Food Research International 121 (2019) 568–576

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Spoilage microbial community profiling by 16S rRNA amplicon sequencing of modified atmosphere packaged live mussels stored at 4°C

Olumide A. Odeyemi[a,*], Christopher M. Burke[a], Christopher C.J. Bolch[a], Roger Stanley[b]

[a] Ecology and Biodiversity Centre, Institute for Marine and Antarctic Studies (IMAS), University of Tasmania, Launceston, Australia
[b] Centre for Food Innovation, Tasmania Institute of Agriculture (TIA), University of Tasmania, Launceston, Australia

ARTICLE INFO

Keywords:
Depuration
Microbiota
Modified atmosphere packaging
Spoilage

ABSTRACT

There is little information on the microbial communities associated with modified atmosphere (MA)-packaged live mussels. There is also a dearth of information on how pre-packaging depuration modifies the microbial communities and spoilage of live mussels. Amplicon sequencing was used to describe spoilage microbial succession in MA-packaged live mussels during storage at 4 °C. Proteobacteria, Cyanobacteria and Firmicutes were the three major phyla observed in the mussel meat and pouch water of undepurated and depurated mussels. Among these phyla, Cyanobacteria was more predominant on day 0 in mussel meat of undepurated and depurated mussels while Proteobacteria was predominant in commercially-depurated mussels. *Synechococcus* was apparently dominant on days 0–7 in the meat of undepurated mussels and days 0–10 in depurated mussels. *Shewanella* was dominant on day 0 in commercially-depurated mussels and dominant on day 15 in undepurated while *Acidaminococcus* was dominant in depurated mussels on day 15. *Psychromonas* was observed to be dominant in commercially-depurated mussels on day 7 and further shifted to *Acinetobacter* by day 10 and 15. In the pouch water, *Acinetobacter* was dominant throughout the storage days in undepurated mussels while *Psychrobacter* was predominant in both depurated and commercially-depurated mussels. This study demonstrated the impact of depuration on the microbiota and the spoilage mechanism of MA-packaged live mussels. *Shewanella* was easily removed through depuration. However, spoilage bacteria such as *Acidaminococcus* could not be easily removed although they are not important at the beginning but grew at the end. Pouch water contributed suitable biological medium for the growth of *Acinetobacter* and *Psychrobacter* and both enhanced the growth of spoilage bacteria such as *Shewanella* and *Acidaminococcus*.

1. Introduction

There is an increasing demand for live mussels due to freshness and palatability. Modified atmosphere (MA) packaging that involves the change of gas composition in a product has been used for the preservation of live mussel till consumption (Pastoriza, Bernárdez, Sampedro, Cabo, & Herrera, 2004). Farming environment, post-harvest handling and storage impact the microbiota of shellfish such as mussels (Lee, Lee, Chung, Choi, & Kim, 2016). Depuration is one of the post-harvest processing methods applied to shellfish (Barile et al., 2009). It involves allowing the shellfish to purge themselves of gut content and associated microorganisms in static seawater over a period of time prior to MA-packing (Ramos et al., 2012). However, during storage, changes in the metabolic state of the shellfish, microbial community and/or physiochemical conditions within the MA-products lead to spoilage (Lee et al., 2016).

Culture-dependent methods such as agar culturing and biochemical characterisation have been used for microbial analysis of mussels (Caglak, Cakli, & Kilinc, 2008; Goulas, Chouliara, Nessi, Kontominas, & Savvaidis, 2005; Pastoriza et al., 2004). However, these methods could not give a complete understanding of microbial communities succession during storage due to the limitations such as the preferential growth of certain bacteria over others thereby underestimating the microbial diversity and or provide an incomplete view of the microbial communities that contributes to spoilage of the product (Parlapani & Boziaris, 2016; Powell & Tamplin, 2012). To overcome these limitations, culture-independent methods are often used. For example, Fernandez-Piquer, Bowman, Ross, and Tamplin (2012) used terminal restriction fragment length polymorphism (T-RFLP) targeting 16S rRNA gene to investigate the impact of postharvest storage temperature (4–30 °C) on the microbial community in live Pacific oysters (*Crassostrea gigas*). They reported high microbial diversity and that *Psychrilyobacter*, a genus from the

* Corresponding author.
E-mail address: olumide.odeyemi@utas.edu.au (O.A. Odeyemi).

https://doi.org/10.1016/j.foodres.2018.12.017
Received 19 June 2018; Received in revised form 10 December 2018; Accepted 12 December 2018
Available online 13 December 2018
0963-9969/

phylum Fusobacteria was more dominant at 4 °C while Bacteroidetes were dominant at 30 °C indicating the effect of storage on microbial diversity. Madigan et al. (2014) reported the use of V1 - V3 regions of the 16S rRNA gene to study the spoilage microbiota of half-shell oysters stored at 4 °C. They observed that *Pseudoalteromonas* and *Vibrio* from the phylum Proteobacteria were the dominant genera at the end of storage.

Several studies have concluded that packaging and storage conditions play significant roles in the microbial diversity of shellfish (Fernandez-Piquer et al., 2012; Madigan et al., 2014). However, there is little information on the microbial communities associated with MA-packaged live mussels or how pre-packaging depuration modifies the microbial communities and spoilage. This study, therefore, aimed to use 16S rRNA amplicon sequencing (Next Generation Sequencing – NGS) to investigate microbial communities' succession of MA-packaged depurated and undepurated live mussels stored at 4 °C for 15 days.

2. Materials and methods

2.1. Sample collection

Freshly harvested mussels (*Mytilus galloprovincialis*) were collected from a commercial mussel farm in the east coast of Tasmania, Australia. The live mussels were harvested from 10 to 15 m deep (Lat: 42° 32.2021′, Long: 147°58.7809′) and were of standard commercial size (average weight: 21.91 ± 0.52 g; average length: 8.25 ± 0.61 cm and average width: 3.73 ± 0.25 cm) with slightly brittle shells. Water temperature at harvest was 13.1 ± 0.14 °C. The mussels were washed, cleaned and debearded before grading and then transported into the laboratory in iced Styrofoam boxes. It should be noted that commercially-packaged mussels (1 kg) were depurated for 1–2 h and packed with an average of 35–40 individual mussels compared with 6–8 mussels (250 g) of undepurated and depurated packs that were packed in the laboratory.

2.2. Mussel packaging and storage

The mussels were divided into two sub-samples. The first sub-sample was subjected to 8 h depuration overnight in an aerated static tank at 4 °C. Thereafter, 100 mL of sterile fresh water was added to 6–8 mussels in 9 × 7 × 40 cm (BT97/40) black barrier tray (Alto packaging Ltd., Australia) and then transferred to a semi-automatic MA-packaging machine (Multivac T100, Germany) that was equipped with vacuum pump for air flushing before filling with pre-set headspace oxygen gas composition – 80% O_2 and 20% N_2 (Food grade, BOC, Australia). Sealing was carried out at 140–142 °C. The oxygen transmission rate (OTR) of the cover film was 22 cc/m^2/day/atm at 23 °C with 0% relative humidity (RH) and the water vapour transmission rate (VPR) was 22 g/m^2/day/ at 37 °C with 90% RH. The second sub-sample was not depurated but stored at 4 °C for 8 h and then packed like the depurated mussels. All the samples (treatments) were stored at 4 °C for 15 days.

2.3. Microbial diversity profiling

The MA - packaged mussels and pouch water were sampled on day 0, 4, 7, 10 and 15. Random triplicate packs of both treatments (depurated and undepurated) and commercially-packed samples were opened and sacrificed at each time point. Samples for NGS-based microbial diversity profiling using 16S rDNA sequencing were collected at the same time. Briefly, 20 g of mussel meat was homogenised with 180 mL sterile alkaline peptone water for 60 s. The pouch water was mixed before collecting 40 mL in a sterile Falcon tube (50 mL). Meat and pouch water samples were immediately frozen and stored at −80 °C until further analysis. The genomic DNA was extracted using a bead milling step with the PowerLyzer Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) following manufacturer's instruction and as used by Nielsen et al. (2018). The polymerase chain reaction (PCR) - based quality control (QC) was performed on the extracted DNA samples to ascertain sufficiency of at least 30,000 reads. Sample with insufficient reads was re-extracted from the supplementary sample. The DNA extraction, PCR amplification and sequencing were performed by the Australian Genome Research Facility, Queensland.

The V1- V3 hypervariable and flanking regions of the 16S rRNA gene was amplified using primers 27F (AGAGTTTGATCMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG) (Madigan et al., 2014). AmpliTaq Gold 360 master mix (Life Technologies, Australia) was used for the primary PCR amplification based on 29 cycles of initial heating at 95 °C for 7 min, dissociation at 94 °C for 45 s, annealing at 50 °C for 60 s, extension at 70 °C for 60 s and finished at 72 °C for 7 min. A secondary amplicon indexing PCR (TaKaRa Taq DNA Polymerase - Clontech) was also used. The amplicon concentration was then normalised by fluorometry (Invitrogen Picogreen) and combined into equimolar pools prior to sequencing on an Illumina MiSeq instrument (San Diego, CA, USA) with 2 × 300 bp paired-end chemistry. Image analysis was performed in real-time by the MiSeq Control Software (MCS) v 2.6.2.1 and Real Time Analysis (RTA) v1.18.54. Then, Illumina bcl2fastq 2.19.1.403 pipeline was used to generate the sequence data. Paired-end reads were assembled by aligning the forward and reverse reads using PEAR, version 0.9.5 (Zhang, Kobert, Flouri, & Stamatakis, 2014).

2.4. Bioinformatics

Priming sequences were identified, trimmed and processed using Quantitative Insights into Microbial Ecology - QIIME 1.8 (Caporaso et al., 2010), USEARCH, version 8.0.1623 (DeSantis et al., 2006; Edgar, 2010) and UPARSE software (Edgar, 2013). The sequences were clustered followed by chimera filtering using “RDP_gold” database as a reference. Reads in each operational taxonomic unit (OTU) were mapped back to OTUs with a minimum identity of 97% (Liu & Tong, 2017). Taxonomy was assigned using the Greengenes database, version 13_8 (DeSantis et al., 2006). Venn diagram was used to show shared and unique OTUs (Li, Li, Qu, & Wang, 2017). Heat-maps were constructed to visualize the change in the microbial communities over the storage time (Zhang & Wang, 2017).

2.5. Diversity analyses

The microbial species richness, diversity indices (Chao 1 estimator and Shannon index) and rarefaction curves were analysed using QIIME (Caporaso et al., 2010; Song et al., 2016). The dataset for metagenomic sequencing was deposited in Sequence Read Archive (SRA) database and are available under NCBI Submission-ID: SRA accession: SRP 144357, BioSample accession: SAMN 09011654 and Bio Project ID: PRJNA 454672. (https://www.ncbi.nlm.nih.gov/bioproject/454691).

2.6. Statistical analysis

To identify the main taxa that are changing in the samples, two-way analysis of variance (ANOVA) based on Tukey's multiple comparison tests (data not normally distributed) was carried out at 95% confidence interval (Xia & Sun, 2017). The analysis was carried out using GraphPad Prism 7.05 version.

3. Results

3.1. Operational taxonomic units (OTU) and taxonomic species richness in mussel meat

The rarefaction curves of mussel meat from undepurated mussels at the sampling points indicated sufficient sampling reads (Fig. 1A). Day 0 had the highest microbial diversity. Days 7 and 10 showed similar microbial diversity but lower diversity was observed on day 15. The

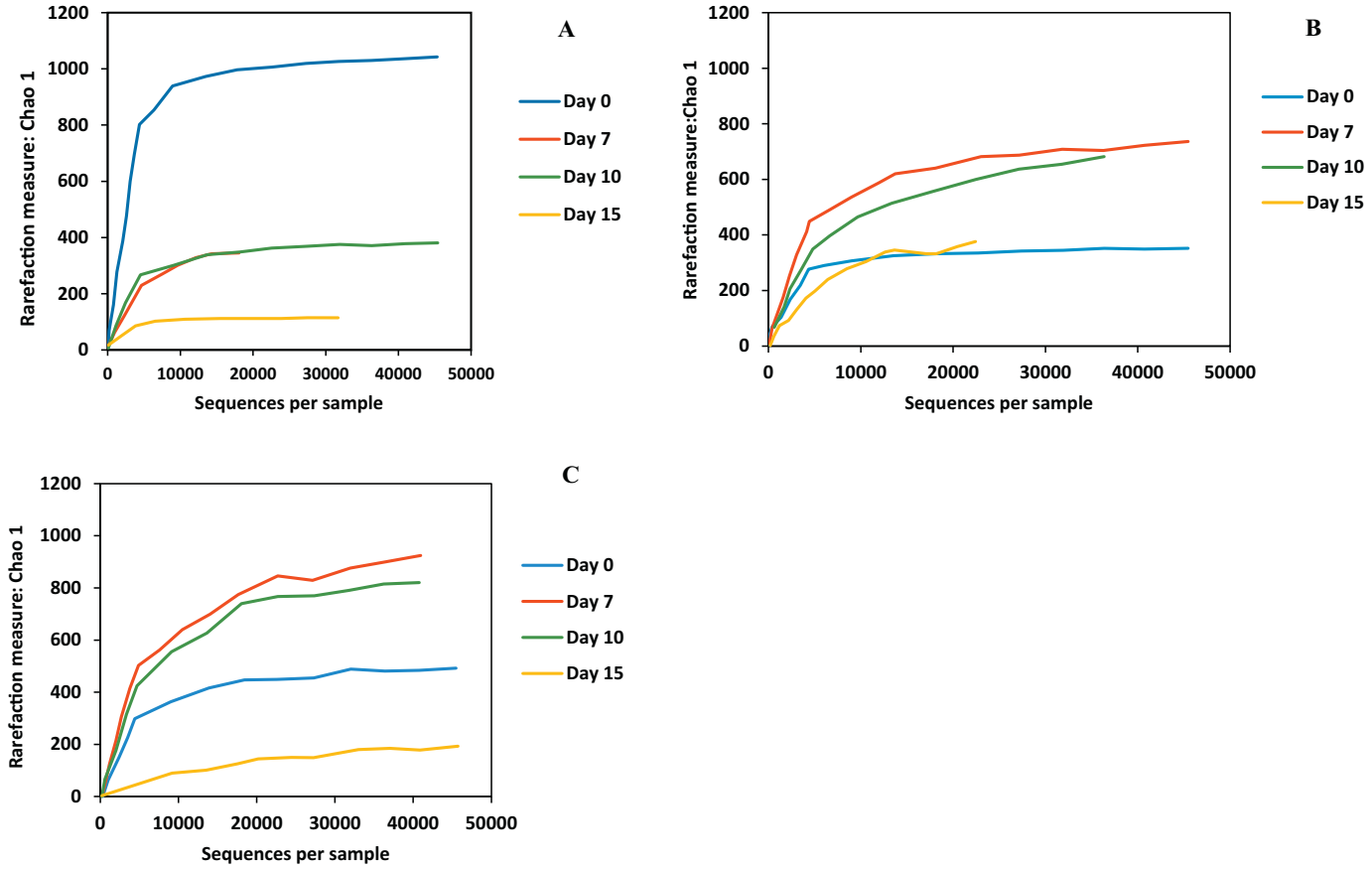

Fig. 1. Rarefaction curve indicating the number of OTUs in mussel meat of undepurated (A), depurated (B) and commercially-depurated mussels (C) based on V1-V3 of 16S rRNA gene sequencing at > 97% sequence similarity. Different colours indicate sampling days. The Chao1 estimator (y- axis) indicates the number of OTUs (taxa) while (x-axis) represents the population of bacteria in the sample.

Table 1
Operational taxonomic units (OTU) and species richness estimation (at minimum identity of 97% similarity) of mussel meat of undepurated, depurated and commercially-depurated mussels stored at 4 °C for 15 days.

Sample	Storage days	Sequences (n)	Average length (bp)	Reads (n)	Chao1 estimator	Shannon index
Undepurated	Day 0	220,470	494	90,291	1059	5.59
	Day 7	94,073	530	24,422	346	2.34
	Day 10	117,356	518	47,926	380	2.75
	Day 15	117,828	527	35,424	110	2.78
Depurated	Day 0	2,353,634	488	114,090	373	3.78
	Day 7	134,084	505	55,332	768	3.79
	Day 10	101,671	498	39,474	718	3.74
	Day 15	139,290	536	24,469	398	2.06
Commercial	Day 0	102,907	512	37,040	392	2.71
	Day 7	81,062	514	23,462	715	4.37
	Day 10	90,623	517	24,501	639	4.62
	Day 15	89,328	526	37,294	182	1.33

curves on days 0, 7 and 15 in depurated mussels plateaued, indicating enough sampling reads. When compared with undepurated mussels, there were fewer OTUs and diversity in depurated mussels on day 0. The curve on day 10 was still approaching a plateau, indicating insufficient sampling reads of the different bacteria in the original population (Fig. 1B). Microbial communities observed on days 7 and 10 were diverse but that of day 7 was more diverse than day 10. In the commercially-depurated mussels, the curves showed a similar pattern to depurated mussels (Fig. 1C). Day 15 levelled off with fewer OTUs when compared with depurated mussels but higher than undepurated mussels. The total valid sequence reads obtained in the mussel meat was 549,727 in undepurated mussels (Table 1). Depurated and commercially-depurated mussel meat showed lower initial taxon richness and diversity, increased taxon richness at day 7, declining on day 10 and day 15. Diversity was lowest on day 15 in both depurated samples (Table 1).

3.2. Operational taxonomic units (OTU) and taxonomic species richness in pouch water

The rarefaction curve of pouch water from undepurated mussels plateaued on day 0 indicating sufficient sampling reads while day 15 had lowest sequences per sample (Supplementary Fig.1A). Day 0 had the most OTUs while day 7 showed fewest OTUs. An increase in the OTUs was observed on day 10 but slightly decreased on day 15. In the depurated mussels, the pattern of curves showed similar numbers of

Table 2
Operational taxonomic units (OTU) and species richness estimation (at the minimum identity of 97% similarity) of pouch water of undepurated mussels, depurated mussels and commercially-depurated mussels stored at 4 °C for 15 days.

Sample	Storage days	Sequence (n)	Average length (bp)	Reads (n)	Chao1 diversity	Shannon index
Undepurated	Day 0	119,996	512	43,690	1279	4.71
	Day 7	83,986	525	40,953	70	1.09
	Day 10	89,643	524	31,108	132	2.00
	Day 15	57,907	530	14,236	79	2.42
Depurated	Day 0	69,410	524	25,625	120	1.57
	Day 7	71,753	525	31,428	93	1.28
	Day 10	138,138	522	52,222	111	1.79
	Day 15	100,653	524	39,776	118	1.76
Commercial	Day 0	72,105	524	25,798	250	1.23
	Day 7	86,686	525	37,308	335	1.26
	Day 10	127,402	523	45,230	303	2.09
	Day 15	94,830	530	23,176	208	2.85

OTUs except that the curve did not plateau on day 0 and day 7 compared to other days (Supplementary Fig.1B). Unlike other samples, the curves in commercially-packed mussels did not plateau (Supplementary Fig.1C). In the pouch water of undepurated mussels, a total of 351,532 valid sequence reads were obtained (Table 2). Microbial community richness was least on day 7 and highest on day 0. Microbial communities was least diverse on day 7 and highest on day 0 as shown by the Shannon index.

The pouch water of both depurated and commercially-depurated mussel showed lower initial taxon richness and diversity, decreased taxon richness on day 7 in depurated but increased in commercially-depurated mussels, increasing till the end of storage in depurated but declining on day 10 and day 15 in commercially-depurated mussels. Diversity increased with storage in both treatments and was highest on day 15 in commercially-depurated mussels.

3.3. Bacterial community diversity and succession in mussel meat at phylum and genus levels

In the undepurated mussel meat samples on day 0, 22 phyla were observed but decreased to 15 on day 7 and subsequently remained approximately the same number (Table 3). Most phyla detected were unique to each sampling day. Only 1–3 shared phyla between sampling days for undepurated mussel meat samples was observed (Fig. 2A). There was no OTU shared across all sampling days at the phylum level in the depurated mussels (Fig. 2B). In the commercially-depurated mussels, few phyla were shared among some of the sample days, but mostly the phyla were unique to the sample day, with the total number of phyla declining with storage (Fig. 2C).

The heat-map shows the change in microbial communities' structure in the mussel meat at the phylum level during storage of live mussels as shown by the colour intensity of relative abundance (Fig.3). In the meat of undepurated mussels, a total of 12 known phyla were observed (Fig.3A). Among these phyla, 5 (Proteobacteria, Cyanobacteria, Actinobacteria, Planctomycetes and Bacteroidetes) occurred throughout the storage period. Fig. 3B shows the relative abundance of identifiable phyla in depurated mussels. Unknown phyla represented only 0.27–1.35% of the community present in depurated mussel meat across the storage period. Microbiota found in mussels after depuration (day 0) had a different group of dominant phyla than did the mussels during storage. A total of 11 known phyla were observed in the mussel meat of commercially-depurated mussels namely: Proteobacteria, Cyanobacteria, Bacteroidetes, Planctomycetes, Firmicutes, Tenericutes, Fusobacteria, Actinobacteria, Chloroflexi, Verrucomicrobia, Spirochaetes and Acidobacteria (Fig. 3C). A complete shift in dominant phylum was observed on day 15. Proteobacteria became dominant in undepurated mussels and Firmicutes in depurated mussels. However, Proteobacteria remained increasingly dominant in commercially-depurated mussels from the start to the end of storage.

Table 3
The distribution of OTUs at different levels in the mussel meat of undepurated, depurated and commercially-depurated mussels stored at 4 °C for 15 days.

Sample	Storage days	Phylum	Class	Order	Family	Genus
Undepurated	Day 0	22	55	105	177	243
	Day 7	15	29	52	84	118
	Day 10	16	34	64	102	136
	Day 15	14	25	39	54	64
Depurated	Day 0	16	37	71	113	144
	Day 7	19	48	94	155	210
	Day 10	17	40	76	127	172
	Day 15	13	32	57	89	116
Commercial	Day 0	25	33	62	95	124
	Day 7	18	40	79	128	172
	Day 10	17	39	74	116	158
	Day 15	13	21	33	49	54

The heat-map (Fig. 4) shows the change in microbial community structure in the mussel meat at the genus level during storage of live mussels as shown by the colour intensity of relative abundance. A diverse microbial community was observed in the undepurated mussel meat (Fig. 4A). A total of 37 genera from the following 9 phyla were identified: Actinobacteria (3 genera), Bacteroidetes (4 genera), Cyanobacteria (1 genus), Firmicutes (4 genera), Fusobacteria (1 genus), Planctomycetes (1 genus), Proteobacteria (18 genera), Tenericutes (1 genus) and Verrucomicrobia (4 genera). The changes in microbial community composition of the meat of depurated mussels at genus level during storage are shown by the heat map in Fig. 4B. Twenty-one known genera from 7 phyla (Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria and Tenericutes) were observed in the meat of commercially-depurated mussels (Fig. 4C). In comparison, *Synechococcus* was observed to be changing in the mussel meat of depurated and undepurated mussels. In both samples, there was a significant difference ($p < .05$) in the occurrence of *Synechococcus* between day 0 and 15. *Acidaminococcus* was significantly different ($p < .05$) on day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15 in the depurated mussel meat. However, *Shewanella*, was significantly different ($p < .05$) on day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15 in the undepurated mussel meat. In the meat samples of the commercially depurated mussels, *Acinetobacter* was significantly different ($p < .05$) on day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15.

3.4. Bacterial community diversity and succession in pouch water at phylum and genus levels

In the pouch water, the least number of OTUs in undepurated mussels was on day 0 and day 7 in both depurated and commercially-depurated mussels (Table 4). Four OTUs were shared in the pouch water of undepurated mussels across the sampling days (Supplementary

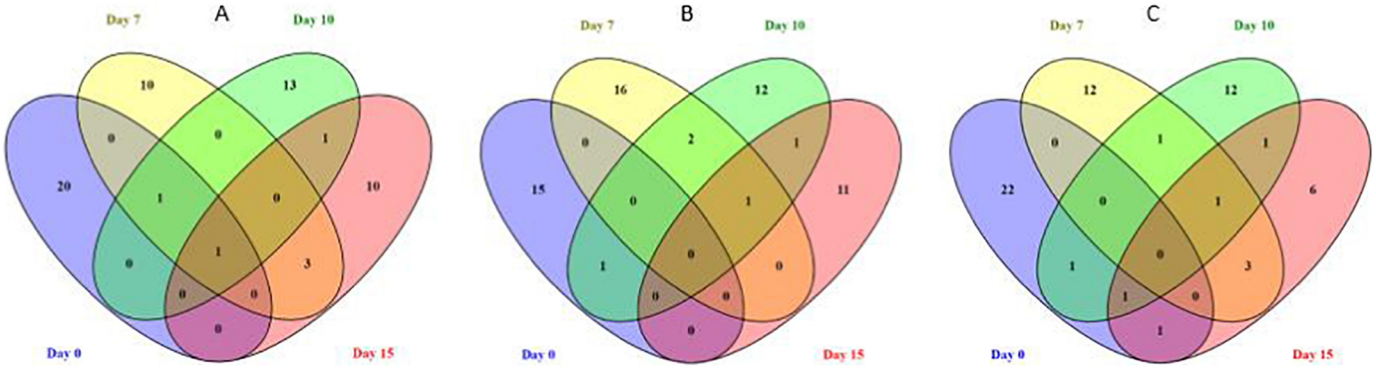

Fig. 2. Venn diagram showing the distribution (97% similarity) of the OTUs at phylum level present in the mussel meat samples of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. Each number indicates shared or unique OTUs in the samples in each sampling day. Different colour indicates different sampling days (day 0 = blue, day 7 = yellow, day 10 = green and day 15 = pink).

Fig.2A). Only a single OTU (Proteobacteria) was shared in depurated mussels throughout the sampling days at phylum level (Supplementary Fig.2B) unlike 4 in undepurated mussels. In the pouch water of commercially-depurated mussels, 2 OTUs were shared in all the sampling days (Supplementary Fig. 4C) almost similar to depurated (1 OTU) but different from undepurated mussels (4 OTUs). The OTUs obtained at other levels are summarised in Table 4 and clearly, there were far fewer OTUs in the pouch water than in the mussel meat. Overall, the number of OTUs in mussel meat were more than those in pouch water. Across the storage time, 12 known phyla were observed in the pouch water of undepurated mussels (Supplementary Fig.3A). However, only 11 phyla were prevalent on day 0 among which Proteobacteria was the most dominant (79%). The relative abundance of known phyla present in the pouch water of depurated mussels represents 98% of all the phyla (Supplementary Fig.3B). A total of 4 dominant phyla comprising of Cyanobacteria, Proteobacteria, Tenericutes and Firmicutes were observed on day 0. This was less than the total known phyla observed in the undepurated mussels. Eight phyla were observed to be dominant in pouch water of commercially-depurated mussels across the storage days (Supplementary Fig.3C). Only two phyla were dominant on day 0 and 7. However, more phyla were observed on days 10 and 15. Proteobacteria was more dominant (99%) on day 0, day 7 (99%), day 10 (98%) and 73% on day 15. Firmicutes suddenly increased from 0.4% on day 10 to 23% on day 15. A total of 31 known genera from 6 phyla were observed in the pouch water of undepurated mussels among which 26 were dominant on day 0 (Supplementary Fig. 4A). *Acinetobacter* which is a genus from the phylum Proteobacteria was the most apparently dominant (55% relative abundance). There was a sudden decrease in the total genera on day 7 as only 2 known genera were observed to be dominant (*Acinetobacter* – 99% and *Pseudoalteromonas* – 0.6%). A total of 22 known genera from 7 phyla were observed in the pouch water of depurated mussels (Supplementary Fig. 4B). The number of dominant genera was observed to double on day 10 with *Psychrobacter* still dominant (80%) and *Arcobacter* increased to 11%. Like undepurated mussels, only one genus was dominant although the genus observed was different (*Psychrobacter*). In the pouch water of commercial mussels, 20 known genera were observed (Supplementary Fig. 4C). In the pouch water of depurated mussels, *Psychrobacter* was significantly different ($p < .05$) on day 0 vs. day 7, day 7 vs. day 10 and *Pseudoalteromonas* was significantly different ($p < .05$) on day 0 vs. day 7, day 0 vs. day 10, day 0 vs. day 15. Similarly, *Arcobacter* was significantly different ($p < .05$) on day 0 vs. day 10, day 7 vs. day 10. Only *Acinetobacter* was significantly different ($p < .05$) on day 0 vs. day 7, day 7 vs. day 10, day 7 vs. day 15, day 10 vs. day 15 in the pouch water of undepurated mussels. In the pouch water of commercially-depurated mussels, *Psychrobacter* was significantly different ($p < .05$) on day 0 vs. day 7, day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15 while *Pseudoalteromonas* was significantly different ($p < .05$) on day 0 vs. day 7, day 0 vs. day 10, day 0 vs. day 15.

4. Discussion

This study aimed to describe the microbial communities in live mussels packed with high initial oxygen (80%) and stored at 4 °C for 15 days. The study sought to describe the impact of depuration procedures on the microbiota present in these live mussels during storage with a view to understanding the spoilage mechanisms of MA-packed mussels. In this current study, huge changes were observed in the microbial community at both phylum and genus levels present in the mussel meat of depurated mussels stored at 4 °C for 15 days in this study. The initial indigenous microbiota was diverse though dominated by a small proportion of taxa. The dominant taxa changed radically over time, suggesting that storage conditions were different from the normal environment of the mussels. The longer the storage, the less diverse were the dominant taxa. The wide diversity at the phylum level observed in this current study reflects the marine environment in which the mussels were harvested. Similarly, gradual reduction of the phyla from highly abundant to extremely low abundance shows the progression of a succession of the microbial community. The shift in phyla observed in this study was like the observation of Fernandez-Piquer et al. (2012) who reported shifts from Spirochaetes, Proteobacteria, Planctomycetes, Verrucomicrobia and Cyanobacteria to Fusobacteria during storage of live oysters although over 70 genera were detected. They reported that *Psychrilyobacter* which is a genus of the phylum Fusobacteria dominated (43.8%) live oysters stored at 4 °C and the overall microbial community diversity in oysters indicated the influence of storage temperature at postharvest storage conditions. Trabal et al. (2012) studied the microbial community in depurated oysters using V3–V5 regions of the 16S rRNA gene. They obtained 13 phyla and over 200 genera while Proteobacteria was the most dominant phylum with variations in the dominant genera. Chen, Wang, Lin, Shi, and Liu (2017) studied the bacterial community profiling of gills of MA-packed oysters stored at 4 °C based on the analysis of V3 region of the 16S rRNA gene using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). They reported that both *Lactobacillus* and *Lactococcus* were dominant bacteria. Temporal Temperature Gel Electrophoresis (TTGE) was used to study the microbial communities in MA-packed scallop meat (*Pecten maximus*) stored at 4 °C for 8 days (Coton et al., 2013). *Shewanella*, *Pseudomonas* and *Brochothrix* were reported as dominant bacteria at the end of storage in their study.

In this current study, the fewer phyla observed in the pouch water on day 0 was because sterile freshwater was added to the pouch and the only source of bacteria in the pouch water was from the surface microbiota and gut contents of mussels. At the start of the storage (day 0) of the samples, Cyanobacteria and Proteobacteria were the dominant phyla present in the mussel meat and pouch water of the treatments and

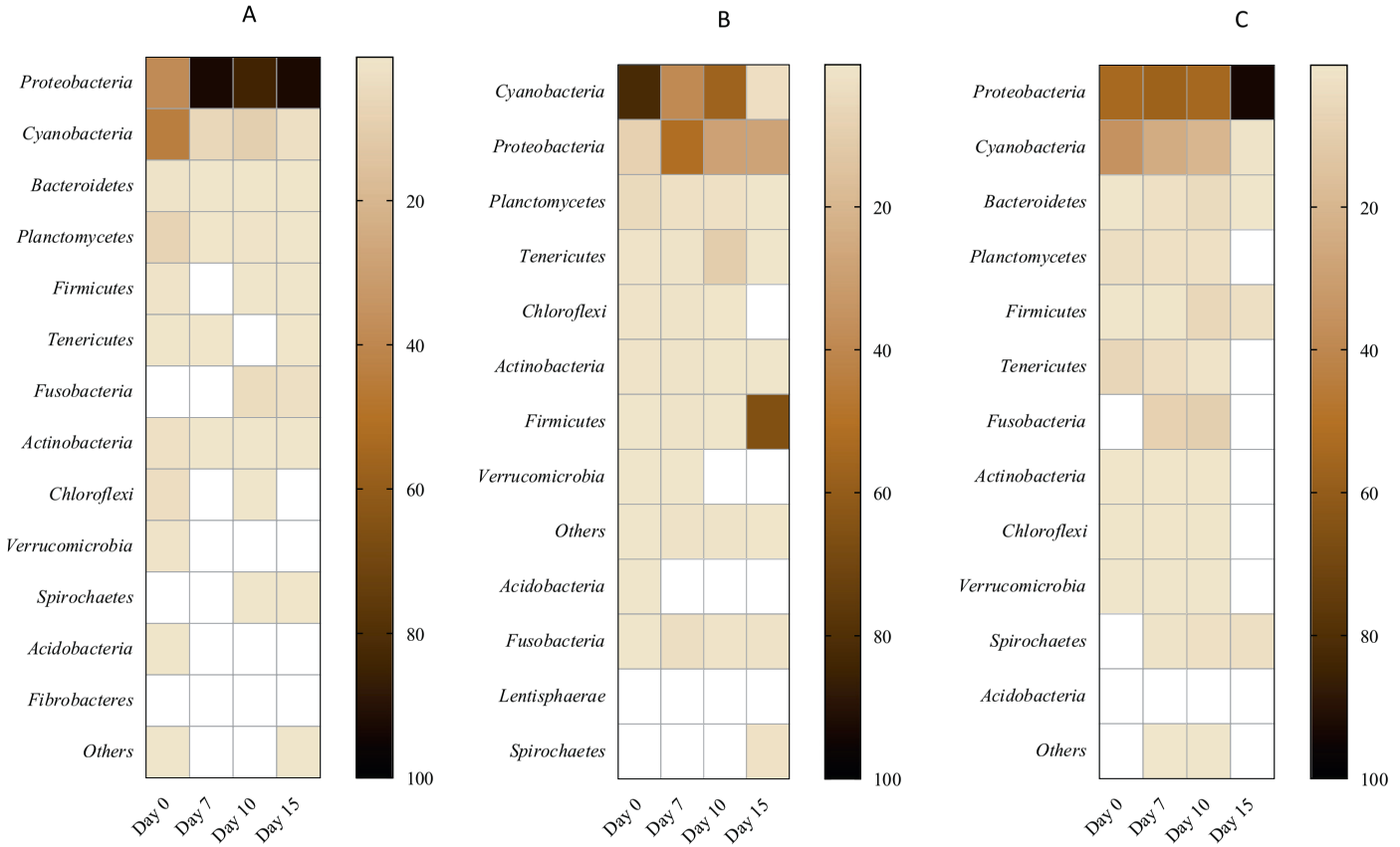

Fig. 3. Heat-map (threshold ≥ 0.1%) of microbial community succession at phylum level observed in the mussel meat of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. The change in relative abundance (%) within the microbial community of each phylum is shown by colour intensity. White indicates extremely low (< 0.1%) abundance, yellow indicates very low (0.1- 10%), pale brown indicates moderate (10 – 49%), brown indicates (50% - 79%) and dark brown indicates high (80 - 100%) abundance.

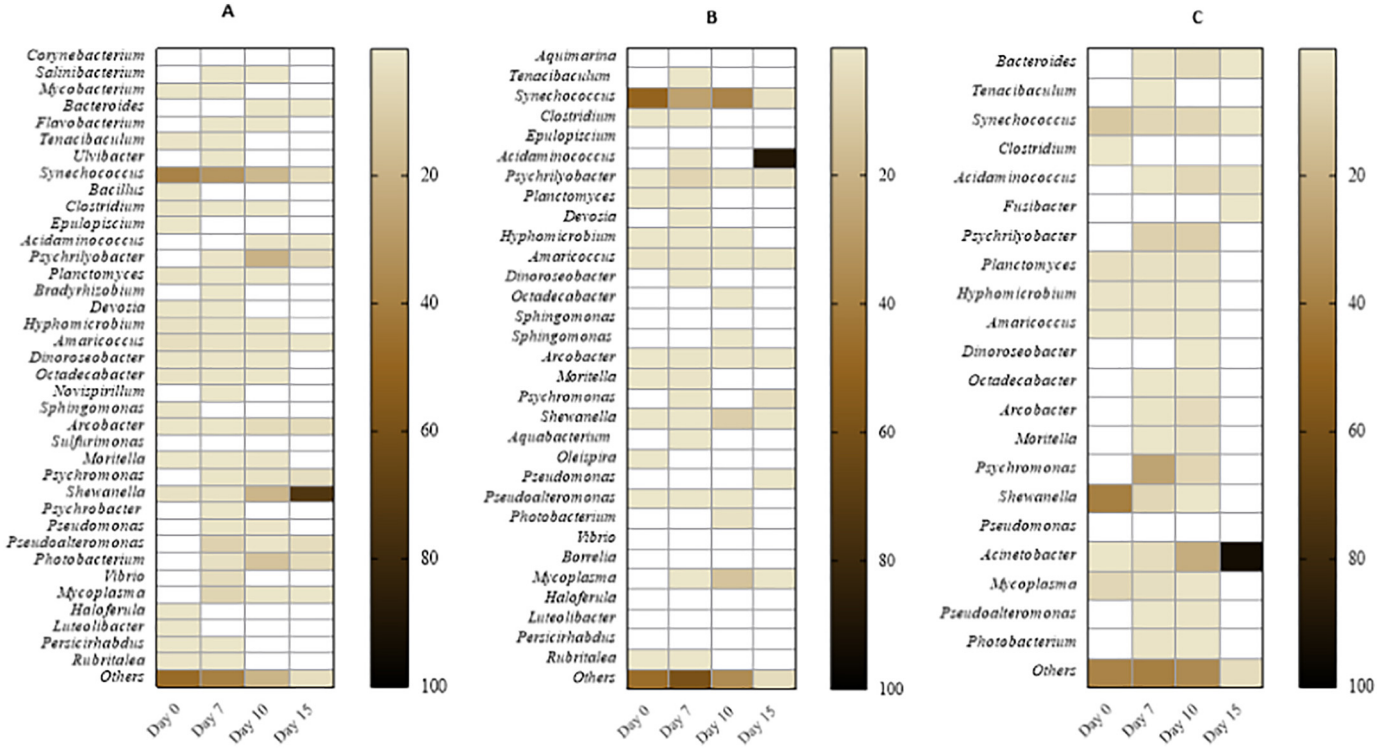

Fig. 4. Heat-map (threshold ≥ 0.1%) of microbial community succession at genus level observed in the mussel meat of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. The change in relative abundance (%) within the microbial community of each phylum is shown by colour intensity. White indicates extremely low (< 0.1%) abundance, yellow indicates very low (0.1- 10%), pale brown indicates moderate (10 – 49%), brown indicates (50% - 79%) and dark brown indicates high (80 - 100%) abundance.

Table 4
The distribution of OTUs at different levels in the pouch water of undepurated mussels (A), depurated mussels (B) and commercially-depurated mussels (C) stored at 4 °C for 15 days.

Sample	Storage days	Phylum	Class	Order	Family	Genus
Undepurated	Day 0	23	62	116	197	284
	Day 7	9	12	20	26	31
	Day 10	12	18	25	37	52
	Day 15	9	15	21	28	36
Depurated	Day 0	5	9	16	23	26
	Day 7	4	11	19	26	33
	Day 10	10	17	26	36	44
	Day 15	9	15	22	34	46
Commercial	Day 0	15	19	32	44	57
	Day 7	18	24	37	57	73
	Day 10	17	24	40	58	78
	Day 15	13	22	37	52	70

commercially-packed mussels. Cyanobacteria were found to be dominant in the mussel meat of both undepurated and depurated mussels. *Synechococcus* was the dominant genus from day 0–7 (undepurated) and day 0–10 (depurated) and was not dominant in commercially-packed mussels. This could indicate the fact that *Synechococcus* was from the marine environment either in the gut or on the external surfaces of the mussels. *Synechococcus* constitutes a large portion of mussels' diet, which is supported by the fact that their relative abundance declined with storage – they aren't growing in or on mussels during storage.

Acidaminococcus was dominant on day 15 in the mussel meat of depurated mussels. *Acidaminococcus*, members of the Firmicutes, are Gram-negative, nonmotile, anaerobic diplococci that utilise amino acids as sole energy sources. They are mostly found in the gastrointestinal tract (GIT) and are oxidase and catalase-negative (Chang et al., 2010; Jumas-Bilak et al., 2007; Russell, 2015). Micro-oxic or even anoxic microenvironments are quite likely to occur in association with animals, even when they are stored in high levels of atmospheric oxygen. This is because of the limitations of diffusion (from the environment into mussels) as a means of transport of oxygen in comparison to potentially high rates of oxygen respiratory consumption by either the animal or the microbiota. The utilization of amino acids suggests that *Acidaminococcus* is associated with the presence of readily available amino acids and the most likely source of these would be from mussel protein degradation. This suggests that *Acidaminococcus* are associated with spoiled mussels.

Shewanella was apparently dominant on day 15 in the mussel meat of undepurated mussels and day 0 in commercially-packed mussels. *Shewanella* is a genus comprising of Gram-negative, oxidase positive, rod-shaped and catalase positive bacteria that cannot ferment glucose and are present in the marine environment (Odeyemi, Burke, Bolch, & Stanley, 2018a; Odeyemi, Burke, Bolch, & Stanley, 2018b). Members of this genus such as *Shewanella baltica* have been associated with spoilage of seafood such as fish (Ge, Zhu, Ye, & Yang, 2017), shrimp (Macé et al., 2013) and oyster (Madigan et al., 2014) and are evident with the production of H_2S production or spoilage volatile metabolites such as dimethyl disulphide in fish (Parlapani, Mallouchos, Haroutounian, & Boziaris, 2017). *Shewanella* have been reported to be able to use anaerobic respiration using electron acceptors such as s trimethylamine-N-oxide (TMAO) in place of oxygen and could survive in oxic/anoxic conditions (Brettar, Christen, & Höfle, 2002; Venkateswaran et al., 1999). Such oxic – anoxic conditions may be found in MA-packed mussels especially towards the end of the storage (day 10 onwards) as the packs have stagnant water in the pouch. This suggests a reason why *Shewanella* was more dominant in mussel meat rather than the pouch water. It should be noted that *Shewanella* species such as *S. baltica* are motile and could produce biofilms (attachment to surfaces) (Zhao, Zhu, Ye, Ge, & Li, 2016). Similarly, like other seafood, mussels are rich in cysteine this enhances the growth of *Shewanella* because of its biochemical actions of producing of H_2S from cysteine. It can also produce

methyl mercaptan (CH_3SH) and dimethylsulphide - $(CH_3)_2S$ from amino acids such as methionine (Lopez-Caballero, Sanchez-Fernandez, & Moral, 2001).

Acinetobacter was observed to be dominant throughout the storage period in the pouch water of undepurated mussels. *Acinetobacter* is a genus of Gram-negative, non-fermenter, diplococci, non - motile, non-pigmented, aerobic, oxidase negative and catalase positive bacteria that are found in the aquatic environment (Bouvet & Grimont, 1986; Clemmer, Bonomo, & Rather, 2011; Kozinska, Pazdzior, Pekala, & Niemczuk, 2014). At the early stationary phase, *Acinetobacter* produces enzymes such as lipase and are capable of producing multiple quorum sensing (QS) signalling molecules (Gonzalez, Nusblat, & Nudel, 2001). QS signalling molecules mediate spoilage gene expression in spoilage bacteria at increased population density (Gu, Fu, Wang, & Lin, 2013). Members of the genus *Acinetobacter* have been isolated from various seafood such as the skin and flesh of Turbot (*Scophthalmus maximus*) at cold storage conditions (Zhang et al., 2016), tropical red drum (*Sciaenops ocellatus*), (Silbande et al., 2018) and sea bream (Parlapani, Meziti, Kormas, & Boziaris, 2013). However, the presence of *Acinetobacter* in mussels has not been previously reported. Recently, it was reported that although *Acinetobacter* has low spoilage potential, it enhances the growth of other spoilage bacteria by means of quorum sensing (QS) signalling molecules such as N-acyl homoserine lactones (AHLs). Zhang et al. (2016) reported that when the total viable count (TVC) reaches 8 log CFU g^{-1}, or Enterobacteriaceae reaches 6 log CFU g^{-1}, QS signalling molecules were triggered in spoilage bacteria present in turbot, thereby causing spoilage of the fish. Hence, this shows that the presence of *Acinetobacter* in mussel meat of commercial mussels (depurated for few hours) and pouch water of undepurated mussels could enhance spoilage of mussels by other bacteria, especially *Shewanella* identified in this study. *Psychrobacter* was dominant in the pouch water of depurated and commercially-packed mussels throughout the storage period. *Psychrobacter* is non-motile, psychrotolerant or psychrophilic, oxidase positive, non-pigmented, Gram-negative rod or coccobacilli that can survive low temperatures (Ayala-del-Río et al., 2010; Bozal, Montes, Tudela, & Guinea, 2003; Juni & Heym, 1986). *Psychrobacter* has been isolated from the marine environment, the flesh and gut content of seafood such as fish, oyster and lobster (Bakermans et al., 2006; Romanenko, Lysenko, Rohde, Mikhailov, & Stackebrandt, 2004).

The fact that they adapt and survive in fatty acid-rich environment shows why they could be dominant in the pouch water especially on day 15 as the mussels have spoiled, degraded and the pouch water became a slurry. *Psychrobacter* could metabolise lipids and also hydrolyse amino acids resulting in off-odours such as trimethylamine - TMA (Broekaert, Noseda, Heyndrickx, Vlaemynck, & Devlieghere, 2013). *Psychrobacter* has been identified as spoilage bacteria in other seafood such as brown shrimp stored at 4 °C for 9 days (Broekaert et al., 2013). This spoilage and lipolytic potential of *Psychrobacter* could contribute to the objectionable smell observed from day 10 when the packs where opened. However, it is noteworthy that in this current study, *Psychrobacter* was not observed in the mussel meat of depurated mussels. This implies that *Psychrobacter* survived in the aquatic environment of the pouch water or if present on the mussels as it is a transient organism that can be easily removed by depuration. The observed shift in the taxa on day 15 across the treatments indicated the synergistic contribution of other spoilage bacteria. For example, amino acids required by *Acidaminococcus* were readily available on day 10 in undepurated and day 15 in depurated mussels. At the end of storage (day 15), a different taxon was observed to be dominant in the treatments.

Acinetobacter in the pouch water of undepurated mussels could have provided a synergistic effect on the dominance of *Acidaminococcus* on day 10 in the mussel meat. *Psychrobacter* potentially provided a similar effect in depurated and commercially-packed mussels. This implies that pouch water contributes significantly to the spoilage of the mussel meat, especially in undepurated and commercially-packed mussels because the later only had short (1 − 2h) duration of depuration. Therefore, inefficient (short time depuration of 1–2 h) and lack (no depuration) of depuration contributes to the faster rate of spoilage compared with effective depuration duration (8 h).

5. Conclusion

This study demonstrated the impact of depuration on the microbiota and the spoilage mechanism of MA-packaged live mussels. As the consumption of oxygen increases, this resulted in hypoxic conditions towards the end of storage and contributed to the change in the microbial community as indicated by the dominance of aerobic or facultative bacteria (Proteobacteria). *Shewanella* was easily removed through depuration. However, spoilage bacteria such as *Acidaminococcus* could not be easily removed although they are not important at the beginning but grew at the end during storage. Pouch water contributed suitable biological medium for the growth of *Acinetobacter* and *Psychrobacter* and both enhanced the growth of spoilage bacteria such as *Shewanella* and *Acidaminococcus* which could be through the production of QS signalling molecules. Effective depuration will, therefore, help in reducing spoilage bacteria and thus reduce the rate of spoilage of live mussels. However, there is a need to study the spoilage potential of either axenic and mixed cultures of these spoilage bacteria.

Acknowledgement

The University of Tasmania is appreciated for the provision of PhD scholarship, living allowance and research operational cost to the first author for the purpose of this study. All mussels used in this study were graciously provided by Spring Bay Seafoods, Tasmania, as was valuable industry advice on the processing of live mussel. Thanks to Mr. P. Lamb and Ms. C. Ooms of Spring Bay Seafoods for their support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.12.017.

References

Ayala-Del-Río, H. L., Chain, P. S., Grzymski, J. J., Ponder, M. A., Ivanova, N., Bergholz, P. W., ... Tiedje, J. M. (2010). The genome sequence of *Psychrobacter arcticus* 273-4, a psychroactive siberian permafrost bacterium, reveals mechanisms for adaptation to low-temperature growth. *Applied and Environmental Microbiology, 76*, 2304–2312.

Bakermans, C., Ayala-Del-Rio, H. L., Ponder, M. A., Vishnivetskaya, T., Gilichinsky, D., Thomashow, M. F., & Tiedje, J. M. (2006). Psychrobacter cryohalolentis sp. nov. and Psychrobacter arcticus sp. nov., isolated from Siberian permafrost. *International Journal of Systematic and Evolutionary Microbiology, 56*, 1285–1291.

Barile, N. B., Scopa, M., Nerone, E., Mascilongo, G., Recchi, S., Cappabianca, S., & Antonetti, L. (2009). Study of the efficacy of a closed cycle depuration system on bivalve molluscs. *Veterinaria Italiana, 45*, 555–566.

Bouvet, P. J. M., & Grimont, P. A. D. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *International Journal of Systematic and Evolutionary Microbiology, 36*, 228–240.

Bozal, N., Montes, M. J., Tudela, E., & Guinea, J. (2003). Characterization of several Psychrobacter strains isolated from Antarctic environments and description of Psychrobacter luti sp. nov. and Psychrobacter fozii sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 53*, 1093–1100.

Brettar, I., Christen, R., & Höfle, M. G. (2002). *Shewanella denitrificans* sp. nov., a vigorously denitrifying bacterium isolated from the oxic-anoxic interface of the Gotland Deep in the central Baltic Sea. *International Journal of Systematic and Evolutionary Microbiology, 52*, 2211–2217.

Broekaert, K., Noseda, B., Heyndrickx, M., Vlaemynck, G., & Devlieghere, F. (2013). Volatile compounds associated with *Psychrobacter* spp. and *Pseudoalteromonas* spp., the dominant microbiota of brown shrimp (*Crangon crangon*) during aerobic storage. *International Journal of Food Microbiology, 166*, 487–493.

Caglak, E., Cakli, S., & Kilinc, B. (2008). Microbiological, chemical and sensory assessment of mussels (*Mytilus galloprovincialis*) stored under modified atmosphere packaging. *European Food Research and Technology, 226*, 1293–1299.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E.

K., ... Gordon, J. I. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7*, 335–336.
Chang, Y.-J., Pukall, R., Saunders, E., Lapidus, A., Copeland, A., Nolan, M., ... Klenk, H.-P. (2010). Complete genome sequence of *Acidaminococcus fermentans* type strain (VR4(T)). *Standards in Genomic Sciences, 3*, 1–14.
Chen, H., Wang, M., Lin, X., Shi, C., & Liu, Z. (2017). Bacterial microbiota profile in gills of modified atmosphere-packaged oysters stored at 4 °C. *Food Microbiology, 61*, 58–65.
Clemmer, K. M., Bonomo, R. A., & Rather, P. N. (2011). Genetic analysis of surface motility in *Acinetobacter baumannii*. *Microbiology Microbiology, 157*, 2534–2544.
Coton, M., Joffraud, J. J., Mekhtiche, L., Leroi, F., & Coton, E. (2013). Biodiversity and dynamics of the bacterial community of packaged king scallop (Pecten maximus) meat during cold storage. *Food Microbiology, 35*, 99–107.
Desantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., ... Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology, 72*, 5069–5072.
Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England), 26*, 2460–2461.
Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat Meth, 10*, 996–998.
Fernandez-Piquer, J., Bowman, J. P., Ross, T., & Tamplin, M. L. (2012). Molecular analysis of the bacterial communities in the live Pacific oyster (*Crassostrea gigas*) and the influence of postharvest temperature on its structure. *Journal of Applied Microbiology, 112*, 1134–1143.
Ge, Y., Zhu, J., Ye, X., & Yang, Y. (2017). Spoilage potential characterization of Shewanella and Pseudomonas isolated from spoiled large yellow croaker (Pseudosciaena crocea). *Letters in Applied Microbiology, 64*, 86–93.
Gonzalez, R. H., Nusblat, A., & Nudel, B. C. (2001). Detection and characterization of quorum sensing signal molecules in Acinetobacter strains. *Microbiological Research, 155*, 271–277.
Goulas, A. E., Chouliara, I., Nessi, E., Kontominas, M. G., & Savvaidis, I. N. (2005). Microbiological, biochemical and sensory assessment of mussels (*Mytilus galloprovincialis*) stored under modified atmosphere packaging. *Journal of Applied Microbiology, 98*, 752–760.
Gu, Q., Fu, L., Wang, Y., & Lin, J. (2013). Identification and characterization of extracellular cyclic dipeptides as quorum-sensing signal molecules from shewanella baltica, the specific spoilage organism of pseudosciaena crocea during 4 °C storage. *Journal of Agricultural and Food Chemistry, 61*, 11645–11652.
Jumas-Bilak, E., Carlier, J. P., Jean-Pierre, H., Mory, F., Teyssier, C., Gay, B., ... Marchandin, H. (2007). Acidaminococcus intestini sp. nov., isolated from human clinical samples. *International Journal of Systematic and Evolutionary Microbiology, 57*, 2314–2319.
Juni, E., & Heym, G. A. (1986). *Psychrobacter immobilis* gen. Nov., sp. nov.: Genospecies composed of gram-negative, aerobic, oxidase-positive coccobacilli. *International Journal of Systematic and Evolutionary Microbiology, 36*, 388–391.
Kozinska, A., Pazdzior, E., Pekala, A., & Niemczuk, W. (2014). *Acinetobacter johnsonii* and *Acinetobacter lwoffii* - the emerging fish pathogens. *Bulletin of the Veterinary Institute in Pulawy*, 193.
Lee, M.-J., Lee, J.-J., Chung, H. Y., Choi, S. H., & Kim, B.-S. (2016). Analysis of microbiota on abalone (Haliotis discus hannai) in South Korea for improved product management. *International Journal of Food Microbiology, 234*, 45–52.
Li, H., Li, Z., Qu, J., & Wang, J. (2017). Bacterial diversity in traditional Jiaozi and sourdough revealed by high-throughput sequencing of 16S rRNA amplicons. *LWT - Food Science and Technology, 81*, 319–325.
Liu, D., & Tong, C. (2017). Bacterial community diversity of traditional fermented vegetables in China. *LWT - Food Science and Technology, 86*, 40–48.
Lopez-Caballero, M. E., Sanchez-Fernandez, J. A., & Moral, A. (2001). Growth and metabolic activity of Shewanella putrefaciens maintained under different CO2 and O2 concentrations. *International Journal of Food Microbiology, 64*, 277–287.
Macé, S., Joffraud, J., Cardinal, M., Malcheva, M., Cornet, J., Lalanne, V., ... Dousset, X. (2013). Evaluation of the spoilage potential of bacteria isolated from spoiled raw salmon (Salmo salar) fillets stored under modified atmosphere packaging. *International Journal of Food Microbiology, 160*, 227–238.
Madigan, T. L., Bott, N. J., Torok, V. A., Percy, N. J., Carragher, J. F., de Barros Lopes, M. A., & Kiermeier, A. (2014). A microbial spoilage profile of half shell Pacific oysters (*Crassostrea gigas*) and Sydney rock oysters (*Saccostrea glomerata*). *Food Microbiology, 38*, 219–227.
Nielsen, L. N., Roager, H. M., Casas, M. E., Frandsen, H. L., Gosewinkel, U., Bester, K., ... Bahl, M. I. (2018). Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels. *Environmental Pollution, 233*, 364–376.
Odeyemi, O. A., Burke, C. M., Bolch, C. C. J., & Stanley, R. (2018a). Seafood spoilage microbiota and associated volatile organic compounds at different storage temperatures and packaging conditions. *International Journal of Food Microbiology, 280*, 87–99.
Odeyemi, O. A., Burke, C. M., Bolch, C. J. S., & Stanley, R. (2018b). Evaluation of spoilage potential and volatile metabolites production by Shewanella baltica isolated from modified atmosphere packaged live mussels. *Food Research International, 103*, 415–425.
Parlapani, F. F., & Boziaris, I. S. (2016). Monitoring of spoilage and determination of microbial communities based on 16S rRNA gene sequence analysis of whole sea bream stored at various temperatures. *LWT - Food Science and Technology, 66*, 553–559.
Parlapani, F. F., Mallouchos, A., Haroutounian, S. A., & Boziaris, I. S. (2017). Volatile organic compounds of microbial and non-microbial origin produced on model fish substrate un-inoculated and inoculated with gilt-head sea bream spoilage bacteria. *LWT - Food Science and Technology, 78*, 54–62.
Parlapani, F. F., Meziti, A., Kormas, K. A., & Boziaris, I. S. (2013). Indigenous and spoilage microbiota of farmed sea bream stored in ice identified by phenotypic and 16S rRNA gene analysis. *Food Microbiology, 33*, 85–89.
Pastoriza, L., Bernárdez, M., Sampedro, G., Cabo, M. L., & Herrera, J. J. R. (2004). Elevated concentrations of oxygen on the stability of live mussel stored refrigerated. *European Food Research and Technology, 218*, 415–419.
Powell, S. M., & Tamplin, M. L. (2012). Microbial communities on Australian modified atmosphere packaged Atlantic salmon. *Food Microbiology, 30*, 226–232.
Ramos, R. J., Miotto, M., Squella, F. J., Cirolini, A., Ferreira, J. F., & Vieira, C. R. (2012). Depuration of Oysters (Crassostrea gigas) contaminated with Vibrio parahaemolyticus and Vibrio vulnificus with UV light and chlorinated seawater. *Journal of Food Protection, 75*, 1501–1506.
Romanenko, L. A., Lysenko, A. M., Rohde, M., Mikhailov, V. V., & Stackebrandt, E. (2004). Psychrobacter maritimus sp. nov. and Psychrobacter arenosus sp. nov., isolated from coastal sea ice and sediments of the Sea of Japan. *International Journal of Systematic and Evolutionary Microbiology, 54*, 1741–1745.
Russell, J. B. (2015). Acidaminococcus. In W. B. Whitman, F. R. P. Kämpfer, M. Trujillo, J. Chun, P. DeVos, B. Hedlund, & S. Dedysh (Eds.). *Bergey's manual of systematics of archaea and bacteria*.
Silbande, A., Adenet, S., Chopin, C., Cornet, J., Smith-Ravin, J., Rochefort, K., & Leroi, F. (2018). Effect of vacuum and modified atmosphere packaging on the microbiological, chemical and sensory properties of tropical red drum (*Sciaenops ocellatus*) fillets stored at 4°C. *International Journal of Food Microbiology, 266*, 31–41.
Song, W., Li, L., Huang, H., Jiang, K., Zhang, F., Chen, X., ... Ma, L. (2016). The gut microbial community of antarctic fish detected by 16S rRNA gene sequence analysis. *BioMed Research International, 2016*, 7.
Trabal, N., Mazon-Suastegui, J. M., Vazquez-Juarez, R., Asencio-Valle, F., Morales-Bojorquez, E., & Romero, J. (2012). Molecular analysis of bacterial microbiota associated with oysters (Crassostrea gigas and Crassostrea corteziensis) in different growth phases at two cultivation sites. *Microbial Ecology, 64*, 555–569.
Venkateswaran, K., Moser, D. P., Dollhopf, M. E., Lies, D. P., Saffarini, D. A., MacGregor, B. J., ... Nealson, K. H. (1999). Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 49*, 705–724.
Xia, Y., & Sun, J. (2017). Hypothesis testing and statistical analysis of microbiome. *Genes & Diseases, 4*, 138–148.
Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics (Oxford, England), 30*, 614–620.
Zhang, L., & Wang, S. (2017). Bacterial community diversity on in-shell walnut surfaces from six representative provinces in China. *Scientific Reports, 7*, 10054.
Zhang, C., Zhu, S., Wu, H., Jatt, A. N., Pan, Y., & Zeng, M. (2016). Quorum sensing involved in the spoilage process of the skin and flesh of vacuum-packaged farmed turbot (Scophthalmus maximus) stored at 4 degrees C. *Journal of Food Science, 81*, M2776–M2784.
Zhao, A., Zhu, J., Ye, X., Ge, Y., & Li, J. (2016). Inhibition of biofilm development and spoilage potential of Shewanella baltica by quorum sensing signal in cell-free supernatant from Pseudomonas fluorescens. *International Journal of Food Microbiology, 230*, 73–80.