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Bacterial communities and potential spoilage markers of whole blue crab (*Callinectes sapidus*) stored under commercial simulated conditions

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ABSTRACT

Bacterial communities composition using 16S Next Generation Sequencing (NGS) and Volatile Organic Compounds (VOCs) profile of whole blue crabs (*Callinectes sapidus*) stored at 4 and 10 °C (proper and abuse temperature) simulating real storage conditions were performed. Conventional microbiological and chemical analyses (Total Volatile Base-Nitrogen/TVB-N and Trimethylamine-Nitrogen/TMA-N) were also carried out. The rejection time point was 10 and 6 days for the whole crabs stored at 4 and 10 °C, respectively, as determined by development of unpleasant odors, which coincided with crabs death. Initially, the Aerobic Plate Count (APC) was 4.87 log cfu/g and increased by 3 logs at the rejection time. The 16S NGS analysis of DNA extracted directly from the crab tissue (culture-independent method), showed that the initial microbiota of the blue crab mainly consisted of *Candidatus* Bacilloplasma, while potential pathogens e.g. *Listeria monocytogenes, Pseudomonas aeruginosa* and *Acinetobacter baumannii,* were also found. At the rejection point, bacteria of Rhodobacteraceae family (52%) and *Vibrio* spp. (40.2%) dominated at 4 and 10 °C, respectively. TVB-N and TMA-N also increased, reaching higher values at higher storage temperature. The relative concentrations of some VOCs such as 1-octen-3-ol, *trans*-2-octenal, *trans,trans*-2,4-heptadienal, 2-butanone, 3-butanone, 2-heptanone, ethyl isobutyrate, ethyl acetate, ethyl-2-methylbutyrate, ethyl isovalerate, hexanoic acid ethyl ester and indole, exhibited an increasing trend during crab storage, making them promising spoilage markers. The composition of microbial communities at different storage temperatures was examined by 16S amplicon meta-barcoding analysis. This kind of analysis in conjugation with the volatile profile can be used to explore the microbiological quality and further assist towards the application of the appropriate strategies to extend crab shelf-life and protect consumer's health.

1. Introduction

Callinectes sapidus is an allochthonous crab species originated from the West Atlantic Ocean which probably colonized Greek coastal areas, especially the Thermaikos Gulf, in 1940 (Serbetis, 1959). Although, for many decades the population of blue crab remained at low numbers, an unexpected increase was recorded in 2009 (Kevrekidis, 2010) which resulted in a noteworthy increase of the population to date. The increase of blue crab population has reinforced the local economy as well as created new jobs and export opportunities. Local fishermen transfer the caught blue crabs to seafood companies for further handling and/or processing. The healthy, live crabs are placed in baskets for direct sale or they are stored in water tanks in order to survive for longer periods of time and be sold at a later date. Blue crabs are sold live or frozen to retail seafood markets, hotels and restaurants in Greece, other European countries and Asia e.g. Japan.

In contrast to other seafood, crabs are usually transported and distributed alive stored at 4–8 °C (Jacklin and Combes, 2007). Crustaceans have the ability to survive out of the water for an extended period of time. Nevertheless, due to the fact that they cannot keep their gills moist, damage can occur to their gills as a result of oxygen reduction which can eventually lead to death (Jacklin and Combes, 2007). Crabs can be stored alive in domestic and commercial refrigerators at 4 °C. The chill-stored crabs become unacceptable (characterized as 'spoiled') for human consumption when still alive (Robson et al., 2007). Crabs and other crustaceans spoilage is mainly due to the microbial growth

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(Boziaris et al., 2011; Robson et al., 2007). The exploration of microbial communities provides information on microbiological spoilage and quality/freshness status of seafood.

Alternatively, crabs can be cooked and subsequently can be stored chilled or frozen (Lorentzen et al., 2014). To date, using conventional culture dependent microbiological approaches, bacteria of the genus *Pseudomonas* have been recognized as the dominant microorganisms during the spoilage of cooked and chill stored crabs, followed by lactic acid bacteria, H_2S producing bacteria and Enterobacteriaceae (Anacleto et al., 2011; Lorentzen et al., 2014, 2016; McDermott et al., 2018). Bacterial communities have also been explored in fresh blue crab meat stored at 4 °C under various packaging conditions (Gutierrez Tyler et al., 2010). In their study, spoilage associated bacteria such as *Pseudomonas putida*, *Shewanella putrefaciens* and *Carnobacterium piscicola* and bacteria such as various *Staphylococcus* species maybe associated with the handling conditions, were isolated and identified using a culture dependent technique.

In the last decades, the use of culture-independent molecular methodologies e.g. denaturing gradient gel electrophoresis (DGGE), thermal gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), single-strand conformation polymorphism-SSCP, and cloning followed by sequencing of the 16S rRNA gene, can identify food microbiota without the need to grow microorganisms on culture media (Cocolin et al., 2007; Delbés et al., 2007; Jaffrès et al., 2009; Macé et al., 2012; Parlapani et al., 2013; Rudi et al., 2004). Regarding crabs, the microbial diversity of blue crab parts such as carapace, gut, and hemolymph has been studied through 16S rRNA gene analysis, cloning and Sanger sequencing (Givens et al., 2013). Various potential pathogens such as *Acinetobacter junii*, *Alteromonas* sp., *Bacillus* sp., *Escherichia coli*, *Photobacterium damselae* subsp. *damselae*, *Photobacterium damselae* subsp. *piscida*, *Pseudoalteromonas* sp. and *Vibrio harveyi*, were detected.

However, the information derived by the aforementioned approaches is often limited (Nisiotou et al., 2014). On the other hand, the development of next generation sequencing technology (NGS) allows the sequencing of thousands 16S rRNA gene amplicons from DNA directly obtained from the samples. In contrast to other molecular methodologies, NGS may be used to investigate the bacterial taxa including non-culturable bacteria and bacteria present in low numbers (Mayo et al., 2014). NGS technology has been applied to understand the microbial diversity during spoilage of smoked salmon, cooked peeled shrimp, salmon fillet, cod fillet (Chaillou et al., 2014), gilt-head sea bream (Parlapani et al., 2018b), common cuttlefish (Parlapani et al., 2018a) and raw Atlantic cod (Kuuliala et al., 2018).

A plethora of Volatile Organic Compounds (e.g. alcohols, aldehydes, ketones, esters) are produced due to metabolic activity of spoilage microorganisms or chemical oxidations during seafood storage (Boziaris and Parlapani, 2016; Odeyemi et al., 2018). VOCs have been studied as potential spoilage markers, considering that some of them usually vary significantly between the initial and rejection day of fish and exhibit an increasing profile. Sarnoski et al. (2010) using SPME-GC/MS found that TMA, ammonia and indole correlated well with spoilage of blue crab meat. Additionally, other volatile markers such as TVB-N and TMA-N are produced due to microbial activity in various crustaceans, mollusks and fish (Anacleto et al., 2011; Boziaris et al., 2011; Lapa-Guimarães et al., 2005; Olafsdottir et al., 1997).

Notwithstanding the increasing economical exploitation of blue crabs (*C. sapidus*) in Northern Greece during the last decade, there are no studies to our knowledge regarding blue crab microbiota using NGS approach and potential spoilage markers. In this context, the aim of the present study was to (i) explore the composition of bacterial communities through 16S amplicon meta-barcoding by sequencing the V3–V4 region of the 16S rRNA gene, (ii) investigate the VOCs profile using SPME-GC/MS and (iii) determine the microbiological, TVB-N (Total Volatile Base-Nitrogen) and TMA-N (Trimethylamine-Nitrogen) changes, in order to assess the quality of blue crabs simulating commercial (e.g. super markets, restaurants and hotels) cold storage conditions.

2. Materials & methods

2.1. Blue crabs provision, storage, handling and sampling

Two (2) different batches of 40 live blue crabs each (totally N = 80 individuals) were taken from a seafood processing and trading company in Chalastra region (Thessaloniki, Greece). The blue crabs were caught in Thermaikos Gulf, North Aegean Sea, Greece (FAO subarea 37.3.1) in September 2017. The crabs were transferred to the laboratory within 3 h and stored in incubators operating at 4 and 10 °C to simulate commercial conditions of sale (4 °C corresponds to the normal refrigeration temperature, whereas 10 °C simulates the temperature increase due to the continuous opening of the refrigerator door).

At each sampling point (day 0, 2, 4, 6, 8, 10, 12, 14 and 16), four (4) individuals (n = 2 × 2 = 4, two individuals from each batch per temperature) were humanely killed taking into account the recommendations for the animal welfare by Torry Advisory Note 26 – revised (Edwards and Early, 1967), Good Manufacturing Practice Guidance - Shellfish Cooking (Watson and Denton, 2007) and the instruction of Royal Society for the Prevention of Cruelty to Animals, on humane killing of crustaceans for human consumption (Anonymous, 2018). More specifically the crabs were immersed in ice-slurry water to induce insensibility and then were spiked into their brain using a sterile knife. After that, each crab carapace was sterilized with 70% alcohol and was placed on a Teflon board previously sterilized with 70% alcohol and separated into two halves using a sterile chopper knife.

Afterwards, the half cut individuals (4 individuals) were taken for analyses. From each individual (weight of edible parts was about 30–35 g per individual), sample of 5 g were used for microbiological analysis, 10 g for TVB-N and TMA-N and 10 g for VOCs analyses.

For the identification of bacterial diversity using 16S amplicon meta-barcoding analysis, pooled samples (edible tissues containing white muscle and internal edible organs excluding digestive gland and stomach) of 25 g from four (4) individuals (two individuals from each batch per temperature) were taken at the beginning of the experiment and rejection time point (determined by development of unpleasant odors), and were subsequently stored at −20 °C.

2.2. Determination of unpleasant odor development

The off-odors development (fresh, marine, musty, ammoniacal, sour, putrid etc) was determined according to FAO/WHO (1999) by five trained panelists. Appearance and color were not evaluated since they did not change significantly during storage as determined by preliminary experiments. The aim of this evaluation was only to determine the rejection time point of the product. The rejection time point was set when at least one panelist indicated unpleasant off-odors.

2.3. Classical microbiological analysis

All microbiological media were supplied from LAB M (Lancashire, UK), apart from Streptomycin-thallous acetate-actidione (STAA) medium which was provided by Biolife Italiana srl (Milano, Italy). Iron Agar (IA) was prepared according to Gram et al. (1987).

Five grams (5 g) sample from raw crab edible tissues (white muscle and internal edible organs excluding digestive gland and stomach) were aseptically transferred to stomacher bags with 45 ml MRD (Maximum Recovery Diluent, 0.1% w/v peptone, 0.85% w/v NaCl) and homogenized for 2 min using a Stomacher (Bug Mixer, Interscience, London, UK). Volumes of 0.1 ml (from 10-fold serial dilutions) were spread on the surface of appropriate culture media in Petri dishes for enumeration of the following microorganisms (a) total microbial population as aerobic plate counts (APC) on TSA (Tryptone Soy Agar) and incubated

at 25 °C for 48–72 h, (b) *Pseudomonas* spp., on cetrimide-fucidin-cephaloridine agar (CFC) and incubated at 25 °C for 48 h and (c) *Brochothrix thermosphacta*, on STAA and incubated at 25 °C for 48 h. Volumes of 1 ml (from 10-fold serial dilutions) were used for the pour plate with overlay technique for enumeration of (a) H_2S producing bacteria on IA by counting only black colonies, after incubation at 25 °C for 72 h, (b) Lactic Acid Bacteria (LAB) on De Man, Rogosa, Sharpe agar (MRS) after incubation at 25 °C for 72 h and (c) Enterobacteriaceae on Violet Red Bile Glucose agar (VRBGA), incubated at 37 °C for 24 h. The results were expressed as mean log cfu g^{-1} ± standard deviation of 4 replicates (2 × 2 = 4, 2 batches x 2 crabs per batch). TSA was used for enumeration of APC instead of other non-selective media such as PCA, IA or Long and Hammer Agar. After preliminary determinations counts on TSA were about 0.5–1 log cfu/g more compared to the other non-selective media.

2.4. 16S rRNA meta-barcoding analysis

2.4.1. DNA extraction, quality evaluation and sequencing

Twenty five (25) grams of each pooled sample (n = 4 individuals, 2 from each batch) were transferred aseptically to stomacher bags with 50 ml sterile deionized H_2O (1:2 dilution) and homogenized for 2 min in a stomacher. Forty (40) ml of homogenized seafood suspension were transferred aseptically to sterile centrifuge tubes and centrifuged for removing the homogenized seafood solid matter and collection of bacteria as described in Parlapani et al. (2018b). Finally, the collected pellet was diluted in 3 ml of sterile deionized H_2O. For each sample, 200 μl of each diluted pellet were used for bacterial DNA extraction with ZR Soil Microbe DNA MicroPrep (ZYMO RESEARCH; Irvine, CA, USA) according to the manufacturer's instructions. DNA concentration was measured on a Qubit 2.0 Fluorimeter using the Qubit® dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA) and its integrity was evaluated by electrophoresis on a 0.8% agarose gel.

Bacterial diversity was assessed by sequencing the V3–V4 region of the 16S rRNA gene (≈460 bp) using the Illumina's 16S Metagenomic Sequencing Library Preparation (15,044,223 B) protocol. For the amplification of the V3-V4 region, gene-specific primers were selected based on the Klindworth et al. (2013), by adding Illumina overhang adapter nucleotide sequences at the 5′ end. The sequence of the primers used were 16S_F: 5′- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′ and 16S_R: 5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3'. All PCR reactions were performed in a 36-well rotor carousel on a Rotor-Gene Q Thermocycler (Qiagen, Hilden, Germany) in a final volume of 25 μl, containing 12.5 μl of 2× KAPA HiFi HotStart Ready enzyme mix (KAPA BIOSYSTEMS, Woburn, MA, U.S.A.), 10 μl of 1 μM 16S primer mix and 2.5 μl (~5 ng/μl) genomic DNA. PCR conditions were: an initial denaturation at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 5 min.

PCR products were purified to remove unincorporated primers and primer-dimer species using NucleoMag® NGS Bead Suspension (Macherey-Nagel, Düren, Germany). A second limited cycle index PCR was performed so as to attach dual indices and Illumina sequencing adapters in all PCR fragments. Each index PCR reaction was performed in 50 μl final volume, consisting of 5 μl PCR product, 25 μl of 2× KAPA HiFi HotStart Ready enzyme mix (KAPA BIOSYSTEMS, Woburn, MA, U.S.A.), 5 μl of each Nextera XT index primer (N7XX and S5XX) (Nextera XT Index kit, Illumina Inc, San Diego, California) and 10 μl PCR grade water. PCR reactions were performed using the following program: initial denaturation 95 °C for 3 min, 8 cycles of 95 °C for 30sec, 55 °C for 30 s, 72 °C for 30 s, and a final extension period of 72 °C for 5 min. Indexed PCR reactions were purified using NucleoMag® NGS Bead Suspension, stained with ethidium bromide and visualized on a 1% agarose gel under UV light.

All libraries were quantified with fluorometric quantification using Qubit® dsDNA BR assay kit and their molarity was calculated in relation to the size of DNA amplicons after indexing. Quantitative PCR (qPCR) was conducted on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) with the KAPA Library Quantification kit for Illumina sequencing platforms (KAPA BIOSYSTEMS, Woburn, MA, U.S.A.).

Libraries were loaded at 8 pM in a MiSeq platform according to the manufacturer's instructions. Libraries were sequenced using the MiSeq® reagent kit v3 (2 × 300 cycles) (Illumina, San Diego, California).

2.4.2. Bioinformatics and data analysis

Raw reads (.fastq files) were quality – trimmed with a local installation of the Trim Galore wrapper version 0.4.1 (Krueger, 2015) using default parameters. Analysis was conducted using Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). In brief, forward and reverse reads were joined using the fastq-join method (*join_paired_ends.py*) and demultiplexing of sequence data was performed with *split_libraries_fastq.py* script. OTU picking was executed with *pick_open_reference_otus.py* script with the default clustering algorithm UCLUST (Edgar, 2010). Sequences were clustered into operational taxonomic units (OTUs) with 99% sequence similarity and aligned to SILVA 128 release for QIIME. OTU tables and. biom files generated by QIIME were imported in R version 3.4.2 (R Development Core Team, 2010), so as to further process and visualize results. OTU counts and taxonomic assignments were merged to a phyloseq object with phyloseq R package (McMurdie and Holmes, 2012) and analyzed using the ampvis2 R package (Albertsen et al., 2015).

Several alpha diversity indices were measured to estimate species richness. In particular, diversity was measured as species richness through the Chao1 and ACE indices (Chao and Chiu, 2016) and based on the proportional abundances and frequencies of species through the Shannon, Simpson, Inverse Simpson and Fisher indices (Lande, 1996).

All plots were visualized by combining functions provided by the ggplot2 R package (Wickham, 2009). All barplots were normalized to 100% as abundance estimations within each sample, thus, percentages do not reflect the true biomass fraction of each sample. Furthermore, the observed OTUs for each sample were submitted to Venny 2.1.0 (Oliveros, 2015) to depict the pairwise comparisons of shared, common and/or unique OTUs at species level. When the species level was unknown, bacteria were classified according to the genus taxonomic rank.

2.5. Determination of pH, TVB-N and TMA-N

All chemicals were supplied by Sigma-Aldrich (Steinheim, Germany). The pH of the crab tissue was evaluated by inserting a pH electrode (Inolab WTW pH meter, Weilheim, Germany) in a 1 / 10 tissue /MRD homogenate at 20 °C.

For TVB-N and TMA-N, ten (10) g sample were homogenized in trichloracetic acid (TCA) 6% and filtered through Whatman No.1 filter paper in a 100 ml volumetric flask. TVB-N analysis was performed by using the steam-distillation procedure according to Vyncke et al. (1987), while TMA-N analysis using the spectrophotometric determination (using picric acid), according to Dyer (1945). From the 100 ml, forty (40) ml in duplicates were used for TVB-N analysis and the remaining 10 ml were used for TMA-N analysis. The results were expressed as mean mg N/100 g ± standard deviation of 4 replicates.

2.6. VOCs determination by headspace SPME-GC/MS analysis

A total amount of 40 g of crab edible tissues were obtained from 4 individuals (2 individuals per batch) and pooled. Five (5) g of the pooled sample were transferred into a 20 ml glass vial. Headspace SPME-GC/MS analysis and the identification of the compounds was carried out according to Parlapani et al. (2015a). Each value is presented as the mean of duplicate measurement of pooled sample. The amount of volatile compounds was expressed in arbitrary unit of the peak area of deconvoluted component multiplied by 10^{-6}.

2.7. Statistical analysis

Differences of means in viable counts, TVB-N and TMA-N were statistically tested by performing t-tests or Analysis of Variance followed by Tukey's significant difference test, using STATISTICA 6.0. A probability level of $P \leq 0.05$ was considered statistically significant. Statistical analysis of VOCs was not applicable, because measurements were conducted only in duplicates. Additionally, the determination of their concentration did not take place, since our aim was to monitor their tendency to increase or not during the storage until the rejection time point.

3. Results

3.1. Rejection time of blue crabs

Initially, crab odor was excellent (fresh, marine). The pleasant odor diminished gradually but strong unpleasant odors (ammoniacal, putrid) were developed after 10 days at 4 °C and 6 days at 10 °C, which coincided with crabs' death. Indeed, during the storage at 4 °C, all crabs remained alive until the 10th day, while the majority of crabs died between d 10 and d 16. A similar pattern on commence of death was observed in crabs stored at 10 °C. Crabs were alive until the 6th day and thereafter they started to die (between d 6 and d 12).

3.2. Microbiological analysis

Initially, APC was 4.87 ± 0.72 log cfu/g, whereas the population of *Pseudomonas* spp. and H_2S producing bacteria was 2.73 ± 0.59 and 2.28 ± 0.33 log cfu/g, respectively (Fig. 1). Bacteria grew faster at higher temperature. At rejection, APC reached the levels of 7.64 ± 0.29 and 7.12 ± 0.43 log cfu/g at 4 and 10 °C, respectively (Fig. 1). *Pseudomonas* spp. reached 4.31 ± 0.89 and 3.85 ± 0.40 log cfu/g, while H_2S producing bacteria 4.43 ± 0.56 and 4.00 ± 0.37 at the rejection time at 4 and 10 °C, respectively (Fig. 1). At the end of experiment (the time point in which all crabs were dead), APC was 8.71 ± 0.19 and 8.95 ± 0.27 log cfu/g, while *Pseudomonas* spp. was 4.58 ± 0.96 and 5.75 ± 0.11 and H_2S producing bacteria 5.57 ± 0.83 and 6.16 ± 0.41 log cfu/g for crabs stored at 4 and 10 °C, respectively (Fig. 1). In all cases *Pseudomonas* spp. and H_2S producing bacteria populations were more than 3 logs cfu/g lower compared to APC ($p \leq 0.05$). Lactic acid bacteria, Enterobacteriaceae and *B. thermosphacta* remained below the detection limit of 1 or 2 logs cfu/g at both temperatures throughout the experiment.

3.3. NGS analysis

3.3.1. Illumina MiSeq data analysis

In total, 408,832 raw reads were obtained after Illumina's filtering; 110,238 for d 0, 159,302 for 4 °C and 139,292 for 10 °C (Table S1). Of them, after processing and clustering against the reference database (open-reference OTU picking), 89,506, 121,784 and 113,581 sequences were generated for taxonomy assignment.

Alpha diversity indices were calculated separately for each sample and are presented in Table S2. Regarding the Chao1 index analysis revealed that the observed richness was slightly lower in all samples than the aforementioned index, indicating that our analysis captured the most of the bacterial diversity that is present in the samples. In total, 3535 unique OTUs were obtained from the three samples (Table S1). The absolute number of identified OTUs didn't vary among samples; 1603, 1777 and 1691 OTUs were found for d 0, 4 °C and 10 °C, respectively. Clustering overlaps as well as common and unique OTUs per sample are illustrated at species level in Fig. 2. From the 3535 unique OTUs, 314 were common in all samples, while 746, 849 and 718 OTUs were found only in d 0, 4 °C and 10 °C, respectively.

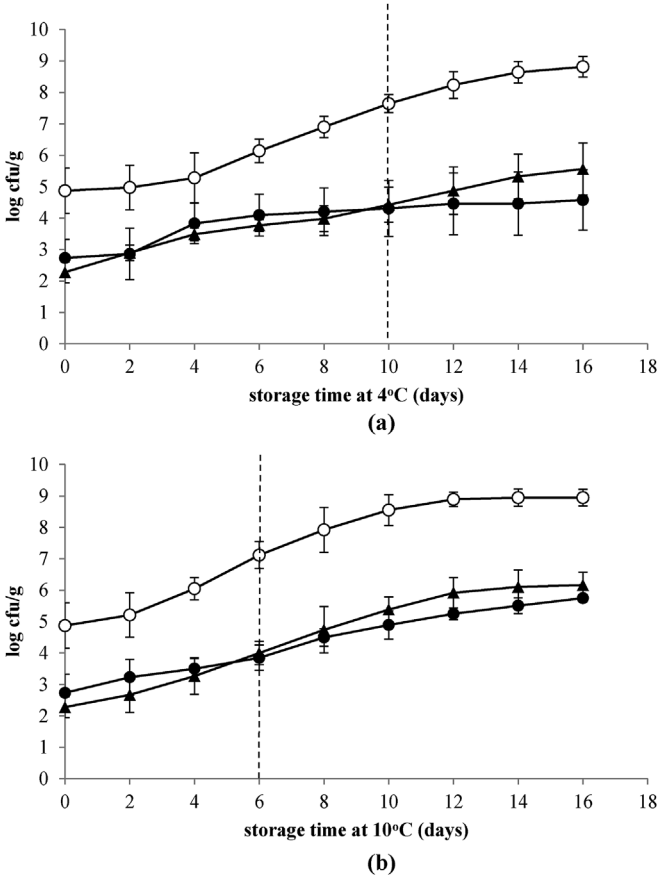

Fig. 1. Microbiological changes during storage of whole blue crab stored at 4 °C and 10 °C. APC (○), *Pseudomonas* spp. (●), H_2S producing bacteria (▲). Each data point and the error bars show the mean ± st. dev. of 4 replicates. The vertical dashed lines indicate the time point of rejection.

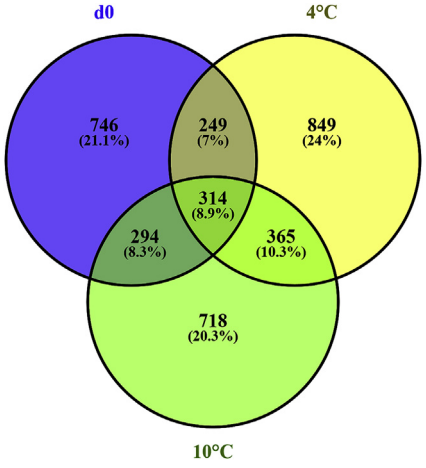

Fig. 2. Venn's diagrams indicating the number of OTUs in blue crabs among different storage temperatures and d 0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3.2. Bacterial diversity

Proteobacteria and Tenericutes, followed by Firmicutes and Actinobacteria, were the main bacterial phyla in fresh raw crabs (d 0). Firstly Proteobacteria and secondly Actinobacteria were found to dominate in stored crabs at the end of shelf-life. Firmicutes were also found at noteworthy abundances in crabs stored at 4 °C (Fig S1.1). Mollicutes (37.0%), Alphaproteobacteria (54.4%) and

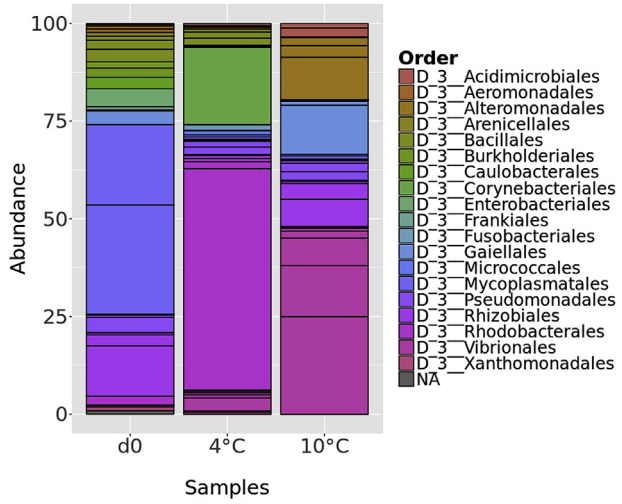

Fig. 3. Relative abundances of top 20 dominant orders assigned to 16S rRNA sequences detected in whole blue crabs. Scale in y axis reflects the normalized abundance percentages (%). Black lines within each bar separates each order into lower taxonomic levels. NA: not available information about their taxonomy at the order level. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

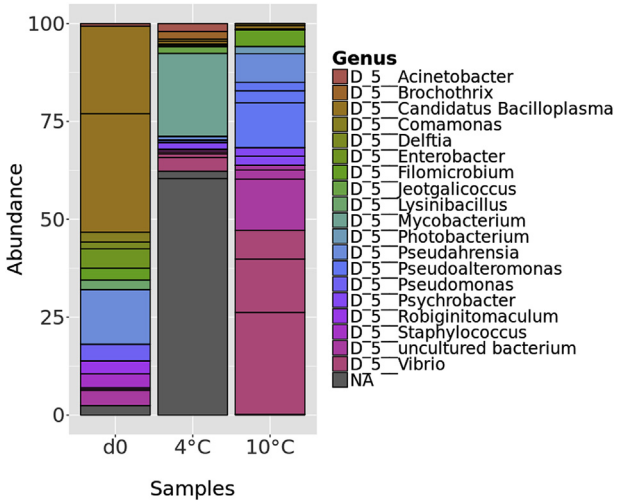

Fig. 4. Relative abundances of top 20 dominant genera assigned to 16S rRNA sequences detected in whole blue crabs. Scale in y axis reflects the normalized abundance percentages (%). Black lines within each bar separates each genus into lower taxonomic levels. NA: not available information about their taxonomy at the genus level. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Gammaproteobacteria (65.4%) were the most abundant taxonomic group at class level in fresh (d 0) and chilled crabs at 4 °C (d 10) and 10 °C (d 6), respectively (Fig S1.2). Alpha-proteobacteria (20.9%) and Gammaproteobacteria (17.0%) were also dominated in fresh crabs, Actinobacteria (21.3%) and Gammaproteobacteria (13.2%) in chilled crabs at 4 °C (d 10), while Thermoleophilia (12.1%) and Alphaproteobacteria (11.5%) dominated at 10 °C (Fig S1.2).

Mycoplasmatales, Rhodobacterales, Vibrionales, Rhizobiales, Gaiellales Alteromonadales, Corynebacteriales were the most abundant orders in crabs (Fig. 3). Mycoplasmataceae (37.0%), Rhodobacteraceae (53.0%) and Vibrionaceae (43.0%) were the most abundant families in crabs at d 0, d 10 and d 6 respectively (Fig S1.3). Bacteria of the Phyllobacteriaceae family were also found at noteworthy abundances in crabs at d 0, while Mycobacteriaceae and Pseudoalteromonadaceae were found in crabs stored at 4 and 10 °C, respectively (Fig S1.3).

Candidatus Bacilloplasma, unknown bacteria of Rhodobacteraceae family and *Vibrio* were the most abundant genera found on fresh (d 0) and chilled crabs at 4 (d 10) and 10 °C (d 6), respectively (Fig. 4). *Pseudahrensia* (10.1%), *Pseudomonas* (4.4%), *Enterobacter* (3.6%), *Staphylococcus* (3.4%) and other genera e.g. *Delftia*, *Filomicrobium*, *Shewanella, Acinetobacter, Comamonas, Stenotrophomonas* (< 3.5%) were also detected in crabs at d 0 (Fig. 4). Under cold storage conditions, *Mycobacterium* (17.9%), *Vibrio* (4.7%) and other genera e.g. *Psychrobacter, Pseudomonas, Acinetobacter, Photobacterium* (< 3.5%) were detected at 4 °C, while unknown bacteria (16.0%), *Pseudoalteromonas* (15.0%), *Pseudahrensia* (6.20%), *Psychrobacter* (5.30%) and other genera e.g. *Shewanella, Photobacterium* were found at lower abundances at 10 °C (Fig. 4).

At species level, the highest percentage of bacteria was defined either as uncultured (70.3% in crabs at d 0 and 64.7% in crabs stored at 10 °C) or unknown bacteria (unknown bacteria of *Mycobacterium* and Rhodobacteraceae family in crabs stored at 4 °C) (Fig S1.4). Among others, *Listeria monocytogenes, Pseudomonas aeruginosa, Enterobacter hormaechei* and *Stenotrophomonas maltophilia* were detected at d 0 (Fig S1.4). All bacteria at species level are presented in Table S3.

3.4. Determination of pH, TVB-N & TMA-N changes

The initial pH was 7.14 ± 0.02. At the time point of rejection (d 10 and d 6 for 4 and 10 °C, respectively), the pH value did not differ ($p > 0.05$) between crabs stored at 4 °C (7.35 ± 0.10) and 10 °C (7.28 ± 0.18). At the end of the experiment, pH reached the levels of 7.98 ± 0.09 (d 16) and 8.52 ± 0.12 (d 12) for 4 and 10 °C, respectively ($p \leq 0.05$).

TVB-N and TMA-N changes are shown in Fig. 5. TVB-N and TMA-N were initially found at the levels of 50.79 ± 2.88 and

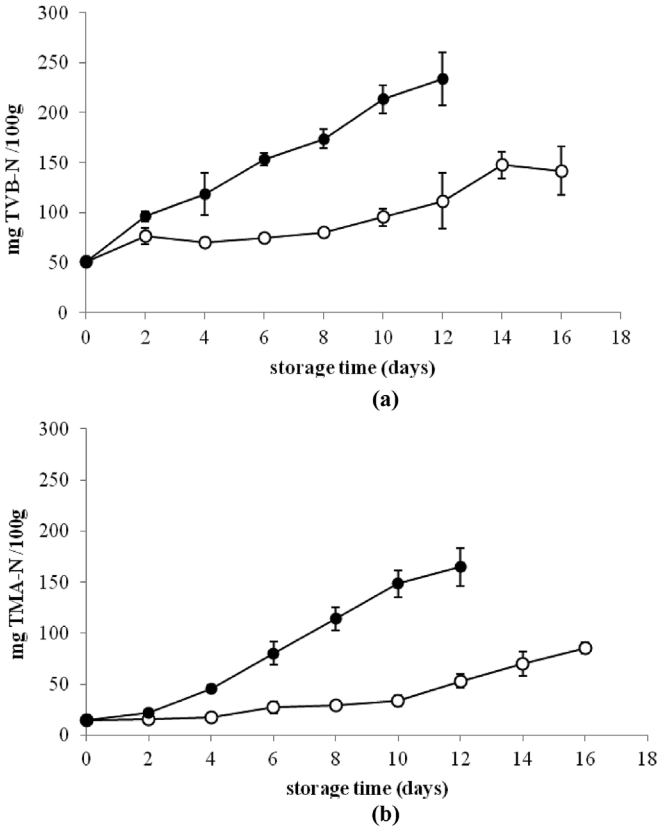

Fig. 5. TVB-N (a) and TMA-N (b) changes of whole blue crab stored at 4 (○) and 10 °C (●). Each data point and the error bars show the mean and ± st. dev. of 4 replicates.

Table 1
Detected VOCs and their relative concentrations (surface x 10^{-6} under the chromatographic peak) in whole blue crab (in days) stored at 4 °C and 10 °C. Each value is the mean of duplicate measurement.

VOCs	relative concentration (area X 10^{-6})				
	4 °C			10 °C	
	d 0	**d 4**	**d 10**	**d 4**	**d 6**
Alcohols					
ethanol	145.8	45.24	129.0	151.8	188.9
3-methyl-1-butanol	3.26	0.43	1.60	5.12	0.78
2-methyl-1-butanol		0.71	1.16		
2-ethyl-1-hexanol	0.29	0.78	1.56	1.95	0.17
1-octen-3-ol	16.8	49.06	124.3	110.8	140.8
1-penten-3-ol	11.7	66.85	67.2	61.50	15.7
2-penten-1-ol	0.37	2.49	5.46		0.71
2-octen-1-ol	0.16	1.33	7.36	11.84	10.54
1-pentanol	3.97	10.88	19.8	11.54	4.34
1-hexanol	0.08	0.20	20.4	4.30	4.11
1-heptanol	0.11	12.0	2.97	45.41	0.23
1-octanol		10.36	0.36	67.88	2.00
2-hexen-1-ol (*trans*)		1.39	0.46	2.84	0.10
cis-6-nonen-1-ol				7.61	0.17
Aldehydes					
2-methylbutanal		0.26	0.44	0.28	1.29
3-methylbutanal		0.25	1.39	0.33	0.11
hexenal		5.64	20.5	4.97	4.22
heptanal		0.24	3.00	0.50	
octanal		0.31		1.65	
nonanal			3.83		
cis-4-heptenal			2.55		1.30
trans-2-heptanal	1.28	9.65			1.43
trans-2-octenal	6.05	2.11	26.7	44.42	63.12
trans,trans-2,4-hexadienal					0.44
trans,trans-2,4-heptadienal	1.05	5.98	7.01	15.87	14.18
trans,trans-2,4-decadienal	0.86			4.74	1.25
Ketones					
acetone	1.63	3.01	6.70	1.58	4.83
acetophenone	0.18		0.17	0.26	0.45
2-butanone	0.31	1.23	3.38	1.26	6.53
2-pentanone	0.20	0.99	1.67	1.09	0.69
3-pentanone		1.00	2.67	2.21	6.29
2-hexanone	0.07	0.08	0.24		0.52
2-heptanone		1.44	5.96	6.29	6.85
2-octanone	0.16	0.20	3.65	3.40	1.34
2-nonanone	3.32	4.00	10.3	24.39	5.13
2-undecanone				5.64	1.87
3-octanone	0.12	0.86	3.39	3.21	1.61
2,3-pentanedione	0.10	2.22	1.20	2.05	0.96
2,3-octanedione	1.26	5.48	0.10	7.10	10.64
4-methyl-2-pentanone	0.07	0.08	0.16		
3,5-octadien-2-one	0.53	15.5	15.1	12.42	2.30
trans,trans-3,5-octadien-2-one				21.63	4.45
3-methyl-2-pentanone			0.42		
Esters					
ethyl propionate	1.02	0.44	0.20	1.55	5.15
ethyl isobutyrate	0.09	0.19	0.66	0.19	4.10
ethyl acetate			5.63		35.4
ethyl butyrate	0.06	0.20	4.19	0.17	0.63
ethyl-2-methylbutyrate	1.35	2.36	5.85	4.23	23.50
ethyl isovalerate	2.67	4.19	20.02	9.02	37.7
ethyl valerate	0.03	0.37		0.65	2.20
ethyl heptanoate			1.30	2.65	2.31
ethyl octanoate	0.09			4.16	5.13
ethyl nonanoate				0.60	1.39
Hexanoic acid, ethyl ester	0.84	1.84	7.21	4.64	23.9
Sulphur compounds					
dimethyl disulfide	0.35	0.11	0.66	0.05	0.51
Organic acids					
Acetic acid			1.04		0.28
Aromatic compounds					
Indole		2.73	22.0	10.89	19.91

14.7 ± 3.01 mg N/100 g respectively. At 4 °C, TVB-N was 95.26 ± 8.85 after 10 days, while at 10 °C TVB-N reached levels as high as 152.8 ± 6.37 mg N/100 g after 6 days of storage period ($p \leq 0.05$). At the end of experiment, TVB-N at 4 °C was 141.74 ± 24.12 mg N/100 g while at 10 °C was 233.78 ± 26.47 mg N/100 g ($p \leq 0.05$) (Fig. 5). At 4 °C, TMA-N value reached the levels of 34.1 ± 5.23 and 85.5 ± 5.14 mg N/100 g at d 10 and d 16, respectively, while at 10 °C the levels were as high as 80.2 ± 11.49 (d 6) and 164.8 ± 18.61 mg N/100 g (d 12) (Fig. 5).

3.5. Headspace SPME-GC/MS analysis of VOCs

The profile of 57 VOCs, consisted of 14 alcohols, 12 aldehydes, 17 ketones, 11 esters, one sulphur compound (dimethyl disulfide), one organic acid (acetic acid) and one aromatic compound (indole) during storage, is shown in Table 1.

The majority of the compounds were detected initially (d 0) and increased during storage. In particular, some alcohols such as 2-methyl-1-butanol, 3-methyl-1-butanol, 1-penten-3-ol, aldehydes such as 3-methylbutanal, heptanal and ketones such as 2-pentanone, 2-hexanone, 2-nonanone, increased during the storage at 4 °C, while the esters ethyl propionate, ethyl valerate, ethyl heptanoate, ethyl octanoate, ethyl nonanoate were increased at 10 °C. The molecules, 1-octen-3-ol, 2-octen-1-ol, 1-hexanol from alcohols, 2-methylbutanal, *trans*-2-octenal, *trans,trans*-2,4-heptadienal from aldehydes, acetone, 2-butanone, 3-pentanone, 2- heptanone from ketones, ethyl isobutyrate, ethyl butyrate, ethyl-2-methylbutyrate, ethyl isovalerate, hexanoic acid ethyl ester from esters and indole from aromatic compounds showed a tendency to increase at both storage temperatures (Table 1).

Some other compounds such as 3-methyl-1-butanol, 1-heptanol, 1-octanol, dimethyl disulfide, 2,3-pentanedione fluctuated during storage at both temperatures, while others appeared exclusively at the rejection time of crabs stored at 4 °C (e.g. nonanal, 3-methyl-2-pentanone, ethyl heptanoate), at 10 °C (e.g. *trans, trans*-2,4-hexadienal) or both cases (e.g. *cis*-4-heptenal, ethyl acetate, acetic acid).

In many cases, VOCs relative values at the rejection time for crabs stored at 10 °C were two or more times higher than those stored at 4 °C, particularly in the case of esters and some other compounds such as 2-methylbutanal, *trans*-2-octenal, *trans, trans*-2,4-heptadienal, 2-butanone, 3-pentanone, 2-hexanone and 2,3-octanedione (Table 1).

4. Discussion

The suppression of crab immune system, which usually occurs due to the oxygen deprivation or other causes e.g. starvation and stress (Jacklin and Combes, 2007), is probably responsible for bacterial growth and spoilage of alive crabs (Robson et al., 2007). According to our findings, the storage temperature affected microbial growth, composition of bacterial communities and VOCs profile.

APC has been found to range from 2.6 to 2.8 log cfu/g in the muscle of live crabs *Carcinus maenas*, *Necora puber* and *Cancer pagurus* while microbial population of 5.0 logs cfu/g has been found at their rejection (Robson et al., 2007). In our study, the APC in the edible tissues (white muscle and internal edible organs excluding digestive gland and stomach) of whole blue crabs was initially 4.87 log cfu/g, and increased during the chill storage to levels as high as 7.5 logs at the rejection time point. The microbiological spoilage level usually varies from 7 to 9 logs cfu/g in fish (Gram and Huss, 1996) and 5 to 6 log cfu/g in crustaceans (Boziaris et al., 2011; Robson et al., 2007). Seafood spoilage depends on spoilage potential and activity of the Specific Spoilage Organisms (SSOs) (Parlapani and Boziaris, 2016). However, in this study, using classical microbiological analysis the usual spoilage microorganisms of seafood e.g. *Pseudomonas*, H_2S producing bacteria, lactic acid bacteria, Enterobacteriaceae and *B. thermosphacta* did not reach such high levels. In addition, the abundances of these microorganisms based on the results derived by NGS analysis were extremely low (less than 1%) at both

temperatures tested. Bacteria of the Rhodobacteraceae family, which dominated on chilled crabs at low temperature and had been found in intestines of Chinese mitten crab *Eriocheir sinensis* (Chen et al., 2015; Li et al., 2007), have never been reported as spoilage microorganisms for seafood or other foods. On the other hand, microorganisms such as *Pseudoalteromonas* and *Psychrobacter,* which spoil chilled brown shrimp (*Crangon crangon*) (Broekaert et al., 2013) and Norway lobster (*Nephrops norvegicus*) (Bekaert et al., 2015), and *Vibrio* (the most abundant at the crab's rejection time at 10 °C) which spoil Nile perch *(Lates niloticus)* stored at elevated temperatures (Gram et al., 1987, 1990), were found to be a major part of the crab microbiota at the rejection time point at 10 °C. *Vibrio* spp. is the most abundant bacterial species present in blue crabs *C. sapidus* (Shields and Overstreet, 2003; Sizemore et al., 1975), and cannot grow at temperatures below 8 °C, but they can grow at higher temperatures such as 10 °C (Burnham et al., 2009). Regarding 4 °C, other spoilage mechanisms or bacteria that have not been studied so far, might be responsible for the spoilage of blue crabs.

The initial microbiota of blue crab consisted mainly of *Candidatus* Bacilloplasma. This microorganism has been also found in intestinal tract of farmed Chinese mitten crab *Eriocheir sinensis* (Chen et al., 2015; Zhang et al., 2016) and in the gut of other crustaceans such as *Porcellio scaber* (Kostanjsek et al., 2007), *Armadillidium vulgare* (Dittmer et al., 2016), shrimps (Chen et al., 2017) and Norway lobster (Meziti et al., 2010). In addition, some species found herein are opportunistic pathogens involved in various human diseases e.g. bacteraemia, diarrhea, or associated with nosocomial infections (mainly *A. baumannii* and *P. aeruginosa*) with high mortality rates (Doughari et al., 2011; Kim et al., 2016). *E. hormaechei* (which was found at high abundances at d 0) and *E. cloacae* are clinically important species. The outbreak of *E. hormaechei* can occur among vulnerable, low-birth-weight premature infants (Wenger et al., 1997), while *E. cloacae* is responsible for a number of bloodstream infections (Fernandez-Baca et al., 2001). Among the detected microorganisms, *L. monocytogenes* is one of the most important food-borne pathogen due to its ability to grow well in crab meat stored under refrigerated temperatures (Rawles et al., 1995). Although *L. monocytogenes* contaminates foods at low population numbers, a high number of listeriosis outbreaks and high mortality rate occurs mainly in immunocompromised individuals (Jami et al., 2014). All these bacteria can be inactivated by applying the adequate cooking temperatures. The heating process has to succeed a minimum internal temperature of 74 °C while the flesh has to become pearly and opaque. A likely consumption of undercooked crabs can cause serious foodborne infections. Foodborne infections from undercooked crabs occurred in the USA. The first confirmed food-borne outbreak (gastroenteritis) due to *Vibrio parahaemolyticus* in the USA, involved in consumption of crabs, was recorded in Maryland during August 1971 (Molenda et al., 1972). *Vibrio cholerae* 01 has also been isolated from a patient who consumed crab meat, again in Maryland during October 1984 (Lin et al., 1986). Other cases of cholera have been identified in patients who consumed cooked crab meat in Texas and Louisiana until 1981 (Lin et al., 1986). None of the aforementioned pathogenic *Vibrio* was found in our study. However, species such as *V. parahemolyticus*, *V. cholerae* and *Vibrio vulnificus* have frequently been found in blue crabs (Davis and Sizemore, 1982; Rodgers et al., 2014; Shields and Overstreet, 2003; Sizemore et al., 1975; Wang, 2011).

Other microorganisms found herein, have also been found in crabs in previous studies. *Acinetobacter* spp. have been found in *C. sapidus* collected from Florida (Shields and Overstreet, 2003), farmed Chinese mitten crab *Eriocheir sinensis* (Li et al., 2007), *Chionoecetes opilio* and *Chionoecetes* sp. (Kim et al., 2013). *Acinetobacter* belongs to the main part of the initial microbiota of fish from sea waters such as sea bream (Parlapani et al., 2013). *Pseudomonas* spp. have also been found in crab *Chionoecetes opilio* (Kim et al., 2013) and farmed Chinese mitten crab *Eriocheir sinensis* (Chen et al., 2015; Li et al., 2007). *Pseudomonas* spp. (e.g. *P. fragi*) are the major spoilers in aqua-cultured fish e.g. sea bream, sea bass, from the Mediterranean Sea, stored aerobically at low temperatures (Parlapani and Boziaris, 2016; Parlapani et al., 2013, 2015a, 2015b). Other bacteria present in fresh or spoiled fish/seafood have also been isolated from crabs e.g. *Psychrobacter* spp. from *C. sapidus* (Shields and Overstreet, 2003) and *Chionoecetes* sp. (Kim et al., 2013), *Pseudoalteromonas*, *Photobacterium* and/or *Shewanella* from horseshoe crabs *Tachypleus gigas* and *Carcinoscorpius rotundicauda* (Ismail et al., 2015) and mud crab *Scylla paramamosain* (Li et al., 2012). *Pseudomonas*, *Psychrobacter*, *Photobacterium*, *Pseudoalteromonas* and *Shewanella* have been associated with spoiled fish (Bekaert et al., 2015; Broekaert et al., 2013; Dalgaard et al., 1993; Parlapani and Boziaris, 2016; Parlapani et al., 2015a, 2015b; Rudi et al., 2004). In this study, the aforementioned bacteria were found at low abundances (< 3.5%), apart from *Pseudoalteromonas* (15% of the total microbiota at 10 °C), which have been reported as spoilage microorganisms in cooked shrimps (Broekaert et al., 2013; Macé et al., 2014).

In contrast to fish products, TVB-N and TMA-N levels in crab tissues often exceed the legislation limit of 35 and 10 mg N/100 g (EC, 1991, 1995). However, no defined limits regarding TVB-N and TMA-N have been set for crustaceans. In our study, TVB-N and TMA-N were approx. 51 and 15 mg N/100 g in whole blue crab at the beginning of the experiment, respectively, while they reached high levels during the storage. According to the literature, initial TVB-N ranges from ≈50 to 80 mg N/100 g and TMA-N from ≈25 to 30 mg N/100 g muscle in processed crab meat *Cancer pagurus* (Anacleto et al., 2011). In precooked crab meat of *Portunus pelagicus*, TVB-N initially has been found at the level of 25.67 mg N/100 g, and reached values between 46.12 and 89.21 mg N/100 g after 38–40 days and 214.25 mg N/100 g after 57 days of storage at 4 °C (Ayas et al., 2012). The high TVB-N and TMA-N values occur due to the metabolic activity of spoilage microorganisms, which metabolize the high quantities of free amino acids and nitrogenous compounds present in crustacean meat (Anacleto et al., 2011). In the present study, TVB-N and TMA-N values increased during storage of whole crabs. However, spoilage microorganisms such as *Pseudomonas* and *Shewanella,* which produce the aforementioned compounds, were found at low population levels. This indicates that other microorganisms or other mechanisms e.g. enzymatic activity, might be responsible for the production of TVB-N and TMA-N, which is something that has to be elucidated in a future study.

Production of VOCs such as 1-penten-3-ol, 1-octen-3-ol, *trans*-2-octenal, *cis*-4-heptenal, *trans, trans*-2,4-heptadienal, 1-hexanol, heptanal, is associated with chemical activity (Duflos et al., 2006; Leduc et al., 2012; Parlapani et al., 2017). On the other hand, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-methylbutanal, 2-methylbutanal, 2-butanone, 2-pentanone, 3-pentanone, 2-hexanone, 2-heptanone, acetone and ethyl esters such as ethyl acetate, ethyl butyrate, ethyl propionate, ethyl isobutyrate, ethyl-2-methylbutyrate, ethyl isovalerate, are involved with metabolic activity of fish spoilage microorganisms during storage (Boziaris and Parlapani, 2016). The majority of the aforementioned compounds were increased during crab storage. Many of them such as ethanol, 2-methylbutanal, 3-methylbutanal, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-butanone and ethyl esters have been proposed as potential spoilage markers in various fish/seafood (Boziaris and Parlapani, 2016). Indole has also been found to increase in blue crab meat caught from the Chesapeake Bay (East coast, USA) and stored at 4 °C, making it a useful spoilage marker for the product (Sarnoski et al., 2010). In the present study, a plethora of volatiles e.g. 1-octen-3-ol, *trans*-2-octenal, *trans,trans*-2,4-heptadienal, 2-butanone, 3-butanone, 2-heptanone, ethyl isobutyrate, ethyl acetate, ethyl-2-methylbutyrate, ethyl isovalerate, hexanoic acid ethyl ester and indole could be proposed as spoilage markers of chill-stored blue crab considering that they changed significantly between the initial and rejection time point.

5. Conclusions

The storage temperature affected the microbial growth, the composition of bacterial communities and the profile of VOCs in blue crabs.

Potential pathogens such as *L. monocytogenes, P. aeruginosa* and *E. hormaechei* which are usually present in low abundances in foods, and seafood spoilage associated bacteria such as *Pseudomonas*, *Pseudoalteromonas, Shewanella, Psychrobacter* and *Photobacterium*, were detected. At the point of rejection, the dominant bacteria in blue crabs were the Rhodobacteraceae family at 4 °C and *Vibrio* spp. at 10 °C. Thus, the 16S amplicon meta-barcoding NGS provided valuable information of the microbiological quality and safety of blue crabs. Moreover, apart from the development of TVB-N and TMA-N, a plethora of specific VOCs such as 1-octen-3-ol, *trans*-2-octenal, *trans,trans*-2,4-heptadienal, 2-butanone, 3-butanone, 2-heptanone, ethyl isobutyrate, ethyl acetate, ethyl-2-methylbutyrate, ethyl isovalerate, hexanoic acid ethyl ester and indole, might be used as potential spoilage markers of chill stored blue crab.

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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.2019.03.011.

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