Vilanova & Chaves 1988), these correlations would require a much larger sampling frequency to be clearly established. Nevertheless, a significant correlation between the pH and the presence of *Mycoplasma* was identified, with a reduction of the percentage of this microorganism in alkaline waters. Pereira et al. (2009) stated that the optimum pH for the growth of this genus of bacteria is between 6.5 and 7.5; therefore, there is a tendency for the amount of *Mycoplasma* to decrease at pH levels above 7.5 (the maximum pH reached 8.4 at the monitored collection points).

The pathogenicity of the second most prevalent genus, *Propionigenium*, is still unknown in both oysters and humans. *Propionigenium* has been found in marine sediments (Janssen & Liesack 1995) with an optimum temperature range of 30 to 37 °C (Schink 2006) and has been identified in abalones grown in Hokkaido, Japan (Tanaka et al. 2004); in *Mytilus galloprovincialis* mussels collected at Lake Faro, Italy (Cappello et al. 2015); and in marine urochordates (*Ciona intestinalis*) (Dishaw et al. 2013).

The genus *Psychrilyobacter*, present in high percentages in the Tibau do Sul-RN and Brejo Grande-SE samples, is found in marine sediment and grows at low temperatures (Zhao et al. 2009). However, the pathogenic potential of *Psychrilyobacter* for oysters and humans is still unknown. According to Fernandez-Piquer et al. (2012), the occurrence of *Psychrilyobacter* was associated with post-harvest oyster storage, regardless of the temperature used during that process (between 4 and 30 °C).

Arcobacter was among the most predominant bacteria at the Marcação-PB sampling location. This genus is associated mainly with faecal contamination of marine waters and is considered potentially pathogenic to humans because it causes gastroenteritis, endocarditis, peritonitis and diarrhoea when ingested (Collado & Figueras 2011). The pathogenic potential of *Arcobacter* for oysters remains unknown.

In addition to contaminated environments, *Arcobacter* has been isolated in seafood (fish, oysters, clams, and mussels) (Rathlavath et al. 2016). Fernandez-Piquer et al. (2012) observed high abundance, without dominance, of *Arcobacter* in the oyster *C. gigas*. Romero et al. (2002) reported that this genus is abundant and common in Chilean oysters of the species *Tiostrea chilensis*.

The results obtained in this study show the importance of next-generation genetic sequencing as an analytical tool for microbiological monitoring studies of and programmes for oysters. These studies show that there is a high diversity of bacteria in cultivated oysters, with a prevalence of those bacterial genera found naturally in the environment itself or in marine/estuarine organisms. Finally, these results recommend the systematic monitoring of bacteria of the genus *Mycoplasma* in oysters grown and commercialized in the north eastern region of Brazil. Currently, this genus is not on the list of microorganisms whose analysis and monitoring are required by Brazilian legislation

during the production and/or commercialization of oysters, although *Mycoplasma* was the most prevalent genus at all the sampling locations and has pathogenic potential both for oysters and for consumers.

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