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HRM analysis as a tool to facilitate identification of bacteria from mussels during storage at 4 °C

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ABSTRACT

High-resolution melting (HRM) analysis followed by sequencing was applied for determination of bacteria grown on plates isolated from farmed mussels (*Mytilus galloprovincialis*) during their storage at 4 °C. The V3–V4 region of the 16S rRNA gene from the isolates was amplified using 16S universal primers. Melting curves (peaks) and high resolution melting curves (shape) of the amplicons and sequencing analysis were used for differentiation and identification of the isolated bacteria, respectively. The majority of the isolates (a sum of 101 colonies, from five time intervals: day 0, 2, 4, 6 and 8) from non-selective solid medium plates were classified in four bacterial groups based on the melting curves (peaks) and HRM curves (shape) of the amplicons, while three isolates presented distinct HRM curve profiles (single). Afterwards, sequencing analysis showed that the isolates with a) the same melting peak temperature and b) HRM curves that were > 95% similar grouped into the same bacterial species. Therefore, based on this methodology, the cultivable microbial population of chill-stored mussels was initially dominated by *Psychrobacter alimentarius* against others, such as *Psychrobacter pulmonis*, *Psychrobacter celer* and *Klebsiella pneumoniae*. *P. alimentarius* was also the dominant microorganism at the time of the sensory rejection (day 8). Concluding, HRM analysis could be used as a useful tool for the rapid differentiation of the bacteria isolated from mussels during storage, at species level, and then identification is feasible by the sequencing of one only representative of each bacterial species, thus reducing the cost of required sequencing.

1. Introduction

The determination of seafood quality from their harvest to consumer table is of high importance for the aquaculture sectors, fishery trade associations, food industry and food authorities. The enumeration of Aerobic Plate Counts (APC) or alternatively Specific Spoilage Organisms (SSOs) is a common way to evaluate freshness of stored seafood. SSOs constitute a small fraction of the initial total microbiota which grows faster than the rest microorganisms during storage reaching higher population densities and produces metabolites that cause the organoleptic rejection of the product (Boziaris and Parlapani, 2016; Gram and Huss, 1996). In this context, the monitoring of the diversity and dynamic of SSOs lets us know which microorganisms prevail against others under the specific storage conditions, e.g., temperature (Parlapani et al., 2015b). Using plate-based against culture-independent approach, we have the advantage to isolate microorganisms for further studies regarding their spoilage potential and activity which can lead to the characterization of them as key players of seafood spoilage (Parlapani et al., 2017).

Culture-dependent identification of seafood microbiota has been traditionally studied by phenotypic and biochemical tests, however these are laborious and usually lack discriminatory power giving failed results (Nisiotou et al., 2014). For these reasons, other, alternative, rapid, accurate and reliable methodologies have been developed. The 16S rRNA gene sequence analysis has been conducted by analyzing (amplification and sequencing) numerous isolates e.g., 50% or more of the total colonies grown on plates (Parlapani and Boziaris, 2016; Parlapani et al., 2015a, 2015b). However, sequencing analysis of 16S

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rRNA gene amplicons is a quite expensive method when a large number of isolates has to be sequenced. A plethora of studies uses molecular fingerprinting methodologies, e.g. T-RFLP (Rudi et al., 2004), DGGE (Broekaert et al., 2013; Hovda et al., 2007a, 2007b; Svanevik and Lunestad, 2011), TTGE (Jaffrès et al., 2009; Macé et al., 2012), followed by sequencing of the 16S rRNA gene amplicons, for the differentiation and identification of isolates coming from seafood. In these electrophoresis approaches, T-RFLP separates products by size, whereas DGGE and TGGE separate products by sequence composition (GC-content).

A PCR based technique the High-Resolution Melting (HRM) analysis is a closed-tube method which is used for the detection of DNA sequence variation as low as one nucleotide, by showing fluorescence changes in the melting profile of the amplified double-stranded DNA amplicon (Taylor, 2009). The HRM Curve profile of the PCR product is influenced by the length, the GC content and the actual sequence of the product (Ririe et al., 1997). HRM has been successfully applied for mutation identification, single nucleotide polymorphism (SNP) genotyping and the identification and discrimination of different fungi and bacteria species, including screening for RIF and INH resistance in *Mycobacterium tuberculosis* (Ganopoulos et al., 2012; Pietzka et al., 2009; Ramirez et al., 2010). The advantages of HRM are its simplicity, accuracy, it is closed-tube, low cost and highly sensitive, and specific approach (Vossen et al., 2009).

HRM analysis has been applied for the detection of foodborne pathogens such as *Salmonella* spp. (Liu et al., 2018a; Omiccioli et al., 2009; Pei Hua et al., 2014), *Listeria* spp (Jin et al., 2012), *Listeria monocytogenes* (Omiccioli et al., 2009; Pei Hua et al., 2014), *Shigella*, *Staphylococcus aureus*, *Vibrio parahaemolyticus* (Pei Hua et al., 2014), *Escherichia coli* O157 (Liu et al., 2018b; Omiccioli et al., 2009), diarrhoeagenic *E. coli* (Pei Hua et al., 2014) and emetic and enterotoxin producing *Bacillus cereus* (Forghani et al., 2016). In addition, HRM has been also used for the differentiation and identification of lactic acid bacteria isolated from poultry carcasses (Sakaridis et al., 2014), black aspergilli from grapes (Xanthopoulou et al., 2018) and microbial composition (lactic acid bacteria) during sourdough fermentation (Lin and Gänzle, 2014).

The aim of this study was to differentiate and identify bacteria isolated from mussels during storage at 4 °C. HRM analysis was used for the differentiation of bacteria grown on plates, while sequencing analysis of V3–V4 region of the 16S rRNA gene was used for the identification of bacteria. To the best of our knowledge this is the first time that HRM analysis followed by sequencing was used in order to determine seafood total microbiota grown on general culture media.

2. Materials and methods

2.1. Provision and storage of mussels

Mussels (N ≈ 250, of commercial size) were collected from a Greek farm at Halastra region (Thessaloniki, N. Greece) in June 2017. Samples were placed on a portable refrigerator and transferred at the Lab. of Marketing and Technology of Aquatic Products and Foods of the Department at Volos within 2 h from sampling. The dead mussels were subsequently discarded. The flesh meat from the remaining mussels was removed from the shell with a sterile knife. Then, 200 g flesh from approx. 10 individuals were packaged in plastic pouches and sealed. The samples were stored in incubator operating at 4 °C until the end of the experiment.

2.2. Rejection time of mussels

The rejection time point was set when at least one of five trained panelists indicated unpleasant off-odors, e.g. ammoniacal, sour, spoiled or putrid according to FAO (1999). Sensory evaluation was not performed to describe the sensory quality changes during storage but only to determine the rejection time point for analyzing the microbiota of samples until the end of the product's shelf-life.

2.3. Microbiological analysis

All microbiological media were obtained from LAB M (Lancashire, UK). Iron Agar (IA) was prepared according to Gram et al. (1987).

Twenty-five (25) g samples were mixed with 25 ml of MRD (Maximum Recovery Diluent, 0.1% w/v peptone, 0.85% w/v NaCl) and homogenized for 1 min in a laboratory blender. The homogenates were transferred to stomacher bags with 200 ml of MRD and homogenized for 1 min using a Stomacher (Bug Mixer, Interscience, London, UK) in order to have the 1st dilution. Spread plating method (0.1 ml of serial dilutions in MRD) was used for enumeration of the following microorganisms: (a) aerobic plate count (APC) on TSA (Tryptone Soy Agar), incubated for 48–72 h at 25 °C and (b) *Pseudomonas* spp., on cetrimide-fucidin-cephaloridine agar (CFC), incubated for 48 h at 25 °C. Pour plating method (1 ml of serial dilutions in MRD) was used 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 3 replicates.

The microbial population changes against storage time were fitted using Baranyi equation (Baranyi and Roberts, 1994), $y(t) = y_{\max} - \ln\left[1 + \left(e^{-y_{\max}-y_0} - 1\right) e^{\mu_m A_n(t)}\right]$, where: y(t) is the logarithm of population at time t, y_{max} is the logarithm of maximum population, y_o is the logarithm of initial population, μ_m is the maximum specific growth rate and $A_n(t)$ is a function related to the physiological state of the cells. DMFIT software (Institute of Food Research, Reading, UK) was used for fitting.

2.4. Identification of bacteria isolated from farmed mussels

2.4.1. Colonies isolation and DNA extraction

Colonies isolated from TSA plates during mussels' storage at 4 °C were taken for bacterial genotyping and identification. More than the 50% of the colonies (from 30 to 300 colonies in each plate) were selected randomly from the highest dilution of TSA plates after incubation at 25 °C for 48–72 h. Before experimental use each isolate was subcultured in TSA and incubated at 25 °C for 24–48 h.

Subsequently, culture in Luria-Bertani broth followed for each isolate. One milliliter of each cells suspension was used for DNA extraction by briefly modifying the method described by Cocolin et al. (2002). After a first centrifugation at 14,000 *g* at 4 °C for 10 min, 500 μl of lysozyme buffer solution (25% [wt/vol] sucrose, 5 mg of lysozyme/ml) was added and followed a second centrifugation at 14,000 *g* at 4 °C for 10 min. Then, the supernatant was discarded and the pellet was resuspended in 50 μl of lysozyme (5 mg/ml) followed by incubation at 37 °C for 30 min. The cells were mixed with 300 μl of a lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8), 300 μl phenol-chloroform-isoamyl alcohol (25:25) and 0.3 g of glass beads. Three 30 s treatments followed, with a 15 s break between them, at maximum speed in the Mini-Bead Beater (Retsch MM300, Haan Germany). Furthermore, 300 μl of 10 mM Tris–5 mM EDTA (pH 8) were added and followed by a centrifugation at 12,000 *g* at 4 °C for 10 min. Then, the upper phase was transferred in a clear tube where 1 ml of ethanol was added and followed by a centrifugation at 14,000 *g* for 10 min. After that, the supernatant was discarded and the DNA pellets were dried at room temperature for 5 min. Finally, 100 μl of Tris-EDTA (TE) 1X and 2 μl RNase solution (10 mg/ml) were added and tubes were incubated at 37 °C for 1 h.

Samples were subsequently stored at −20 °C for the 16S rRNA gene analysis by HRM.

2.4.2. Differentiation of bacteria by HRM analysis

The melting curve analysis of the amplicons classifies the colonies into different group species generating distinct HRM curve profiles (Sakaridis et al., 2014). Based on this proven scenario, HRM analysis was used for the differentiation of the colonies isolated from TSA plates. For PCR amplification, V3-V4 region of the 16S rRNA gene was amplified using the bacterial Forward (F) primer S-DBact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and Reverse (R) primer S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) resulting in fragments of approx. 450 bp (Klindworth et al., 2013). PCR amplification was performed in a total volume of 20 μL on a Rotor-Gene 6500 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia). The reaction mixture contained 1 X PCR buffer B (Kapa), 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM F and R primers, 1.2 μM Syto 9 green fluorescent nucleic acid stain, 1U/ml KAPA Taq DNA polymerase (Invitrogen, UK) and 20 ng genomic DNA. A rapid PCR protocol was conducted in a 36-well or 72-well carousel using a pre-PCR step at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and elongation at 72 °C for 20 s. PCR was adjusted to 40 cycles. HRM analysis was performed with a pre-melt step at 70 °C for 30 s followed by a melt step from 75 to 92 °C with 0.1 °C increments every 2 s. The size of the PCR products was analyzed on a 2% agarose gel. All experiments were performed in three technical replicates.

The resulting melting profiles and the % similarity between reference genotype and the other members of each bacterial group (genotypes) were analyzed by the Rotor-Gene Q Series Software (version 2.0.2, QIAGEN).

2.4.3. Sequencing

Sequence data were obtained by ABI Prism 3730 XL Capillary Sequencer (VBC-BIOTECH Vienna, Austria). Each sequence read was ca. 450 bp, and for each individual sample, forward and reverse reads were assembled. For the detection of closest relatives, all sequences were compared with the BLAST function (Zhang et al., 2000), http://www.ncbi.nlm.nih.gov/BLAST/). Sequence data were aligned using the ClustalW2 aligning utility (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and phylotypes were defined as sequences showing ≥98% homology to each other. Sequences of dominant phylotypes found in this study are presented as supplementary data.

3. Results

3.1. Microbial populations

APC of mussels was initially 4.22 ± 0.29 log cfu/g and increased during the storage reaching 7.63 ± 0.34 log cfu/g at the time of minimum acceptability level (day 6) and 7.95 ± 0.34 log cfu/g at the rejection time point (day 8). Counts on CFC, IA and MRS were 3.99 ± 0.23, 3.03 ± 0.35 and 2.48 ± 0.45 log cfu/g respectively, at day 0, while VRBGA counts were below detection limit of 1 log throughout the experiment. Counts on CFC were also higher than IA and MRS throughout the storage reaching the level of 7.45 ± 0.29 and 7.74 ± 0.23 log cfu/g at day 6 and 8, respectively. The population on IA was 5.52 ± 0.42 and 6.37 ± 0.55 log cfu/g, while MRS counts were 4.44 ± 1.34 and 5.32 ± 0.78 log cfu/g at day 6 and 8, respectively (Fig. 1).

3.2. Identification of mussels' microbiota grown on TSA

A sum of 101 isolates, from five time intervals: day 0, 2, 4, 6 and 8 from TSA plates (~30 colonies from each plate) were used for the differentiation and identification of mussels' microbiota. The isolates were classified into four different bacterial groups based on both the melting peak temperature and the shape of normalized melting curves of the amplicons using HRM analysis, while three isolates presented distinct HRM curve profiles as 'single' (Table 1; Fig. 2). The normalized

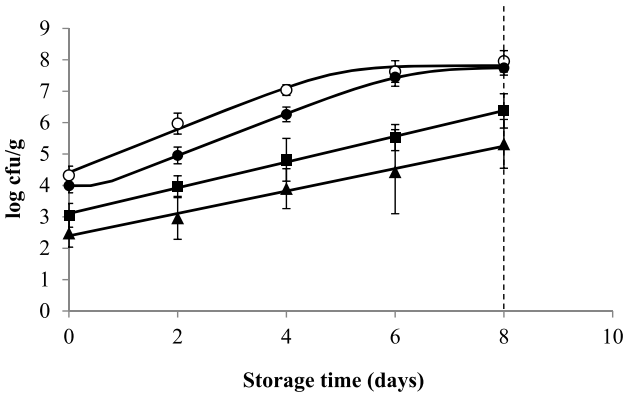

Fig. 1. Microbiological changes during storage of mussels at 4 °C. APC (○), counts on CFC (●), IA (■) and MRS (▲). Each data point and the error bars show the mean and ± st. dev. of 3 replicates. The curves represent the data fitting using Baranyi equation (Baranyi and Roberts, 1994). The vertical dashed lines indicate the time point of organoleptic rejection.

HRM curves showed that isolates can be more easily discriminated and differentiated based on the shape of their HRM curves compared to the conventional melting curves profile (Fig. 2). The amplicons can be differentiated based on the shape of HRM curves (Figs. 2 and 3) even when they present the same melting peak temperature (Table 1). Their identification using 16S rRNA gene sequencing revealed that the amplicons with the same melting peak temperature and same normalized melting curves shape were indeed grouped into the same bacterial species (Table 1; Fig. 2). The seven HRM curves were found to represent seven bacterial species based on 16S rRNA gene sequencing, particularly four *Psychrobacter* species (*Psychrobacter* sp., *P. alimentarius, P. pulmonis* and *P. celer*), *Klebsiella pneumoniae, Oceanimonas smirnovii* and one *unknown* bacterium (named MUSNGR154 phylotype by our team). Twenty representatives e.g., *P. alimentarius* 1, *P. alimentarius* 2, *P. alimentarius* 3 etc. from the seven bacterial species e.g. *P. alimentarius* are indicated in Fig. 3.

Assigning one representative from each *Psychrobacter* group (Table 1) as reference genotype, the % similarity between the representative and the other members of the group was ranged from 95.00 to 99.61% for *P. celer*, 97.00% for *Psychrobacter* sp., 99.00–99.96% for *P. alimentarius* and 96.54–98.67% for *P. pulmonis*. The DNA sequence data were corresponding to the species assigned by the HRM curve profile even in cases where the sample was unknown thus ensuring the correct discrimination of the different species by the HRM. Sequencing analysis was performed for at least 50% of the isolates belong to the same group. Sequences of the seven phylotypes found in this study are presented in Supplementary Data.

Based on the aforementioned approach, *P. alimentarius* was initially found at higher abundances (40% of the microbiota grown on TSA) than other bacteria such as *P. pulmonis* (33.3%), *P. celer* (20.0%) and *K. pneumoniae* (6.67%) (Fig. 4). The abundance of *P. alimentarius* increased during the storage at 56.5, 64.3, 68.8 and 70.6% for the day 2, 4, 6 and 8, respectively. At the time of the minimum acceptability (day 6), *P. alimentarius* was followed by *P. pulmonis* (15.6%), *P. celer* (9.37%) and *Psychrobacter* sp. (6.25%) (Fig. 4). At the time of the rejection (day 8), *P. alimentarius* reached abundances of 70.6%, while *P. pulmonis* and *P. celer* were reduced at 11.8 and 5.88%, respectively. The phylotype MUSNGR154 was found at abundances of 5.88%. *Oceanimonas smirnovii* was also presented (Fig. 4).

4. Discussion

To study the mussels' microbiota grown on plates, HRM analysis was applied to distinguish APC at species level. Amplicons which presented both the same melting peak temperature and the same shape of

Table 1
Melting peak temperature (mean ± st. dev of three replicates), % similarity between reference genotype and the other members of the group and closest relatives (≥98% similarity) of the 101 analyzed isolates.

Isolates	Peaks °C	Similarity (%)	Phylotypes	Closest relative	Identity (%)	GenBank number
13	87.37 ± 0.62	95.00–99.61	MUSNGR105	*Psychrobacter celer* strain G13	100	MH930052
2	87.42 ± 0.53	97.00	MUSNGR112	*Psychrobacter* sp. BA17	99	KP756656
62	87.47 ± 0.38	99.00–99.96	MUSNGR114	*Psychrobacter alimentarius* strain F430	100	MH929851
21	87.59 ± 0.34	96.54–98.67	MUSNGR116	*Psychrobacter pulmonis* strain YT49	100	MH725523
1	87.44 ± 0.06	–	MUSNGR154	Non-described in GENBANK database	–	-
1	89.26 ± 0.37	–	MUSNGR171	*Oceanimonas smirnovii* strain AL2C-4W	98	MH881375
1	89.43 ± 0.33	–	MUSNGR095	*Klebsiella pneumoniae* strain LB-AMP3KSU	100	MH973162

normalized melting curves were sequenced. Sequencing analysis showed that the isolates can be grouped into the same bacterial species if they present a) the same melting peak temperature and b) HRM curves that were > 95% similar.

Species-level differentiation of seafood microbiota is important to monitor the bacteria which are not differentiated nor enumerated at species level on general, or selective culture media. The identification of bacteria grown on a plate is able to give us a picture of seafood microbiota (Alfaro and Hernandez, 2013; Boziaris and Parlapani, 2016; Hovda et al., 2007a, 2007b; Macé et al., 2012; Olofsson et al., 2007). In fish and seafood, the initial microbial diversity usually consists of various bacterial genera, while only few dominate at the end of product's shelf-life (Dalgaard, 2003; Gram and Huss, 1996). HRM methodology used herein differentiated the bacteria of mussels' microbiota nearly close at species level highlighting *P. alimentarius* as the most dominant microorganism against others of the genus *Psychrobacter* (*P. pulmonis*, *P. celer* and *Psychrobacter* sp.). The differentiation and identification of other genera and/or species e.g., of Lactic Acid Bacteria,

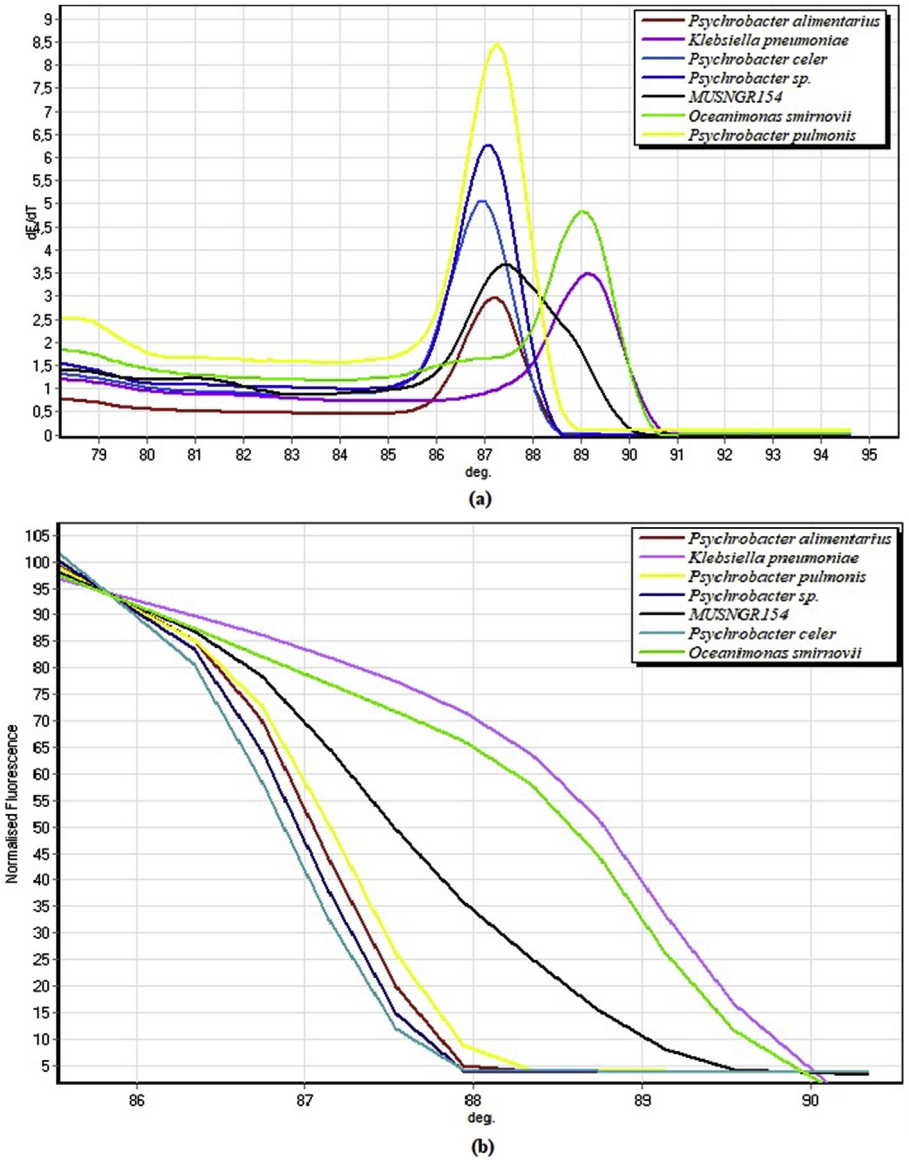

Fig. 2. Conventional (a) and normalized (b) melting curves of the seven bacterial species.

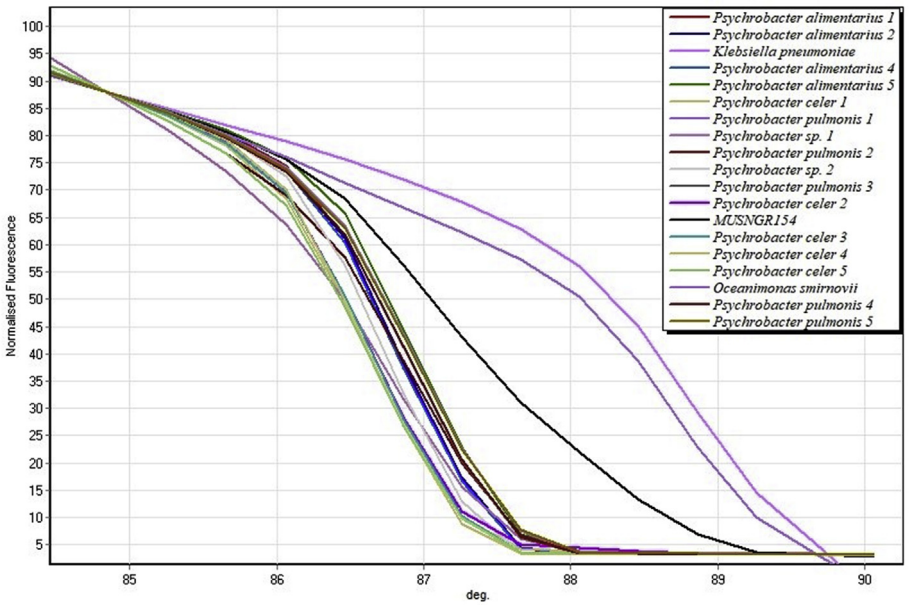

Fig. 3. Normalized HRM curves of 20 representatives of the seven bacterial species.

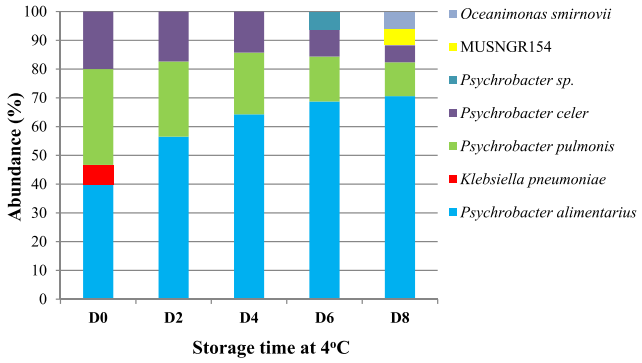

Fig. 4. Changes of the microbiota of mussels stored aerobically at 4 °C grown on TSA plates determined by HRM and sequencing analysis.

has been already checked by other researchers (Iacumin et al., 2015; Lin and Gänzle, 2014; Sakaridis et al., 2014). In their studies, HRM curve profiles were used to classify numerous unknown isolates into ten LAB reference strain groups (Sakaridis et al., 2014); or previously identified, by other approaches, isolates into one or more *Lactobacillus* species (Iacumin et al., 2015; Lin and Gänzle, 2014). Their findings showed that even highly close related species can be distinguished by HRM analysis. Indeed, in our study, the four closest relative phylotypes *P. alimentarius, P. pulmonis*, *P. celer* and *Psychrobacter* sp. presented different high resolution melting curves, thus were easily distinguished by HRM analysis.

During the last decade, the introduction of a plethora of molecular methodologies in the field of Food Microbiology has significantly enhanced our knowledge about spoilage associated bacteria. Not only *Pseudomonas* and H_2S producing bacteria, presumptive *Shewanella*, can be located as dominants in spoilage microbiota of chill-stored seafood, but also other bacteria such as *Psychrobacter*, *Pseudoalteromonas* and/or *Carnobacterium* (Broekaert et al., 2013; Parlapani et al., 2018a, 2018b). Molecular markers have also revealed that fish originating from aquaculture farms from two geographical areas of the Mediterranean exhibit different spoilage microbiota, in particular *Pseudomonas* dominated in chill-stored fish from the Ionian and *Psychrobacter* in fish from the Aegean Sea (Parlapani et al., 2018b). In addition, regarding packaging, various species of LAB, *Photobacterium* or *Brochothrix* dominate alternatively in seafood. For example, *Carnobacterium maltaromaticum* has been found to dominate against *Carnobacterium divergens* and other lactic acid bacteria e.g. *Lactobacillus fuchuensis* and *Vagococcus fluvialis* in sea bream fillets stored under MAP containing $CO_2/O_2/N_2$:60/10/30 at 5 °C (Parlapani et al., 2015a), while *Brochothrix thermosphacta* dominates against *Carnobacterium divergens* and *Carnobacterium piscicola* in salmon stored under MAP with CO_2/N_2:60/40 at 1 or 4 °C (Rudi et al., 2004). HRM analysis could be used as a rapid method for screening of SSOs among seafood species, or seafood from different origin, storage (temperature, atmosphere), or processing conditions.

In this study, high resolution melting curves differentiated bacteria at species level allowing us to track the dominant ones in mussels during storage. The domination of *P. alimentarius* against other *Psychrobacter* species such as *P. pulmonis*, *P. celer* and *Psychrobacter* sp. as well as *K. pneumoniae, O. smirnovii* and the phylotype MUSNGR154 was obvious. *Psychrobacter* and other various genera e.g. *Pseudomonas, Shewanella, Acinetobacter, Flavobacterium* and *Bacillus* belong to the indigenous microbiota of fish and seafood (Gram and Huss, 1996; Parlapani et al., 2015b; Svanevik and Lunestad, 2011), while *K. pneumoniae* can initially be found as contamination microbiota from e.g. terrestrial environment, food contact surfaces, workers etc. Regarding the phylotype MUSNGR154, this is the first time that it is described in GENBANK database (≤92% homologies to GenBank entries/database records) thus no previous information is available. Bacteria such as *Pseudomonas*, H_2S-producing bacteria (including *Shewanella putrefaciens*) and LAB have been previously involved in spoilage of mussels (*Mytilus galloprovincialis*) stored at 4 °C based on classical (enumeration) microbiological approach (Goulas et al., 2005). On the other hand, when using molecular approaches, *Psychrobacter* spp. have been found to dominate in chill-stored Norway lobster (Bekaert et al., 2015), brown shrimp (Broekaert et al., 2013), gilt-head sea bream (Parlapani et al., 2018b) and cuttlefish (Parlapani et al., 2018b). The dominance of one or more microorganisms against others depends on various factors e.g. temperature, nutrients availability, initial abundance, microbial interaction and other implicit factors i.e. pH, aw, Eh (Boziaris and Parlapani, 2016). Herein, HRM analysis followed by the sequencing of V3–V4 region of the 16S rRNA gene highlighted *P. alimentarius* as the most dominant microorganism of mussels stored at 4 °C under aerobic conditions. To elucidate which of the microorganisms revealed herein (*P. alimentarius* or the rest species) contributes in mussels spoilage, we could choose one representative from each group and further study

their spoilage potential and activity in order to characterize them as key players of mussels spoilage (Parlapani et al., 2017).

5. Conclusions

Plate-based approach is essential to isolate the microbiota for further studies on their spoilage potential and activity in order to determine them as SSOs. HRM analysis can successfully distinguish even highly close related bacterial species i.e. *P. alimentarius, P. pulmonis*, *P. celer* and *Psychrobacter* sp.. Isolates can be grouped into the same bacterial species if they present the following criteria a) same melting peak temperature and b) HRM curves that were > 95% similar. Therefore, HRM analysis could be used as a useful tool for rapid differentiation of bacteria isolated from seafood during storage. Then, only one representative from each bacterial species could be sequenced in order to determine seafood microbiota during storage, thus reducing the cost of sequencing.

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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.2019.103304.

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