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Bacterial spoilage profiles in the gills of Pacific oysters (*Crassostrea gigas*) and Eastern oysters (*C. virginica*) during refrigerated storage

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ABSTRACT

Microorganisms harbored in oyster gills are potentially related to the spoilage and safety of oyster during storage. In this study, the microbial activities and pH changes of the gills of the two species, *Crassostrea gigas* and *C. virginica*, harvested from three different sites were determined and sensory evaluation was conducted during refrigerated storage. The bacteria in gills with an initial aerobic plate count (APC) of 3.1–4.5 log CFU/g rose remarkably to 7.8–8.8 log CFU/g after 8-days of storage. The APC of *Enterobacteriaceae* increased from 2.5 to 3.6 log CFU/g to 4.5–4.8 log CFU/g, and that of lactic acid bacteria (LAB) fluctuated in the range of 1.4–3.0 log CFU/g during the whole storage period. The results of sensory analysis indicated that the oysters had 8-days of shelf-life and that the gill presented the fastest deterioration rate. The pH of all samples showed a decrease in the early stages followed by an increased after 4-days of storage. The dynamic changes in microbial profiles were depicted to characterize gill spoilage by Illumina Miseq sequencing to characterize gill spoilage. The results revealed that oysters harvested at different sites showed common bacterial profiles containing *Arcobacter*, *Spirochaeta*, *Pseudoalteromonas*, *Marinomonas*, *Fusobacterium*, *Psychrobacter*, *Psychromonas*, and *Oceanisphaera* when spoiled, especially, among which *Psychrobacter* and *Psychromonas* (psychrotrophic genus) were represented as the most important gill spoiled bacteria during refrigerated storage, and *Arcobacter* with pathogenic potential was the dominated bacteria in all spoiled oysters. The consumption quality and safety of refrigerated oysters could be greatly improved by targeted control of bacteria in oyster gills according to the results the present study provided.

1. Introduction

Oysters, which are excellent sources of protein, polyunsaturated fatty acid (PUFA), vitamins, and minerals, are largely consumed globally (Cruz-Romero et al., 2008a; Rey et al., 2012). Annual oyster yields in the USA are over 16,245 metric tons in the USA (National Marine Fisheries Service, 2015), and over 25,800 metric tons in Canada (Fisheries and Oceans Canada, 2015). Oysters with such huge yields sales, however, just like the most seafood with high pH value, abundant water and nutrient contents, always suffer from deterioration during transport and storage. The deterioration of oysters is mainly attributed to the combined activities of microbiological metabolism and biochemical deteriorations, such as enzymatic decomposition and PUFA oxidation (Ashie et al., 1996; Madigan et al., 2014). In particular, the proliferation of microorganisms takes lead responsibility for seafood spoilage and unacceptable qualities during storage (Boziaris and Parlapani, 2017; Gram and Dalgaard, 2002). By conventional cultivation, *Pseudomonas* and *Vibrio* were identified as the predominant bacteria in spoiled Pacific oysters (Cao et al., 2010; Cruz-Romero et al., 2008b). Today, molecular identification methods, such as DGGE and 16S rRNA pyrosequencing, have been applied to uncovered the highly diverse bacterial communities in oysters (Chen et al., 2016; La Valley et al., 2009; Trabal et al., 2012). This diversity could be influenced by many factors including growing environment, temperature, storage

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Table 1
Freshness guide for oyster.

Parameters	Score			
	0[a]	1	2	3[b]
Odour	Sour and putrid smell	Slight sour smell	Strong sea-weedy	Hay/crisp
Fluids	Cloudy	Clear with large amount of debris	Clear with small amount of debris	Clear
Body colour	Yellow or light brown	Tan or beige	White	Cream white
Texture	Mushy	Slight mushy	Soft and less elastic	Firm and elastic
Gill & mantle	Faded and undefined	Faded and poorly defined	slight fading and less defined	strong colour and well defined

[a] Extremely unacceptable.
[b] Extremely acceptable.

conditions, and residential bacteria in different oyster tissues (Chen et al., 2017; Fernandez-Piquer et al., 2012; Madigan et al., 2014; Manatawee et al., 2011; Trabal et al., 2012; Wang et al., 2014a, b).

Oyster gills as a filtering tissue with rich bacterial diversity and potential pathogens always exhibit highly hygienic risk (Asmani et al., 2016; Wang et al., 2014a, b; Zurel et al., 2011). Highly bacterial counts were pointed out in the gills of fresh oysters or spoiled oysters stored at different temperatures (Chen et al., 2017, 2013). The combined deterioration of bacterial proliferation and the degradation of tissue when oyster gills spoiled could result in a visible liquefied appearance change, which was also reflected in histological observations of the digestive epithelia and gills that undergo degenerative changes to a greater extent than do other tissues in recently deceased oysters. (Aaraas et al., 2006). Therefore, the spoilage of oyster gills played a critical role in determining unacceptable qualities during storage.

Although spoilage bacteria in oysters have been comprehensively examined through culture-dependent and culture-independent methods, little is known about the contribution of spoilage and/or pathogenic bacteria in oyster gills during refrigerated storage. In this study, high-throughput sequencing was performed to reveal the dynamic changes of spoilage bacterial profiles in the gills of Pacific oysters and Eastern oysters from three different sites during refrigerated storage. Sensory assessment was conducted to obtain supplementary descriptions of the quality changes of shucked oysters that underwent refrigeration.

2. Materials and methods

2.1. Oyster and storage

Commercial Pacific oysters (*C. gigas*) (shell length of 11 ± 1 cm) were harvested from Fanny Bay in British Columbia (BC) in Canada; Eastern oysters (*C. virginica*) were harvested from commercial farms in New Brunswick (NB), and Prince Edward Island (PEI), Canada, respectively. Two batches of oysters were harvested respectively in March and April 2016. The oysters packed in polyethylene bags with blue ice bags were placed in a waxed cardboard box and transported to the lab under refrigerated conditions within 48 h according to commercial wholesale distribution practices. The live oysters were scrubbed under running potable water to remove debris, such as mud, sand, and fouling organisms from shells immediately. The shelled oyster meats left on the cupped side were covered with food plastic wraps and stored at 4 ± 1 °C. Aerobic plate count (APC), pH, and sensory evaluation were performed on days 0, 2, 4, 6 and 8. The total bacterial genome DNA of oyster gills was extracted on day 0 (BCg0, NBg0 and PEIg0), day 4 (BCg4, NBg4 and PEIg4), and day 8 (BCg8, NBg8, and PEIg8).

2.2. Microbiological assay

The homogenate of gill tissues was prepared for microbiological tests according to the previously described methods (Chen et al., 2017). Aliquot 0.1 ml of the ten-fold gradient dilution of homogenate was spread on a Plate Count Agar (Oxoid, CM0325B, England) supplemented with 10 g/l NaCl and then incubated at 22 °C for 48 h for APC. The dilution was spread on Violet Red Bile Glucose (VRBG) Agar (Oxoid, CM0485, England) and incubated at 37 °C for 24 h to obtain the colony forming unit (CFU) of *Enterobacteriaceae*. Lactic acid bacteria (LAB) were counted after spread on de Mann, Rogosa, Sharpe (MRS) Agar (Oxoid, CM1153, England) and cultivated under the AnaeroGen W-Zip compact system (Oxoid) for 72 h. Microbiological counts of oyster gills were evaluated every second days, and expressed as mean $\log_{10}$ CFU/g ± standard deviation (SD).

2.3. pH measurement for oyster gills

The pH of oyster gill tissue was measured in triplicate for each group using a pH meter (Fisher scientific AE150, Canada) according to a previous method (Chen et al., 2017).

2.4. Sensory scores of oyster samples

Sensory scores of oyster samples were assessed every 2 days on the basis of the Freshness Guide for Pacific Oysters (He et al., 2002) with modifications (Table 1). In brief, the mantle and gills were assessed together as a gill indicator (including colour, definition, and liquefied appearance). Six trained panellists were invited to evaluate the odour, fluid, body colour, texture, and gills. Each parameter was assigned a score range of 3 to 0 corresponding to the level of acceptance from perfectly acceptable to extremely unacceptable. A sum of the above five parameter scores was calculated as freshness scores ranging from 15 to 0. A score below 7.5 was regarded as unacceptable (Cao et al., 2009).

2.5. DNA extraction

An oyster gill matrix was prepared by reference to a previously described method (Chen et al., 2013). The total bacterial genomic DNA in oyster gills was extracted with a PowerSoil DNA isolation kit (Mobio Laboratories, Inc. Carlsbad, USA) following the manufacturer's protocol. The yield and purity of DNA was assessed by nano spectrophotometer (Thermo Scientific, Massachusetts, USA) and stored at −20 °C for further use.

2.6. PCR amplification and Illumina MiSeq sequencing

Amplifications were performed in two steps: in the first step, the V4-V5 region of bacterial 16S rRNA genes in oyster gills were amplified with a primer pair of 515F/926R (Klindworth et al., 2013); in the second step, the amplified products were purified and multiplexed with AxyPrep DNA Gel Extraction Kit (Axygen, USA) and Illumina Nextera XT Index kit (Illumina Inc., San Diego, USA), respectively. The normalized DNA was quantified using FTC-3000™ real-time PCR. Two batches of amplicons from March and April 2016 were pooled together into a library. Sequencing was carried out on an Illumina MiSeq platform according to standard protocol.

2.7. Illumina data analysis

For all of the samples, paired-end sequencing reads obtained from the Miseq platform were converted to a raw fastq file. After merging and pretreatment, the reads were filtered in terms of quality control to obtain effective tags. The effective sequencing was clustered into operational taxonomic units (OTUs) at an identity level over 97% using the software USEARCH (Edgar, 2010), and the representative sequences were assigned for annotation via Mothur (Version 1.33.3) referring to the workflow of Miseq SOP (Schloss et al., 2009). The obtained representative sequences of the OTUs were taxonomically classified at different levels.

2.8. Statistical analysis

The significant analysis was performed with a One-way ANOVA program (software SPSS17.0), and $P < 0.5$ was considered as significant difference. Alpha diversity was analyzed on the basis of OTU (Chao/ACE/Coverage/Shannon/Simpson index) (Amato et al., 2013). Principal component analysis (PCA) based on the weighted UniFrac distance was carried out to determine the differences in microbiological communities composition (Ashhab et al., 2014). The similarities of bacterial community in the oyster samples were examined by overlapping the OTUs in a Venn diagram.

3. Results and discussion

3.1. Microbial culture

Bacterial counts of oyster gills were calculated on the storage day of 0, 2, 4, 6 and 8 (Fig. 1A, B, and C). The aerobic plate counts (APC) in the gills of the oysters from British Columbia (BCg), New Brunswick (NBg), and Prince Edward Island (PEIg) increased from initial 3.9, 3.1, and 4.5 log CFU/g to 7.8, 7.9, and 8.8 log CFU/g, respectively, after 8 days in refrigerated storage (Fig. 1A). The increase of APC in oyster gills during storage was similar to previous reports (Chen et al., 2017, 2013). Kim et al. (2002) suggested that APC limitation in the oyster deterioration could be defined at 7 log CFU/g. In this study, the APC counts of NBg, BCg, and PEIg on day 6, corresponding to the APC of 7.3, 7.1, and 8.3 log CFU/g, respectively, were higher than suggested limitation of 7 log CFU/g, although the oysters were still acceptable. This might be due to high initial bacterial counts in oyster gill tissue(Chen et al., 2017).

The counts of *Enterobacteriaceae* increased by 2.6 log for NBg, 3 log for BCg, and 2.8 log for PEIg, respectively, during 8 days in storage. The alterations of *Enterobacteriaceae* in gills of the oysters from three collection sites shared a similar trend. *Enterobacteriaceae*, such as *Salmonella* sp. and *Escherichia coli* that were the source of water contamination from human residues, were related to the hygiene-sanitary conditions of the oysters (Anderson Pereira et al., 2006). *Enterobacteriaceae* accumulation was observed accumulated in oyster gills by filtering cultivation water and grew during the storage period in this study.

LAB that are fermenting bacteria and growing in the environments

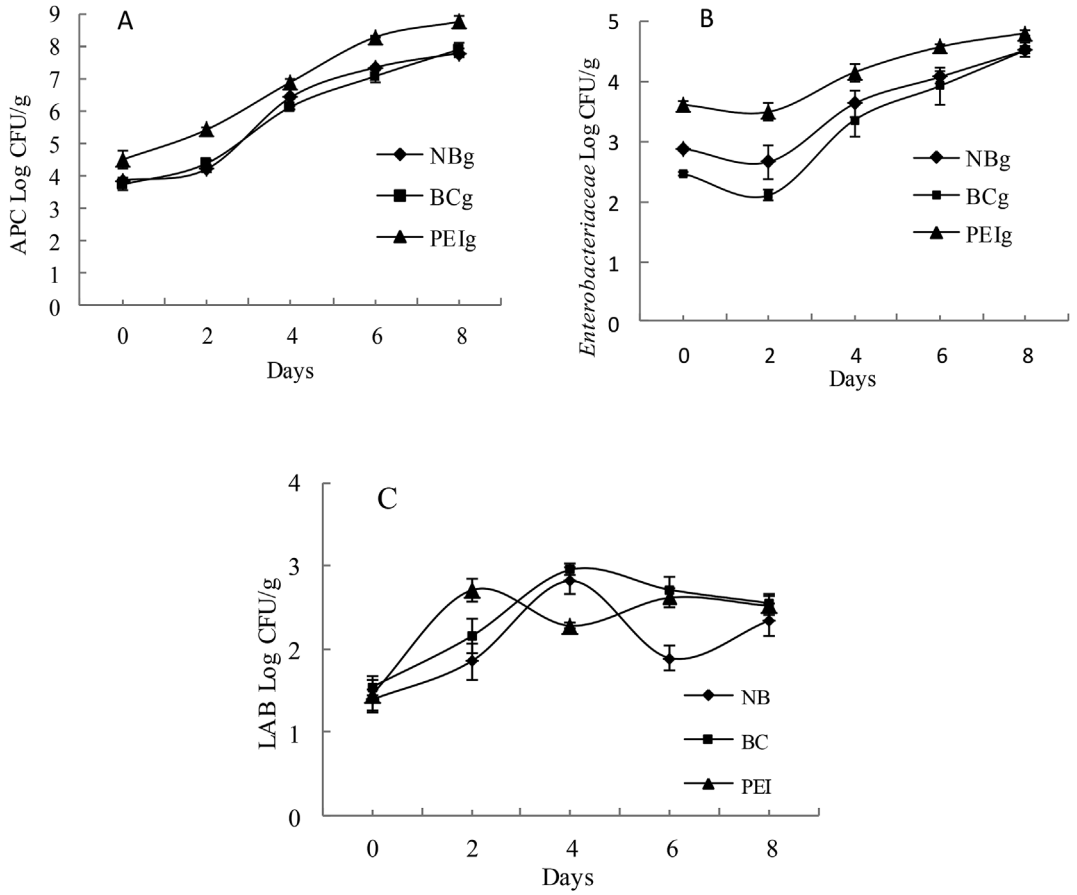

Fig. 1. Changes in APC (A), *Enterobacteriaceae* (B), and LAB (C) in the gills of NB, BC, and PEI oyster under refrigerated storage. Each data point and error bars were showed as mean ± SD. The legend abbreviation of BCg, NBg, and PEIg meant the gill of oyster from British Columbia, New Brunswick, and Prince Edward Island, respectively (the same below).

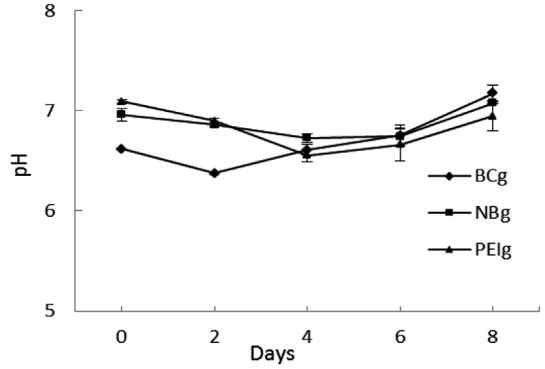

Fig. 2. The pH changes in BCg, NBg and PEIg during storage. Each data point and error bars were showed as mean ± SD.

where the sugars are present are frequently involved in food spoilage through producing a sour flavour and biogenic amines (Remenant et al., 2015). Slight variations of the LAB count were observed in the range of 1.4–3.0 log CFU/g (Fig. 1C) in the present study, suggesting that the LAB was not primarily responsible for the spoilage in oyster gills. Similarly, low LAB counts were also reported for Pacific and Sydney rock oysters during refrigerated storage (Madigan et al., 2014).

The changes in pH were closely related to the acidification caused by the glycogen fermentation of microorganisms and the decarboxylation or deamination of amino acid (Fernandez-Piquer et al., 2012; Madigan et al., 2014). The initial pH values of the three oysters were 6.62, 6.96, and 7.09 for BCg, NBg, and PEIg respectively, which suffered slight decreases after 2–4 days in storage and then increased with the prolonged storage time (Fig. 2). The change of the pH was similar to that found in Chen et al. (2013) study, which reported that the pH of oyster gills decreased from 6.36 to 6.03 in the first 4 days and then increased. On the 8th day of storage, the pH of the BCg, NBg and PEIg were 7.18, 7.08 and 6.95, respectively. The slight decrease and then increase might be related to the low LAB counts in this work. The pH change reported in oyster gills in this study differed from other reports for oyster that suggest that the pH only decreases or increases over the entire storage period (Cao et al., 2009; Madigan et al., 2014).

3.2. Sensory quality

The oysters' degree of freshness was evaluated via sensory scores (Fig. 3). The total scores slightly decreased during the first 4-day storage period and decreased dramatically after day 4 (Fig. 3), which demonstrated that the sensory qualities of oysters dropped rapidly after 4 days of refrigeration. The sensory scores of oysters from PEI and BC were lower than the desirable threshold score of 7.5, NB almost reached this threshold on the 8th day, suggesting the shelf-life of refrigerated oysters was no more than 8 days. It was observed that the scores of body colour and texture were suffered a slight and significant decline in the first four days and the last four days of storage, respectively (Fig. 3). On day 8, moulds appeared in the gill tissues of some tested oysters from BC. The results of sensory assessment also revealed that gill spoilage occurred earlier than spoilage in the other tissues, underlining the vital role of the gills in the spoilage of oyster during storage.

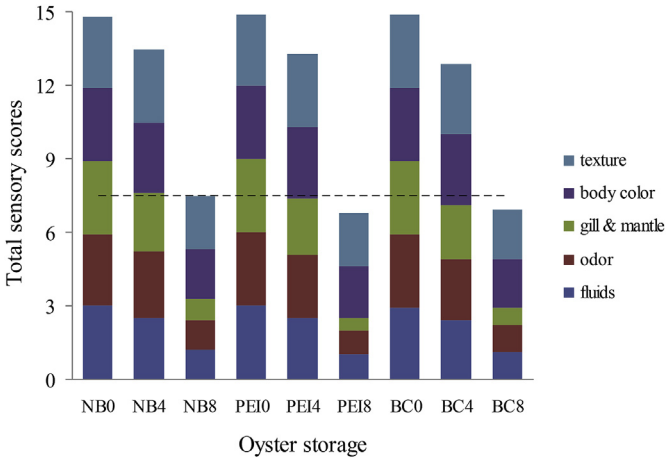

Fig. 3. The alterations of sensory qualities of oysters during refrigeration. Total sensory scores were assessed for refrigerated BC, NB and PEI oysters. Samples detected on the day of 0 (NB0, BC0, and PEI0), 4 (NB4, BC4, and PEI4), and 8 (NB8, BC8, and PEI8). The dash line represents the acceptable limitation scores of 7.5.

Table 2
Sequencing reads and Alpha diversity of the gills of oyster from different sites and storage (0, 4, and 8 days).

Samples	Reads	OTU	ACE	Chao	Coverage	Shannon	Simpson
BCg0	36699	153	156	157	99.98%	2.31	0.32
BCg4	33778	106	113	111	99.96%	2.06	0.25
BCg8	32552	74	127	91	99.94%	1.99	0.25
NBg0	22955	77	85	85	99.96%	1.66	0.37
NBg4	25479	82	128	115	99.90%	1.81	0.24
NBg8	31820	92	99	99	99.96%	1.84	0.27
PEIg0	32185	124	137	141	99.95%	2.97	0.14
PEIg4	33545	96	114	111	99.94%	2.19	0.17
PEIg8	35354	116	130	135	99.95%	2.31	0.20

3.3. Analysis of community diversity and richness

The bacterial diversity and structure of BCg, NBg, and PEIg were analyzed based on Miseq sequencing data. Over 22,000 effective sequence reads were obtained for each sample. The sequencing reads and alpha diversity are shown in Table 2. The OTUs of bacteria decreased from 153 to 74 in BCg during the entire storage period, which increased from 77 to 92 in NBg. The OTUs were 124, 96 and 116 from PEIg on the storage day of 0, 4 and 8, respectively. The Shannon and Simpson indexes are commonly used to represent the alpha diversity of a bacterial community. Both indexes jointly implied that high bacterial diversity in fresh and spoiled oyster gills was obtained (Table 2). The high value of ACE and Chao demonstrated the high bacterial community richness of oyster gills, although their values varied from fresh to spoiled gills. The coverage higher than 99.9% in all samples indicated that the sequences obtained in current study were sufficient to represent the actual bacterial community of samples. The OUT numbers combined with these species richness estimators presented the sufficient information for revealing the microbiota in fresh and spoiled oyster gills.

3.4. The alterations of oyster gills' bacterial community of the oyster gill during storage

The Venn diagram revealed that the total bacterial communities of the oyster gills from different sites shared 99 OTUs (Fig. 4A) in all sampling days. The BCg, NBg and PEIg had unique OTUs of 57, 7 and 59, respectively. The NBg and PEIg shared more OTUs than that of NBg and BCg after storage (Fig. 4A), which suggested that the both eastern oysters showed more similar bacterial communities in their gills. The two eastern oysters, however, collected from NB and PEI also processed distinct microbiota structures due to the differences in living environments. The result of PCA showed that the microbial communities of the oyster gills changed obviously after refrigeration (Fig. 4B). On day 8, this share of BCg, NBg and PEIg accounted for 42 OTUs and *Bacteroidetes* (11 OTUs) and *Proteobacteria* (25 OTUs) were the main bacterial phyla (Fig. 4C).

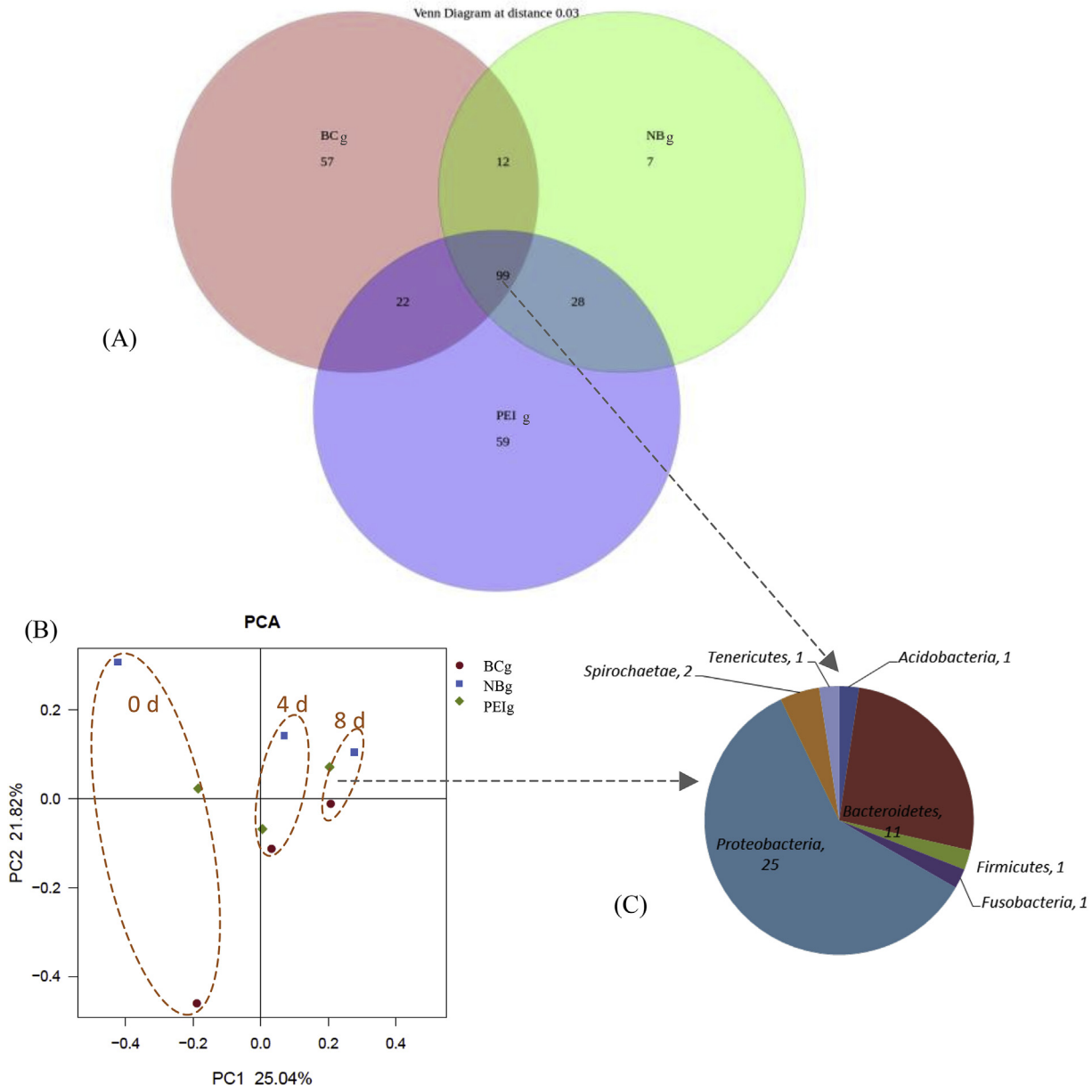

Fig. 4. Bacterial community and share OTUs analysis for the gills of oyster during refrigerated storage. (A) Venn diagram at distance 0.03 for bacterial community in the gill of oysters refrigerated for eight days. (B) PCA based on 16S rRNA gene sequencing at 21.82% and 25.04% of variation for the gill during storage. (C) Common OTUs in the gills of all oyster samples and share phyla on eight days. Arrow meaning is the common OTUs on day 8.

3.5. Microbial diversity in fresh oyster gill

The taxonomy of bacteria in fresh oyster gills at the phylum and class levels are shown in Fig. 5A and B. *Spirochaetes* (61.5%) and γ-*Proteobacteria* (12.3%) were the dominant dominated in the fresh BCg, while *Spirochaetes* (27.6%) and *Bacteroidia* (55.8%) in the fresh NBg, and *Spirochaetes* (33.9%), γ-*Proteobacteria* (18.2%), and *α-Proteobacteria* (21.6%) in the fresh PEIg (Fig. 5B). The main bacterial genera of the gills of fresh Pacific oysters were *Borrelia, Colwellia, Arcobacter* and *Sphingomonas*; and that of the gills of fresh Eastern oysters were *Marinifilum*, *Arcobacter*, *Spirochaeta, Sphingomonas, Bradyrhizobium*, *Caulobacter*, *Pelomonas*, *Psychrobacter*, *Pseudomonas* and *Bryobacter* (Table 3). Most of the above bacteria are the typical bacteria found in fresh oysters (Cao et al., 2010; Trabal et al., 2012; Wang et al., 2014a, b). *Spirochaeta*, *Borrelia*, *Sphingomonas*, and *Colwellia* were also reported to be highly abundant in the oyster gills with high abundance in previous studies (Fernandez-Piquer et al., 2012; Madigan et al., 2014; Wegner et al., 2013).

The bacterial community structures were compared between different species and harvested locations (Fig. 5 and Table 3). The differences in microbiota profiles were usually attributed to multiple factors such as environment, habitat, season, and specimen. Discrepancy in microbiota was verified in a study carried out by Trabal et al. (2012), which reported that the abundant bacteria in oysters (*C. gigas* and *C. corteziensis*) are affected by the conditions of cultivation sites and specimen. Trabal Fernández et al. (2014) also found high bacterial diversity in three harvested oysters (*C. corteziensis*, *C. gigas* and *C. sikamea*) and revealed the microbial profile differences among them. These distinct microbial profiles in fresh oyster gills in this study could be explained by the combined effects of culture environment and oyster species.

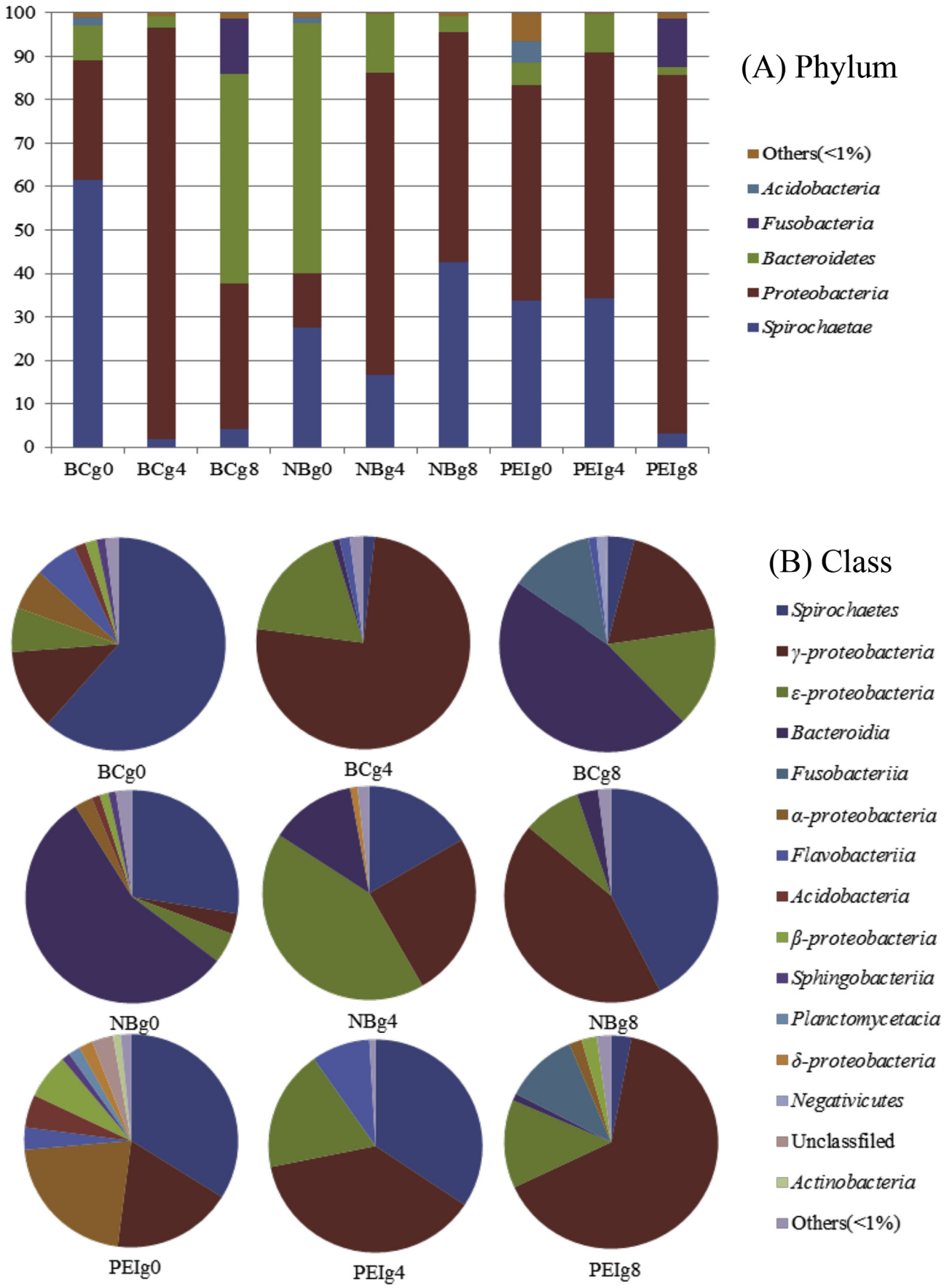

Fig. 5. Taxonomic classification of 16S rRNA gene sequencing of refrigerated BCg, NBg and PEIg at phylum (A) and class levels (B). The percentage abundance of bacteria less than 1% were labelled as others at phylum and class level.

Table 3
Taxonomic classification of the 16S rRNA gene sequences at genus levels for BC, NB, and PEI oyster gills.

Phylum	Family	Genus	BCg0	BCg4	BCg8	NBg0	NBg4	NBg8	PEIg0	PEIg4	PEIg8
Acidobacteria	*Acidobacteria*(class)[a]	*Bryobacter*	**1.1**[b]	0.1	-[c]	0.8	ND[d]	–	**1.7**	–	0.2
Actinobacteria	*Propionibacteriaceae*	*Propionibacterium*	–	–	ND	ND	–	–	0.9	–	0.1
Bacteroidetes	*Marinilabiaceae*	*Marinifilum*	0.1	0.1	0.2	**55.8**	**12.6**	**3.1**	ND	ND	–
Bacteroidetes	*Flavobacteriaceae*	*Polaribacter*	**1.4**	0.3	0.1	–	–	–	ND	–	ND
Bacteroidetes	*Flavobacteriaceae*	*Bizionia*	0.1	0.2	0.5	ND	0.1	–	–	**8.3**	0.1
Bacteroidetes	*Sphingobacteriales*(order)	*Sediminibacterium*	0.6	0.1	–	**1.1**	–	–	**1.3**	0.1	0.1
Fusobacteria	*Fusobacteriaceae*	*Fusobacterium*	ND	–	**12.5**	0.5	0.2	0.8	ND	ND	**11.1**
Proteobacteria	*Campylobacteraceae*	*Arcobacter*	**6.0**	**18.2**	**14.7**	**4.6**	**42.4**	**8.8**	0.6	**18.4**	**13.4**
Proteobacteria	*Sphingomonadaceae*	*Sphingomonas*	**2.1**	0.1	ND	0.8	0.2	0.1	**11.4**	–	0.4
Proteobacteria	*Bradyrhizobiaceae*	*Bradyrhizobium*	0.3	–	–	0.5	0.2	0.1	**3.3**	0.1	0.5
Proteobacteria	*Caulobacteraceae*	*Caulobacter*	0.1	–	ND	0.3	ND	–	**2.3**	–	0.1
Proteobacteria	*Comamonadaceae*	*Pelomonas*	**1.2**	0.2	–	0.9	0.4	0.1	**4.9**	–	0.2
Proteobacteria	*Colwelliaceae*	*Colwellia*	**6.4**	**2.4**	–	0.1	0.1	–	ND	0.1	–
Proteobacteria	*Pseudomonadaceae*	*Pseudomonas*	0.9	–	0.1	0.4	0.4	**1.9**	**1.8**	0.6	0.6
Proteobacteria	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	0.7	**45.4**	**8.7**	–	**5.5**	**29.0**	0.8	**11.4**	0.8
Proteobacteria	*Shewanellaceae*	*Shewanella*	0.7	**3.1**	0.1	ND	–	0.3	–	0.1	–
Proteobacteria	*Psychromonadaceae*	*Psychromonas*	0.3	0.1	0.6	–	–	**2.1**	–	**5.6**	**3.7**
Proteobacteria	*Oceanospirillaceae*	*Marinomonas*	–	–	ND	ND	0.1	**7.7**	0.1	**13.7**	0.7
Proteobacteria	*Vibrionaceae*	*Photobacterium*	–	0.3	0.1	–	**18.5**	**1.2**	0.4	0.9	0.7
Proteobacteria	*Aeromonadaceae*	*Oceanisphaera*	ND	0.1	**4.3**	ND	–	0.1	ND	0.3	**2.8**
Proteobacteria	*Polyangiaceae*	*Sorangium*	0.2	–	–	ND	ND	ND	**1.1**	–	–
Proteobacteria	*Moraxellaceae*	*Psychrobacter*	–	**13.8**	**3.5**	–	0.1	0.4	**11.3**	**3.1**	**53.9**
Proteobacteria	*Desulfobulbaceae*	*Desulfotalea*	ND	ND	0.1	0.3	**1.1**	ND	–	ND	ND
Spirochaetae	*Spirochaetaceae*	*Borrelia*	**61.4**	**1.3**	0.1	0.2	–	ND	0.1	ND	ND
Spirochaetae	*Spirochaetaceae*	*Spirochaeta*	–	0.3	**4.0**	**3.0**	**15.1**	**41.9**	ND	**33.5**	**3.0**
		Unclassfiled	**11.7**	**12.4**	**49.6**	**28.2**	**2.5**	**1.5**	**51.4**	**3.2**	**3.4**

[a] The family is not available and the class or order in the blanket instead.
[b] The bold highlighting means the main bacteria.
[c] "-" means the percentage less than 0.05%.
[d] ND means that the genus is not detected.

3.6. Analysis of the relative abundance of bacteria in the oyster gills during storage

The representative OTUs of the gill tissues were identified at different taxonomic levels (Fig. 5). At the phylum level (Fig. 5A), except for PEIg0, *Spirochaetae*, *Proteobacteria*, and *Bacteroidetes* accounted for more than 95% of the total phyla in all samples, which were previously identified as the three dominant phyla in either fresh or spoiled oysters by high throughput molecular methods previously (Madigan et al., 2014; Prapaiwong et al., 2009; Rong et al., 2018). On day 8, the dominant bacteria in the BCg were *Proteobacteria* (33.7%) and *Bacteroidetes* (48.2%) on the phyla level; *Bacteroidia* (47.0%), γ-*Proteobacteria* (18.6%), and *ε-proteobacteria* (15.0%) on the class level; and *Marinilabiaceae*, *Campylobacteraceae*, and *Fusobacteriaceae* on the family level (Table 3). The dominant bacteria were *Spirochaetae* (42.5%) and *Proteobacteria* (53.2%) on the phyla level; *Spirochaetes* (42.45%) and γ-*Proteobacteria* (43.5%) on the class level; and *Spirochaetaceae* and *Pseudoalteromonadaceae* on the family level in NBg. The dominant bacteria in PEIg were classified as *Proteobacteria* (82.3%) and *Fusobacteria* (11.1%) on the phylum level; γ-*Proteobacteria* (65.0%), *ε-Proteobacteria* (13.4%) and *Fusobacteria* (11.1%) on the class level; and *Moraxellaceae*, *Campylobacteraceae*, and *Fusobacteriaceae* on the family level (Table 3). The relatively high prevalence of *Enterobacteriaceae*, which belong to class γ-*Proteobacteria*, was observed in in vitro cultivation. This result was consistent with the metagenomic results that showed that the γ-*Proteobacteria* was dominant in the three sites' oysters in our study. Interestingly, *Escherichia coli* was the only species of *Enterobacteriaceae* appearing in the sequencing result and was not the dominant species. This bias might be explained by the growth of other bacteria of γ-*Proteobacteria* that could grow other bacteria mentioned in the protocol precaution of media specific.

The bacterial communities in BCg8, PEIg8, and NBg8 became similar as the gills deteriorated (Table 3, Fig. 4). Although the predominant spoilage bacterial structures in the gill of two oyster specimens (*C. gigas* and *C. virginica*) from the three sites were somewhat different, the dominant spoilage OTUs were closely related to *Arcobacter*, *Spirochaeta*, *Pseudoalteromonas*, *Marinomonas*, *Fusobacterium*, *Psychrobacter*, *Marinifilum*, *Psychromonas*, and *Oceanisphaera* (Table 3), most of which were nonfermenting gram-negative bacteria. Nonfermenting gram-negative bacteria were previously reported to be vital in the contribution of oyster spoilage, and most of them were identified as special spoilage organisms (SSOs). Previously reported SSOs or dominant spoilage bacteria in oyster or oyster tissues under low temperature storage are summarized in Supplementary Table 1. The proportion of these bacteria such as *Shewanella*, *Vibrio*, *Photobacterium* and *Pseudomonas*, known as typical SSO in seafood (Gram and Huss, 1996) and reported as dominant in spoiled oyster (Cao et al., 2010; Prapaiwong et al., 2009), were detected at low abundance in day 8 samples in the present study. This finding was similar to findings from Madigan et al. (2014), who described the low abundance of *Shewanella*, *Photobacterium* and *Pseudomonas* were observed in the spoiled Pacific oyster and Sydney rock oysters stored at refrigerated conditions. This might be related to the low abundance of *Shewanella*, *Vibrio*, *Photobacterium* and *Pseudomonas* in fresh oyster gills.

The proportion of *Marinifilum*, *Bradyrhizobium*, *Caulobacter*, *Pelomonas*, *Borrelia*, and *Colwellia* decreased dramatically during storage. It is worth noting that *Colwellia*, psychrotrophic anaerobic bacterial, were dominant in the gills of fresh oysters, but they were not the dominant spoilage bacteria in the gills after 8 days in storage at 4 °C. This result was different from those of previous studies showing that this anaerobe can survive under psychrophilic conditions and can be considered as the spoilage bacteria in oysters (Gram and Huss, 1996; Madigan et al., 2014). This discrepancy might due to the fact that *Colwellia* can survive in the oyster tissues other than gills during refrigerated storage. The decreasing of the other bacteria that were rarely reported as dominant spoiled bacteria in oysters could be attributed to two reasons: one is their poor low-temperature tolerance (Gram, 2009) and their poor competitiveness with some bacteria such as *Arcobacter*, *Pseudoalteromonas*, *Fusobacterium*, and *Spirochaeta* (Madigan et al., 2014; Rong et al., 2018).

Arcobacter, which has been reported to be growing at relatively lower temperatures and aerobic conditions (Kjeldgaard et al., 2009), was identified as being abundant in all current tested gill samples. Similar results were described in previous reports that this bacterium has high abundance in fresh and spoiled Pacific oysters (at 4 °C or 30 °C storage) (Fernandez-Piquer et al., 2012; Madigan et al., 2014). *Arcobacter* spp. are prevalent in food and some of them from shellfish are considered human pathogens (Collado et al., 2009). In terms of the high percentage of *Arcobacter* in fresh and stored oysters, its risk should be further assessed in future studies.

Psychrotrophic microorganisms related to oyster spoilage in low-temperature storage have been examined through the pour plate method at refrigerated incubation for 7–10 days, and high bacterial counts were obtained (Cruz-Romero et al., 2008b; Linton et al., 2003). Owing to the difficulty and time-consuming nature of psychrotroph cultivation, few studies have been carried out on spoiled oysters using culture methods. Only recently was the use of high throughput molecular methods such as Highseq and 454 pyrosequencing proven to be an effective method for the observation of these bacteria (Supplementary Table 1). In this study, the psychrotrophic genera *Psychrobacter* and *Psychromonas* were also confirmed as dominant bacteria in the gills of the spoiled oysters at 4 °C storage through Miseq sequencing. *Psychrobacter* has been reported to be able to produce volatile compounds such as TMA, 2-butanone, and acetone, resulting in seafood spoilage under ice or refrigerated storage (Broekaert et al., 2013).

Pseudoalteromonas are able to produce TMA, H_2S, ammonia, and acetic acid, which are closely related to poor seafood quality (Broekaert et al., 2013). Fernandez-Piquer et al. (2012) stated that *Pseudoalteromonas* was not detected in Pacific oysters stored at 4 °C, 15 °C and 30 °C. In contrast, Romero et al. (2002) claimed that *Pseudoalteromonas* can grow to be the dominant bacteria under 18 °C storage. Madigan et al. (2014) and Rong et al. (2018) reported that multiple *Pseudoalteromonas* OTUs were also detected in Pacific oyster stored at 4 °C. The growth of *Pseudoalteromonas* in spoiled oyster was verified in our experiment. The highly competitive proliferation of this bacterium was observed in most of the gill samples on day 4 and day 8.

In addition, *Spirochaeta* was observed in all gill samples at the end of their shelf life. This genus has been observed in spoiled oysters or oyster gills (Chen et al., 2017; Fernandez-Piquer et al., 2012). Due to the high presence of this genus in spoiled seafood, the mechanism of spoilage resulting from *Spirochaeta* must be clarified in subsequent studies. Some distinguishing dominant bacteria in different oyster species were observed. For example, *Marinomonas* only dominated in NBg8 and PEIg4, and has been identified to be high abundance in the live oyster *C.hongkongensis* (Wang et al., 2014a, b); *Marinifilum* accounted for approximately 50% of the identified OTUs in NBg0 and its abundance decreased to 3.1% after 8 days in storage. Meanwhile, the LAB was not detected as the dominant bacteria using Illumina Miseq sequencing in our experiment, which is consistent with culture method results that could explain why the pH value did not decrease through entire storage period for oyster gills.

In this study, high microbial diversity and clear spoilage bacteria genera were observed in the gills of oysters from three-site oysters using Illumina Miseq sequencing; however, their role, mutual function, and association with specific biochemical indicators of gill deterioration or oyster spoilage have yet to be elucidated. Therefore, future work should focus on the effects of harvest site, specimen and storage conditions on gill microbiota. Further studies should also examine SSO or microbiological indicators of isolates and determine the safety risk of potential pathogens from oyster gills.

4. Conclusions

Illumina Miseq sequencing analysis provided useful insights into the microbiological spoilage in the gills of shucked Pacific oysters (from BC) and eastern oysters (from NB and PEI) at 4 °C ± 1 °C storage. The spoilage bacterial communities became similar for oysters from different sites when the gills deteriorated. The dominant bacteria in the spoiled oyster gills under refrigerated storage were *Arcobacter*, *Spirochaeta*, *Pseudoalteromonas*, *Marinomonas*, *Fusobacterium*, *Psychrobacter*, *Psychromonas*, and *Oceanisphaera* under refrigerated storage. To improve the oyster quality and safety, the controlling of psychrotrophic spoilers and potential pathogens such as *Arcobacter* in gill is vital in the refrigerated storage of oysters.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.02.008.

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