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ORIGINAL ARTICLE

Metagenomic evaluation of the effects of storage conditions on the bacterial microbiota of oysters *Crassostrea gasar* (Adanson, 1757)

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Abstract

Aims: To evaluate the influence of storage conditions on the composition of the bacterial microbiota of living oysters *Crassostrea gasar*.
Methods and Results: The oysters used in this study came from marine farms (Guaratuba Bay, Brazil) and were exposed to two conditions that simulated different storage situations: immersion in water (group I) and exposure to air (group II). The animals were subjected to five different temperatures (5–25°C), for 10 days. The 16S rRNA gene from oysters was amplified and sequenced to determine the taxonomic units and bacterial strains present in the samples. Group I showed higher diversity of bacteria (163 genera) rather than group II (104 genera). In all, 59 bacterial genera potentially pathogenic to humans were identified (n = 56 in group I and n = 45 in group II).
Conclusions: The storage conditions having a direct influence on the oyster microbiota. Live *C. gasar* should be stored exposed to air at 5–25°C, because it favours a lower prevalence of bacteria potentially pathogenic to humans.
Significance and Impact of the Study: During the oyster commercialization process, some conditions of storage, time and temperature must be followed in order to reduce the prevalence of bacteria potentially pathogenic to humans.

Introduction

Oysters are among the most cultivated and marketed bivalve mollusks in the world (Fernandez *et al.* 2014). In most cases, the animals can be stored for several days after collection, and the oysters are consumed while still alive (Aaraas *et al.* 2004; Jiménez-Ruiz *et al.* 2015).

Because they are filtering animals, oysters retain micro-organisms during the feeding process (Wang *et al.* 2014). When exposed to air, they close their valves and, as a consequence, there is a decrease in pH and oxygen concentration and an accumulation of residues in the intervalvar liquid, causing changes in the composition of the bacterial microbiota (Cook 1991). Thus, the microbial composition at the time of consumption is directly influenced by the original microbiota (resident or transient) and the conditions under which the animals are stored and handled (Fernandez-Piquer *et al.* 2012).

During storage of live oysters, depending on the temperature and storage time, there may be a predominance of species of bacteria such as *Vibrio*, *Shewanella*, *Alcaligenes*, *Enterobacter*, *Moraxella*, *Acinetobacter*, *Flavobacterium*, *Bacillus* and *Corynebacterium* (Cao *et al.* 2009). Such micro-organisms can induce the deterioration of the oyster flesh and affect the quality of the product (Chen *et al.* 2013).

As these animals may be consumed whole and alive, potentially pathogenic bacteria can be ingested and cause

illness to the consumer (Cruz-Romero *et al.* 2008). However, the complete identification of the bacterial microbiota and potentially pathogenic bacteria in marketed oysters is not feasible by traditional methods (those based on selective culture media; Fernandez-Piquer *et al.* 2012) or through biochemical tests. For this reason, protocols that do not use bacterial proliferation in culture media have been developed to increase the agility and accuracy of the analyses (Postollec *et al.* 2011).

Metagenomics is a method that allows microbial communities collected directly from the environment or from biological samples to be characterized without the need to isolate and culture specific bacteria (Tringe and Rubin 2005). This method is based on 16S rRNA gene libraries, which are generated through next-generation sequencing and allow the full mapping of the bacteria present in the analysed animals (Hernández-Zárate and Olmos-Soto 2006).

This work aimed at using next-generation sequencing to evaluate the influence of two storage methods (underwater immersion and exposure to air) on the bacterial microbiota of living oysters.

Materials and methods

The experiment used *Crassostrea gasar* oysters ($n = 216$), with an average weight of 79·3 ± 22·3 g and height of 8·3 ± 1·3 cm. The specimens were collected from marine farms located in Guaratuba Bay (25°83′S, 48°57′W) on the coast of the state of Paraná, Brazil.

At the time of collection, the salinity (g l^{-1}) of the cultivation water was measured with a refractometer (Instrutemp™, São Paulo, Brazil). Shortly after collection, the oyster shells were washed with sterile plastic bristle brushes to remove sediment and epibionts. The animals were transported in isothermal boxes at 15–20°C to the Laboratory of Aquatic Organisms Research (LAPOA), part of the Integrated Group for Aquaculture and Environmental Studies (GIA) of the Federal University of Paraná, Curitiba, Paraná, Brazil (25°24′47.70″S, 49°14′52.82″W).

Experiments

The oysters did not undergo any acclimation process and did not have access to food during the experimental period. Two experimental conditions (groups) were defined that simulated the most common storage procedures for the maintenance of live oysters during marketing:

i Oysters immersed in water. The entire volume of water (25 g l^{-1}) was renewed daily. The 'new' water was chlorinated (10% sodium hypochlorite) and neutralized (50% sodium thiosulphate) before being used.

ii Oysters exposed to air. The animals were held in a 27% moisture-controlled environment throughout the experimental period.

Using BOD-type incubators, the two groups were subjected to five treatments (5, 10, 15, 20 and 25°C), in triplicate, for 10 days. Exposure temperatures were set after conducting a pilot test that determined that the best oyster survival range was between 0 and 35°C. Before beginning the experiment, six oysters were collected from each treatment to determine the initial composition of the bacterial microbiota (control group). Subsequently, batches of 30 oysters were placed in 15 experimental units ($n = 900$). Each unit consisted of a plastic tray containing 5 l of water at 25 g l^{-1} (group I) or the same plastic tray without water (group II). From each experimental unit, six oysters were collected on days 2, 4, 6, 8 and 10 for microbiological evaluation ($n = 90$ per treatment).

The experimental units were inspected three times per day. At these times, dead animals were identified and discarded. Oysters were deemed dead when they had open or semiopen valves and showed no reaction to a slight knocking on their shell using a solid object, such as the shell of another oyster. A hollow sound generated by the knocking of an object against the oyster's shell was also used as an indicator of the animal's death.

DNA extraction

On days 0, 2, 4, 6, 8 and 10, six oysters from each experimental unit were collected, and their valves were opened for the removal of the meat and intervalvar liquid. This material was placed in sterile plastic bags and macerated; a 200 μl aliquot was removed and stored in Eppendorf flasks for extraction of total DNA by the Genomic Purelink™ DNA Kit (Invitrogen, Waltham, MA, USA).

After extraction, the quality and quantity of total DNA were evaluated by spectrophotometry using a NanoDrop 2000 spectrophotometer (ThermoScientific™, Waltham, MA, USA), with readings at 260 and 280 nm absorbance.

All analyses were performed based on the methodology proposed by Caporaso *et al.* (2011), with some adaptations. The 16S rRNA gene was amplified with PCR in a 12 μl reaction containing 5 μl of DNA (10 ng l^{-1}), 1 μl of universal primer (515F/806R), 1 μl of the first 'adapter' and 5 μl of the KlenTaq DV ReadyMix enzyme. Genomic expression levels of the 16S rRNA gene were analysed in a thermocycler (Veriti 96 well Applied Biosystems™, Waltham, MA, USA); sequencing conditions were 94°C for 3 min for the initial denaturation of the DNA, followed by 25 cycles of 94°C for 45 s (final denaturation), 50°C for 30 s (annealing) and 68°C for 1 min (initial extension), with a final extension at 72°C for 10 min. After this step, the samples were maintained at between 0 and 4°C.

To confirm gene amplification, the PCR products were tested on a 1% agarose gel in Tris/Borate/1X EDTA buffer (0·09 mol l^{-1} Tris-HCl, 0·09 mol l^{-1} boric acid and 0·002 mol l^{-1} EDTA). A mixture was made with 2 μl of PCR product and 2 μl of FSUDS dye (bromophenol blue); 3 μl of this mixture was added to a well of the gel. After that, electrophoresis was allowed to run for one hour at a constant voltage (70 V) in the presence of 1× TBE buffer. Together with the samples, 3 μl of 1 kb molecular weight marker was applied to the gel. The gel was stained with 1% ethidium bromide for 15 min, washed in water for 10 min and visualized in a UVP 3UV Transilluminator Imaging System (Canada).

Sequencing of PCR products was performed on the next-generation Illumina MiSeq™ platform (San Diego, CA, USA), which is capable of generating information on thousands of base pairs in a single run. The MiSeq Reagent Kit v2 was used for paired-end sequencing (500 cycles).

Data analysis

The 16S rRNA reads 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 carried out using the software Past ver. 2.17.

The taxonomic identities obtained in the genetic analyses were tabulated, sorted and classified according to their prevalence. Prevalent genera were defined as those found in quantities equal to or greater than 5% of the reads in each sample analysed.

The Mann–Whitney test was applied to evaluate differences between the groups subjected to immersion in water and exposure to air. The evaluation of different exposure times and temperatures was performed with the Kruskal–Wallis test. In both cases, the data were analysed with the software Statistica 12.0 (StatSoft™, Tulsa, OK, USA).

Results

Mortality

Bacterial microbiota identification was not performed in tree treatments of the group I (5, 10 and 25°C) and one treatment of group II (5°C), after 6 and 8 days of exposure, respectively, due to the mortality of oysters (Table 1).

Bacterial microbiota

We identified a total of 169 genera of bacteria belonging to 133 families, 77 orders, 44 classes and 22 phyla. Oysters from group I showed a higher number of taxonomic categories of bacteria when compared to oysters from group II (Table 2). The most prevalent phyla in both treatments were Proteobacteria, Tenericutes, Spirochaetes and Bacteroidetes.

Table 1 Survival time (days) and the number of oysters, *Crassostrea gasar*, analysed through the period of exposure to different treatments

Temperature (°C)	Survival time (days)	
	Group I (immersion in water)	Group II (exposed to air)
5	6 (n = 54)	8 (n = 72)
10	6 (n = 54)	6 (n = 54)
15	10 (n = 90)	10 (n = 90)
20	10 (n = 90)	10 (n = 90)
25	5 (n = 54)	1 (n = 54)

Table 2 Number of taxonomic categories of bacteria identified in the different groups

Taxonomic categories	Number of taxonomic categories	
	Group I (immersion in water)	Group II (exposed to air)
Phylum	20	17
Class	43	31
Order	75	57
Family	127	93
Genus	163	104

In all, 59 genera of bacteria potentially pathogenic to humans were identified; 56 of these were found in the oysters of group I and 45 in the oysters of group II. Among these bacteria, eight are considered causes of foodborne disease (Table 3).

A large percentage of 16S gene sequences mapped to bacteria still without specific identification (for example BD2-6, WH1-8 and SC3-56). These sequences were reported here as 'unclassified'. Bacteria that were not adequately identified by the analytical method used were reported as 'undefined'.

Table 4 presents the different indexes quantified in the oysters of the different treatments at the beginning and end of the experiments. For both groups, day 0 oysters exposed to 5°C were not analysed due to the low number of 16S rRNA reads; the same case applies to oysters immersed in water at 25°C for 6 days. The results show some differences and similarities between the different treatments.

In group II oysters (exposed to air), there were fewer bacterial genera present at higher temperatures at both the beginning and end of the experiment. However, in the oysters of group I (exposed to water), there was an

Table 3 The average percentage of phyla and bacterial genera recorded in oysters, *Crassostrea gasar*, exposed to different temperatures, immersed in water (group I) or exposed to air (group II)

Phylum/genera	Average percentage	
	Group I (immersion in water)	Group II (exposed to air)
Acidobacteria	0·2	0·1
Candidatus Solibacter	0·1	0·0
Undefined	0·2	0·1
Actinobacteria	0·2	0·1
Brevibacterium	0·1	0·1
*Corynebacterium**	0·1	0·1
Iamia	0·1	0·0
Micrococcus	0·0	0·1
*Mycobacterium**	0·1	0·0
Pseudonocardia	0·1	0·0
Rubrobacter	0·1	0·0
Sporichthya	0·2	0·0
*Williamsia**	0·1	0·1
Undefined	0·2	0·1
Armatimonadetes	0·5	0·0
Fimbriimonas	0·5	0·0
Bacteroidetes	0·8	1·3
Aquimarina	0·2	0·3
Arenibacter	0·1	0·1
*Bacteroides**	0·3	2·8
*Capnocytophaga**	0·1	0·0
*Chitinophaga**	0·5	0·0
*Chryseobacterium**	0·2	0·3
*Cloacibacterium**	0·3	0·3
Crocinitomix	0·2	0·2
Dyadobacter	0·1	0·0
Flavobacterium†	0·6	0·6
Flexibacter	0·1	0·2
Fluviicola	0·3	0·2
Gaetbulibacter	0·2	0·1
Gramella	0·1	0·1
Kordia	0·3	0·3
Leadbetterella	0·1	0·1
Lewinella	0·2	0·0
Maribacter	0·2	0·1
Mesonia	0·1	0·1
Owenweeksia	0·2	0·2
Paludibacter	0·7	0·6
Pedobacter	0·2	0·1
Persicobacter	1·5	0·0
Polaribacter	0·2	0·2
Psychroserpens	0·1	0·1
Reichenbachiella	0·1	0·0
Roseivirga	0·3	0·3
Saprospira	0·3	0·3
Sediminibacterium	2·0	0·0
Tenacibaculum	0·1	0·2
*Wautersiella**	0·3	0·6
Winogradskyella	0·2	0·3
Undefined	1·2	1·8
Caldithrix	0·1	0·0
Undefined	0·1	0·0
Chlamydiae	0·2	0·1
Candidatus Rhabdochlamydia	0·1	0·0
Undefined	0·2	0·1
Chlorobi	0·3	0·1
Undefined	0·3	0·1
Chloroflexi	0·1	0·1
Undefined	0·1	0·1
Crenarchaeota	0·7	0·1
Nitrosopumilus	0·1	0·1
Undefined	1·8	0·0
Cyanobacteria	1·4	4·0
Chlamydomonas	5·7	0·0
*Cyanobacterium**	0·0	0·1
Synechococcus	0·2	0·2
Undefined	1·3	5·5
Elusimicrobia	0·4	0·0
Undefined	0·4	0·0
Euryarchaeota	0·3	0·0
Undefined	0·3	0·0
Fibrobacteres	0·0	0·1
Undefined	0·0	0·1
Firmicutes	0·3	0·4
*Acidaminococcus**	0·1	0·3
Alkaliphilus	0·3	0·0
*Anaerococcus**	0·1	0·1
Brochothrix	0·1	0·1
Clostridium†	0·3	0·1
*Finegoldia**	0·1	0·0
Fusibacter	0·4	0·5
*Lactobacillus**	0·2	0·2
*Lactococcus**	0·0	0·1
*Leuconostoc**	0·3	0·3
*Peptoniphilus**	0·1	0·0
Sedimentibacter	0·5	0·5
Staphylococcus†	0·1	0·1
*Streptococcus**	0·2	0·1
Tepidibacter	0·0	0·1
Undefined	0·2	0·5
Unclassified	0·2	0·3
Fusobacteria	2·4	1·7
*Cetobacterium**	0·1	0·1
*Fusobacterium**	0·3	2·2
Propionigenium	1·5	0·6
Psychrilyobacter	4·1	2·4
Undefined	0·1	0·1
Gemmatimonadetes	0·0	0·1
Undefined	0·0	0·1
Lentisphaerae	0·1	0·0
Undefined	0·1	0·0
Planctomycetes	0·1	0·3
Gemmata	0·1	0·0

Table 3 (*Continued*)

Phylum/genera	Average percentage	
	Group I (immersion in water)	Group II (exposed to air)
*Planctomyces**	0·1	0·1
Undefined	0·1	0·3
Proteobacteria	1·4	2·6
*Achromobacter**	0·1	0·1
*Acinetobacter**	0·2	0·3
*Agrobacterium**	0·8	0·0
*Aliivibrio**	4·6	9·5
Alteromonas†	0·3	0·1
Amphritea	0·2	0·1
Arcobacter†	18·0	24·9
Arthrospira	2·3	0·0
Asticcacaulis	0·3	0·0
Azospirillum	0·2	0·2
Bacteriovorax	0·1	0·1
Bdellovibrio	0·6	0·1
Bradyrhizobium	0·8	0·0
Burkholderia	0·3	0·5
Cellvibrio	1·1	0·0
Cohaesibacter	0·1	0·0
*Comamonas**	0·1	0·0
*Cupriavidus**	2·1	1·3
Desulfarculus	0·1	0·0
Desulfobacter	0·1	0·0
Desulfococcus	0·2	0·1
Desulfofrigus	0·3	0·5
*Desulfosarcina**	0·1	0·0
*Desulfovibrio**	0·2	0·2
Devosia	0·2	0·0
Dokdonella	0·2	0·0
*Enhydrobacter**	0·0	0·1
Erythrobacter	0·1	0·0
Ferrimonas	0·1	0·1
*Francisella**	0·1	0·0
Glaciecola	0·1	0·0
*Helicobacter**	0·2	0·2
Hylemonella	1·2	0·0
Hyphomicrobium	0·1	0·0
*Janthinobacterium**	0·2	0·2
Kaistia	0·1	0·0
Kaistobacter	0·4	0·0
Leucothrix	0·3	0·2
Magnetospirillum	0·1	0·0
Marinicella	0·1	0·1
Marinobacter	0·2	0·1
Marinobacterium	0·1	0·1
*Marinomonas**	0·3	0·3
Marivita	0·4	0·0
*Massilia**	0·2	0·0
Mesorhizobium	0·3	0·0
Methylibium	1·0	0·0
*Methylobacterium**	0·2	0·2
Methylophaga	0·1	0·0

Table 3 (*Continued*)

Phylum/genera	Average percentage	
	Group I (immersion in water)	Group II (exposed to air)
Methylotenera	0·1	0·0
Moritella	0·3	0·4
Mycoplana	0·1	0·1
Neorickettsia	1·7	0·0
Neptunomonas	0·4	0·4
Nevskia	0·7	0·0
*Novosphingobium**	1·8	0·0
*Oceanospirillum**	0·3	0·1
Oleibacter	0·2	0·4
Oleispira	0·1	0·1
Pedomicrobium	0·1	0·1
Perlucidibaca	0·1	0·0
Phaeobacter	1·3	0·3
*Photobacterium**	0·9	0·7
Plesiocystis	0·3	0·0
Pseudidiomarina	0·2	0·0
*Pseudoalteromonas**	2·6	1·5
Pseudomonas†	0·3	0·2
*Psychrobacter**	0·3	0·3
Psychromonas	0·2	0·2
*Ralstonia**	0·3	0·0
Ramlibacter	0·2	0·0
*Rhizobium**	0·2	0·0
Rhodobacter	0·6	0·0
*Rhodoplanes**	0·2	0·0
Roseateles	0·5	0·1
Salinispora	0·1	0·0
*Shewanella**	2·4	2·8
Sphingobium	0·1	0·1
Sphingomonas†	0·2	0·1
Sphingopyxis	0·2	0·1
Spongiibacter	0·1	0·0
*Stenotrophomonas**	0·1	0·1
Sulfurimonas	0·3	0·1
Sulfurospirillum	0·1	0·2
Thalassomonas	0·2	0·1
*Thalassospira**	0·2	0·1
Vibrio†	0·4	0·5
Xanthobacter	0·2	0·2
Zoogloea	0·2	0·0
Undefined	1·0	1·3
Unclassified	0·4	0·2
Spirochaetes	4·7	3·1
*Borrelia**	0·9	1·0
*Spirochaeta**	0·1	0·1
Undefined	6·1	3·8
Tenericutes	8·2	10·1
*Acholeplasma**	0·2	0·2
*Mycoplasma**	10·9	13·1
Undefined	0·5	0·5
Verrucomicrobia	0·4	0·1
Candidatus Xiphinematobacter	0·1	0·0

Table 3 (*Continued*)

Phylum/genera	Average percentage	
	Group I (immersion in water)	Group II (exposed to air)
Chthoniobacter	0·7	0·0
Coraliomargarita	0·0	0·1
Opitutus	0·5	0·0
Pedosphaera	0·9	0·0
Persicirhabdus	0·1	0·1
Prosthecobacter	0·6	0·0
Undefined	0·4	0·1
Unclassified	0·3	0·4
Unclassified	0·3	0·4
Undefined	3·0	2·9
Undefined	3·0	2·9

*Genera with species potentially pathogenic to humans.
†Genera with species that cause Foodborne Diseases in humans.

increase in the number of genera identified at the end of the experiment at 15 and 20°C.

As quantified by the Shannon–Winner index, the diversity of genera in group II was higher at the end of the experiment for all temperatures, except for 20°C. For the oysters of group I, there was an increase in diversity at different temperatures, except at 15°C.

The highest absolute value related to the dominance of bacterial genera was recorded in oysters exposed to air (group II) at the end of the experiment at 20°C. For the oysters of group I, the highest value was recorded at the end of the experiment at 15°C.

Among the genera of bacteria identified, 13 were most prevalent (those present at a percentage equal to or greater than 5% in each sample analysed). The percentages of the genera *Arcobacter*, *Mycoplasma* and *Aliivibrio* were higher in oysters of group II; *Bacteroides* and *Fusobacterium* were found exclusively in this group. In the oysters of group I, *Arcobacter*, *Mycoplasma*, *Aliivibrio*, *Psychrilyobacter*, *Shewanella* and *Pseudoalteromonas* were the most prevalent. The genera *Chlamydomonas*, *Photobacterium*, *Phaeobacter* and *Propionigenium* were found exclusively in this group. The prevalence of *Cupriavidus* was similar in both groups (Fig. 1).

A significant difference ($P = 0{\cdot}037$) was observed in the number of prevalent genera of bacteria between groups I and II. In contrast, the exposure times and the different temperatures had no influence ($P > 0{\cdot}05$) on the prevalence of bacteria present in the oysters.

The percentage of prevalent bacteria tended to increase throughout the experiment in oysters of group I, mainly at temperatures below 15°C (Fig. 2a). In oysters of group II, this increase occurred at temperatures above 15°C (Fig. 2b).

Discussion

This study represents the first time in Brazil that the 16S rRNA gene has been sequenced for the purpose of

Table 4 The number of genera, Shannon–Winner diversity index and Dominance index at the beginning (day 0) and the end of the experiment (exposing oysters, *Crassostrea gasar*, to different treatments)

Temperature	Group			
	I (immersion in water)		II (exposure to air)	
	Beginning	End	Beginning	End
Number of genera				
5	NA	18 (6 days)	NA	18 (8 days)
10	38 (day 0)	30 (6 days)	38 (day 0)	12 (6 days)
15	20 (day 0)	27 (10 days)	20 (day 0)	19 (10 days)
20	40 (day 0)	47 (10 days)	40 (day 0)	12 (10 days)
25	27 (day 0)	NA	27 (day 0)	12 (6 days)
Shannon–Winner Index				
5	NA	1·81 (6 days)	NA	1·30 (8 days)
10	1·66 (day 0)	2·15 (6 days)	1·66 (day 0)	1·75 (6 days)
15	1·46 (day 0)	1·09 (10 days)	1·46 (day 0)	1·93 (10 days)
20	2·35 (day 0)	2·46 (10 days)	2·35 (day 0)	0·62 (10 days)
25	1·23 (day 0)	NA	1·23 (day 0)	1·30 (6 days)
Dominance Index				
5	NA	0·22 (6 days)	NA	0·44 (8 days)
10	0·41 (day 0)	0·17 (6 days)	0·41 (day 0)	0·25 (6 days)
15	0·40 (day 0)	0·62 (10 days)	0·40 (day 0)	0·25 (10 days)
20	0·19 (day 0)	0·16 (10 days)	0·19 (day 0)	0·76 (10 days)
25	0·54 (day 0)	NA	0·54 (day 0)	0·36 (6 days)

NA: not analysed due to the low number of 16S rRNA sequence reads.

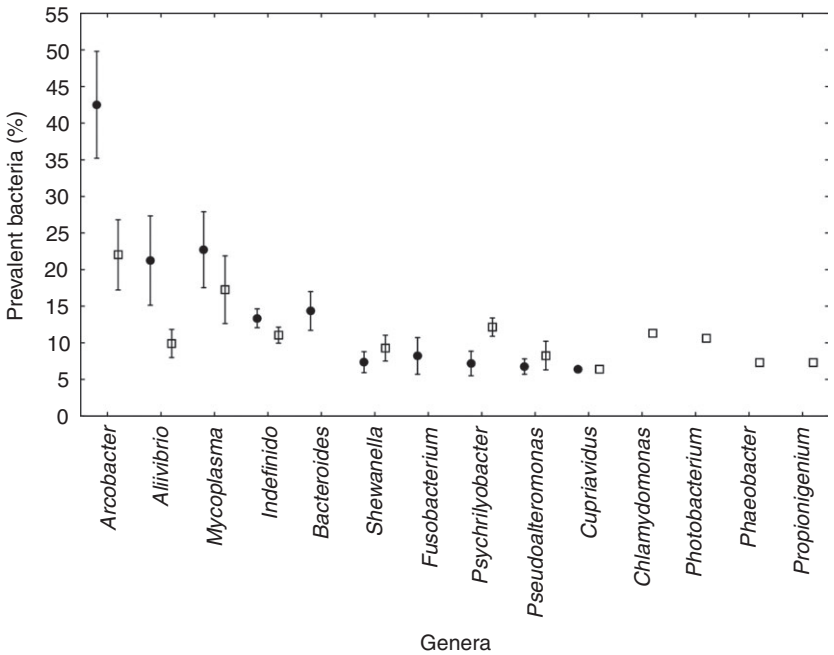

Figure 1 The mean and SE of prevalent (>5%) bacterial genera in oysters, *Crassostrea gasar*, immersed in water (⧲ group I) or exposed to air (⧱ group II). ⧱ Exposed to air; ⧲ Immersion in water

evaluating the influence of two storage conditions on the bacterial microbiota present in live oysters. However, a similar study was carried out by Fernandez-Piquer *et al.* (2012), in Tasmania, Australia, using *Crassostrea gigas*. In their work, the authors reported that traditional culture methods are not efficient for the identification of bacterial strains, demonstrating the importance of using next-generation sequencing techniques for a complete evaluation of the oyster bacterial microbiota.

In our study, mortality of oysters maintained at 5, 10 and 25°C occurred after 6 days of experimentation in the respective groups. This suggests that *C. gasar* does not tolerate storage at temperatures below 10°C or above 25°C for more than 6 days, whether immersed in water or exposed to air. Conversely, Moreira *et al.* (2017) found that oysters *Crassostrea brasiliana* (a synonym of *C. gasar*) were kept alive for 28 days in tanks containing 10 l of water at 29 g l^{-1} and 24°C. Zhang *et al.* (2006), studying *C. gigas*, reported similar temperature tolerance when the oysters were exposed to air. According to the authors, mortalities were higher at temperatures below 5°C and above 25°C. Seaman (1991) reported that *C. gigas* survives longer when stored immersed in water than exposed to air.

We found that the most prevalent phyla in both treatment groups were Proteobacteria, Tenericutes, Spirochaetes and Bacteroidetes. Fernandez-Piquer *et al.* (2012), when evaluating the bacterial microbiota of postharvest *C. gigas* oysters at different temperatures (4, 6, 15, 20 and 30°C), observed that the phylum Proteobacteria predominated in newly collected oysters. However, according to the authors, after the animals were stored exposed to air, the phylum Fusobacteria became dominant in oysters kept for more than 8 days at 4°C and Bacteroidetes in oysters kept at 15°C for 4 days or at 30°C for 1 day.

Arcobacter was one of the most prevalent bacterial genera in the two groups tested. This genus is found naturally in environments contaminated by human faeces and can cause DTA (gastroenteritis, endocarditis, peritonitis and diarrhoea) (Collado and Figueras 2011). This genus was previously found in the typical microbiota of the Chilean oyster *Triostrea chilensis* by Cruz-Romero *et al.* (2008). Fernandez-Piquer *et al.* (2012) observed the presence of this genus in *C. gigas* maintained at temperatures between 4 and 30°C.

The second most prevalent genus in oysters of both groups was *Mycoplasma*. This genus is commonly found in the normal oyster microbiota and is potentially pathogenic to humans; it may cause allergic inflammations, pneumonia, diabetes mellitus and multiple sclerosis (Razin 1996). According to Jaffe *et al.* (2004), the optimal growth temperature for this genus varies between 20 and 37°C, depending on the species.

The genus *Aliivibrio*, present in high percentages in oysters of both groups, is easily found in marine environments. This genus was recently described and includes species that were formerly part of the genus *Vibrio* (Urbanczyk *et al.* 2007). Hjerde *et al.* (2008) reported that it might be pathogenic to fish, but its pathogenicity to bivalve mollusks and humans is still unknown.

Among the pathogenic bacteria found in oysters, only *Staphylococcus*, present at a low percentage and without confirmation of whether it is a positive or negative coagulase strain, is provided for in Brazilian regulations dealing with the

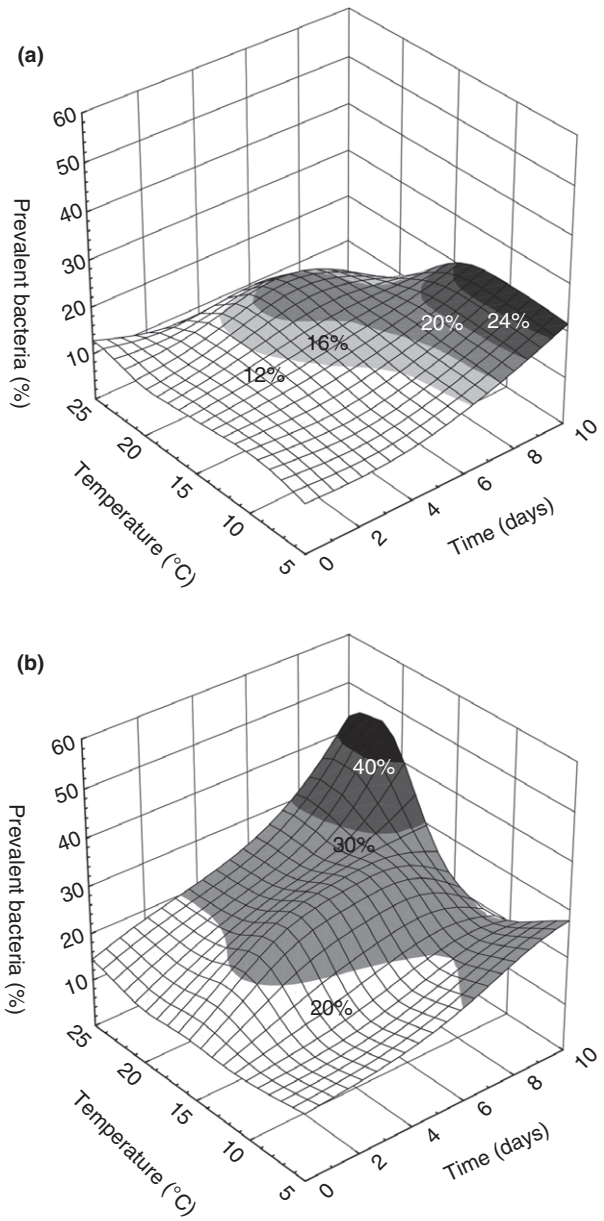

Figure 2 Percentage of prevalent bacteria identified in oysters, *Crassostrea gasar*, at different exposure times and temperatures. (a) Group I – immersion in water; (b) Group II – exposure to air.

microbiological standards that must be respected for food in the country (ANVISA 2001). The presence of *Staphylococcus* in oysters is usually associated with contamination during handling and may cause bacteraemia, endocarditis and cutaneous infections (Tong *et al.* 2015).

In addition to *Staphylococcus*, other bacteria causing foodborne diseases were identified in both groups (*Arcobacter*, *Pseudomonas*, *Flavobacterium*, *Clostridium*, *Sphingomonas*, *Vibrio* and *Alteromonas*). As these bacteria can cause health risks for consumers, we suggest that the occurrence and prevalence of these bacteria should be monitored in marketed oysters to guarantee the quality of the product sold and the food safety of the consumers.

The oysters of group I contained a higher number of bacterial genera along with greater diversity and lower genus dominance. Additionally, the prevalent bacteria developed better at temperatures below 15°C. Thus, we suggest that the bacterial microbiota of oysters is more diverse under favourable conditions for the development of micro-organisms (temperatures above 15°C).

The oysters of group II, on the other hand, contained fewer genera, with lower diversity and, consequently, higher dominance of microbial genera. When exposed to air, oysters keep their valves closed resulting in changes in metabolic pathways (anaerobiosis) an internal pH, as well as accumulation of physiological wastes and carbon dioxide (Gosling 2015). This process can have direct effects on the bacterial microbiota, so we suggest that there is selection of bacteria, with the growth of those most adapted to these conditions and the inhibition of those less adapted. Group II also observed an increase in prevalent bacteria at temperatures above 15°C. Madigan (2008) noted that the total number of bacteria in oysters (*Saccostrea glomerata*, *C. gigas* and *Ostrea angasi*) stored exposed to air at 15°C was lower than in those stored at 8°C. The author postulated that the smaller number of bacteria at this temperature was associated with more active oysters, which allowed their immune defences to be more efficient in eliminating bacteria.

The results obtained in the study with *C. gasar* indicate that the prevalence and composition of the oyster microbiota are directly related to storage conditions. During storage and commercialization, live oysters should be exposed to air at 5–25°C rather than immersed in water. This is because exposure to air is more effective for limiting the prevalence of bacteria which are potentially pathogenic in humans.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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