



Monitoring the succession of the biota grown on a selective medium for pseudomonads during storage of minced beef with molecular-based methods

Agapi I. Doulgeraki*, George-John E. Nychas

Laboratory of Food Microbiology and Biotechnology, Department of Food Science and Technology, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece

ARTICLE INFO

Article history:

Received 14 March 2012

Received in revised form

26 November 2012

Accepted 27 November 2012

Available online 3 December 2012

Keywords:

Meat

Pseudomonas

Enterobacteria

Lack of selectivity

PCR-DGGE

PFGE

ABSTRACT

In the present study, the succession of the biota grown on a selective medium for pseudomonads (pseudomonas agar based medium – PAB) during the storage of meat under different conditions was monitored. Thus, minced beef was stored aerobically and under modified atmosphere packaging in the presence (MAP+) and absence (MAP–) of oregano essential oil at 0, 5, 10 and 15 °C. A total of 267 pure cultures were recovered from PAB throughout the storage period and subjected to PCR-Denaturing gradient gel electrophoresis (PCR-DGGE) for their differentiation. In parallel, the direct analysis of the whole cultivable community (WCC) from the same medium was applied. These two approaches were used in order to indicate the lack of selectivity. Fifteen different DGGE fingerprints were obtained after PCR – DGGE analysis of the isolates, which were assigned to *Pseudomonas putida* (3 fingerprints), *Pseudomonas fragi* and *Pseudomonas fluorescens*, *Pseudomonas* spp., *Serratia liquefaciens* (2), *Citrobacter freundii*, *Serratia grimesii*, *Hafnia alvei* (3), *Rahnella* spp. and *Morganella morganii*. Twelve of them occurred during the direct analysis of the WCC. The biota succession found to be affected from the different storage conditions. However, the outcome of the two strategies was quite different, which is leading to the use of different appropriated molecular approaches in order to widen the knowledge of bacterial succession of meat.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Pseudomonads are Gram negative rods which constitute a large genus of bacteria consisting of five phylogenetic groups based on rRNA similarity studies (Palleroni, 1993). The contribution of pseudomonads in spoilage of animal origin foods e.g. meat fish and dairy is well established in the literature (Nychas et al., 2007). In particular, it has been reported that pseudomonads dominate the microbiota of meat stored aerobically, whilst storage under Modified Atmosphere Packaging (MAP), with or without the addition of natural preservatives, suppressed the counts of this group (Tsigarida et al., 2000; Skandamis and Nychas, 2001, 2002; Nychas and Skandamis, 2005; Ercolini et al., 2006; Argyri et al., 2011). Among the most important meat spoilage species are *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas cichorii*, *Pseudomonas viridiflava* and *Pseudomonas syringae* (Garcia-Lopez et al., 1998), while the phenotypic and molecular characterization of the psychrotrophs isolated from fresh and

spoiled meat revealed the presence of three major species of *Pseudomonas* (*Pseudomonas fragi*, *Ps. fluorescens* and *Pseudomonas lundensis*) (Liao, 2006). Their psychrotrophy, very fast growth rate and high affinity for oxygen have been suggested as the main reasons for the predominant growth of the above pseudomonas species in air packed fresh meats or high-O₂ MAP fresh meats, since these properties presumably lead to rapid glucose uptake (Gill and Newton, 1977; Gill, 1982; Gill and Molin, 1991). These strict aerobes use glucose as their primary substrate and once this nutrient present in the meat system has been totally consumed by the bacteria, amino acids are utilized and malodorous compounds such as sulphides, esters, acids, and others are formed as by-products (Nychas et al., 1988). Since a large proportion of pseudomonads e.g. *Ps. fluorescens*, *Ps. lundensis* and *Ps. fragi* strains are capable of producing extracellular proteases, lipases, the slimy or mushy appearance, production of off odours and partial or complete degradation of animal tissues is mainly attributed to them (Liao, 2006; Odagami et al., 1994).

Although the behavior of different species/strains of pseudomonads on animal origin foods has been extensively reported e.g. they differ in oxygen affinity, glucose consumption (Nychas et al., 1998), limited information is available regarding the effect of storage conditions on pseudomonads diversity as well as on their

* Corresponding author. Tel./fax: +30 210 5294938.

E-mail addresses: adoulgeraki@aua.gr (A.I. Doulgeraki), gjn@aua.gr (G.-J.E. Nychas).

succession at species and/or strain level. Such information can be important since a considerable number of species or strains of the same species that are usually developed might be also affecting the type as well as the rate of deterioration (Ercolini et al., 2010a).

The aims of the present study were (i) to investigate the microbial diversity of the population obtained from a pseudomonas agar based medium isolated from meat stored under different packaging and temperature conditions, (ii) to indicate the lack of selectivity by using different approaches (iii) to check the specificity of the pseudomonas agar based medium.

2. Materials and methods

2.1. Bacterial isolation from pseudomonas agar base growth medium

Bacteria were isolated from PAB (pseudomonas agar base medium) after incubation for 48 h at 25 °C from minced beef according to Doulgeraki et al. (2010). In brief, isolates were recovered from minced beef stored at 0, 5, 10 and 15 °C aerobically and under modified atmosphere packaging (MA) consisting of 40% CO₂–30% O₂–30% N₂ in the presence (MAP+) and absence (MAP–) of volatile compounds of oregano essential oil. Minced beef was sampled at appropriate time intervals, depending on storage temperature; the incubation lasted 650, 482, 386 and 220 h at 0, 5, 10 and 15 °C, respectively (Argyri et al., 2011). Colonies (10%) were selected randomly (Harrigan, 1998) from the highest dilution of PAB from different the different time points. Pure cultures included in this study were stored at –80 °C in Brain Heart Infusion Broth (BHI, Merck, Darmstadt, Germany) supplemented with 20% (v/v) glycerol (Serva, Heidelberg, Germany). Before experimental use each isolate was subcultured twice in BHI for 16 h at 25 °C, while the purity of the culture was always checked on PAB plates.

2.2. Preparation of bulk cells from pseudomonas agar base growth medium

After the microbial counts, the plates were used for bulk formation as previously described (Ercolini et al., 2001). Briefly, all the colonies present on the surface of each countable plate were suspended in a suitable volume of one-quarter-strength Ringer's solution, harvested with a sterile pipette, and stored by freezing at –80 °C supplemented with 20% (v/v) glycerol. When necessary, 100 µL of the bulk was used for DNA extraction.

2.3. DNA extraction

The protocol described by the Wizard DNA purification kit (Promega, Madison, Wiscon.) was applied. One millilitre of cell culture was centrifuged at 17,000 g for 5 min at 4 °C, and the resulting pellet was resuspended in 100 µL of Tris–EDTA buffer (100 mM Tris, 10 mM EDTA). The sample was mixed with 160 µL of 0.5 M EDTA/Nuclei Lysis Solution in 1/4.16 ratio and 15 µL of proteinase K (20 mg mL⁻¹, Sigma, Chemical Co., St. Louis, Mo. USA) and incubated for 90 min at 55 °C. After incubation, 1 volume of potassium acetate 5 M was added to the sample which was then centrifuged for 10 min at 4 °C. The supernatant was precipitated with 0.7 volume of ice cold isopropanol and centrifuged for 10 min at 4 °C. After that, the pellet was resuspended in 0.5 mL ice cold ethanol (70%) and centrifuged for 10 min at 4 °C. The pellet was dried and resuspended in 45 µL of DNA Rehydration Solution by incubation at 55 °C for 45 min. After incubation, 5 µL of RNase (10 mg mL⁻¹, Promega) was added and the sample incubated for 30 min at 37 °C. The samples were stored at –20 °C until further use.

2.4. PCR amplification

PCR amplification was performed according to Ercolini et al. (2006). In brief, primers U968 (AACCGAAGAACCTTAC) and L1401 (GCGTGTGTACAAGACCC) were used to amplify the variable V6–V8 region of the 16S rRNA gene, giving PCR products of about 450 bp. A GC clamp was added to the forward primer according to a method described previously by Muyzer et al. (1993). PCR amplifications were conducted in a final volume of 25 µL containing 2.5 U of thermostable (*Taq*) DNA polymerase (New England Biolabs, Ipswich, MA, USA), 2.5 µL *Taq* buffer, 0.8 mM dNTP's, 0.2 µM of each primer, 1.0 mM MgCl₂ and 20 ng of DNA template. PCR reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles (denaturation at 94 °C, 1 min, primer annealing at 56 °C, 45 s, primer extension at 72 °C, 3 min), and a final extension step at 72 °C for 10 min. Aliquots (5 µL) of PCR products were routinely checked on 1.5% agarose gels by electrophoresis. Reference strains included in this study consisted of *Ps. putida* KT2440, *Ps. fragi* DSM 3456 and *Ps. fluorescens* GTE 015; these strains were used for the construction of a DGGE based identification database.

2.5. DGGE analysis

PCR products were analyzed by DGGE using a DCode apparatus (Biorad) according to Ercolini et al. (2006). Briefly, samples were applied to 7% (w/v) polyacrylamide gels in 1× Tris acetate-EDTA buffer. Parallel electrophoresis experiments were performed at 60 °C by using gels containing a 20–50% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% (w/v) formamide). The gels were run for 10 min at 50 V, followed by 4 h at 200 V. They were then stained with GelRed Nucleic Acid Stain (Biotium, Investment Blvd, Hayward, CA) for 3 min, rinsed for 15 min in distilled water, before being photographed using a Gel-Doc system (Biorad, Hercules, CA, USA).

2.6. Species identification

A representative number of isolates per distinct DGGE cluster were selected and subjected to species identification by sequencing the V6–V8 variable region of the 16S rRNA gene with the primer L1401. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (www.macrogen.com). The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences are to HM536985 to HM536997.

2.7. Multiplex PCR for the *carA* gene

Multiplex PCR amplification was performed for the *Pseudomonas* isolates according to Ercolini et al. (2007). Briefly, primers putF (ATGCTGGTTGCYCGTGGC), fraF (CGTCAGCACCGAAAAGCC), lunF (TGTGGCGATTGCAGGCATT) and carAR (TGATGRCCSAGGCA GATRCC) were used to amplify the *carA* gene, giving PCR products of about 230, 370 and 530 bp, respectively. PCR amplifications were conducted in a final volume of 25 µL containing 1.25 U of thermostable (*Taq*) DNA polymerase (New England Biolabs), 2.5 µL *Taq* buffer, 1 mM dNTP's, 0.2 µM of each primer (0.6 µM in the case of reverse primer), 1.0 mM MgCl₂ and 10 ng of DNA template. PCR reaction consisted of an initial denaturation step at 94 °C for 3 min, followed by 30 cycles (denaturation at 94 °C, 15 s, primer annealing at 63 °C, 30 s, primer extension at 74 °C, 15 s), and a final extension step at 74 °C for 10 min. Aliquots (5 µL) of PCR products were checked on 2% (w/v) agarose gels by electrophoresis.

Table 1

Species identification of isolates from pseudomonas agar base medium recovered from minced beef after sequencing of the variable V6–V8 region of the 16S rRNA genes.

Closest relative	Code	GenBank accession number of closest relative	Identity (%)	GenBank accession number of sequence
<i>Serratia liquefaciens</i>	CK2	AY243097	99	HM536985
<i>Citrobacter freundii</i>	CK19	AB548828	100	HM536986
<i>S. grimesii</i>	CK30	AY789460	100	HM536987
<i>C. freundii</i>	CK33	AB548828	100	HM536988
<i>Hafnia alvei</i>	CK36	FM179943	100	HM536989
<i>H. alvei</i>	CK39	DQ412565	100	HM536990
<i>H. alvei</i>	CK49	AY572428	99	HM536991
<i>Pseudomonas</i> spp.	CK73	AY365075	99	HM536992
<i>Ps. putida</i>	CK119	AY365080	98	HM536993
<i>Ps. putida</i>	CK148	AY599720	99	HM536994
<i>Rahnella</i> spp.	CK153	EU275360	99	HM536995
<i>Morganella morganii</i>	CK265	EF550572	100	HM536997

2.8. Pulsed-field gel electrophoresis (PFGE)

Genomic DNA was prepared from all isolates as described by Doulgeraki et al. (2011). Briefly, after the proteinase K treatment,

Table 2

Distribution of isolates recovered from pseudomonas agar base medium according to PCR-DGGE profiling during storage of minced beef under different packaging (air, MAP– and MAP+) and temperature conditions (0, 5, 10 and 15 °C).

Temperature (°C)	Storage period (h)	Fresh meat	Packaging condition		
			Meat stored aerobically	Meat stored under MAP– ^a	Meat stored under MAP+ ^b
	0	<i>Pseudomonas fragi</i>^c, <i>Serratia liquefaciens</i> (CK2)			
0	69		<i>S. liquefaciens</i> (CK2), <i>Ps. fluorescens</i>	<i>S. liquefaciens</i> (CK2)	<i>Ps. fluorescens</i>, <i>S. grimesii</i> (CK30)
	196		<i>Pseudomonas</i> spp. (CK73), <i>Hafnia alvei</i> (CK39), <i>S. liquefaciens</i> (CK2), <i>Ps. fluorescens</i>, <i>Ps. fragi</i>, <i>Ps. putida</i>	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>Morganella morganii</i> (265)	<i>S. liquefaciens</i> (CK2), <i>Rahnella</i> spp. (CK153)
	291		<i>Pseudomonas</i> spp. (CK73), <i>Ps. fragi</i>, <i>Citrobacter freundii</i> (CK19), <i>Ps. putida</i> (CK119)	<i>M. morganii</i> (265), <i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>M. morganii</i> (265), <i>S. grimesii</i> (CK30), <i>Ps. putida</i> (CK148)
	485		<i>Ps. fragi</i>, <i>Pseudomonas</i> spp. (CK73)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>Pseudomonas</i> spp. (CK73)	<i>M. morganii</i> (265), <i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30)
5	48		<i>Ps. fragi</i>, <i>S. liquefaciens</i> (CK2), <i>Ps. putida</i> (CK119)	<i>S. grimesii</i> (CK30), <i>S. liquefaciens</i> (CK2), <i>M. morganii</i> (265)	<i>S. liquefaciens</i> (CK2)
	69		<i>Ps. putida</i> (CK119), <i>S. liquefaciens</i> (CK2), <i>Ps. putida</i>	<i>S. liquefaciens</i> (CK2), <i>Ps. putida</i> (CK148), <i>Ps. fluorescens</i>, <i>Ps. putida</i>	<i>S. liquefaciens</i> (CK2)
	114		<i>Ps. putida</i> (CK119)	<i>S. liquefaciens</i> (CK2), <i>Rahnella</i> spp. (CK153), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2), <i>Rahnella</i> spp. (CK153)
	196		<i>Ps. fragi</i>, <i>Ps. putida</i> (CK119)	<i>S. liquefaciens</i> (CK2), <i>Rahnella</i> spp. (CK153)	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>
	244		<i>Ps. fragi</i>, <i>H. alvei</i> (CK39), <i>Ps. putida</i> (CK119)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>H. alvei</i> (CK49), <i>Pseudomonas</i> spp. (CK73)	<i>S. liquefaciens</i> (CK2), <i>Ps. putida</i> (CK119), <i>Ps. putida</i>
10	18		<i>S. liquefaciens</i> (CK2), <i>H. alvei</i> (CK39)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>C. freundii</i> (CK33)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>H. alvei</i> (CK39)
	54		<i>S. grimesii</i> (CK30), <i>Ps. fluorescens</i>, <i>Ps. fragi</i>, <i>S. liquefaciens</i> (CK2), <i>Pseudomonas</i> spp. (CK73)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>H. alvei</i> (CK39)
	90		<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>, <i>Pseudomonas</i> spp. (CK73)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30)	<i>S. grimesii</i> (CK30), <i>S. liquefaciens</i> (CK2), <i>H. alvei</i> (CK36), (CK49)
	162		<i>Ps. fragi</i>, <i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>H. alvei</i> (CK39)	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30)
15	12		<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>M. morganii</i> (265), <i>Ps. fluorescens</i>,
	36		<i>S. liquefaciens</i> (CK2)		
	69		<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>C. freundii</i> (CK33), <i>H. alvei</i> (CK36), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2), <i>S. grimesii</i> (CK30), <i>H. alvei</i> (CK39), (CK49)
	110		<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>, <i>C. freundii</i> (CK19)	<i>S. liquefaciens</i> (CK2), <i>H. alvei</i> (CK39),	<i>S. grimesii</i> (CK30), <i>H. alvei</i> (CK49), <i>S. liquefaciens</i> (CK2)

^a Modified atmosphere packaging (40% CO₂/30% O₂/30% N₂).

^b MAP with the presence of volatile compounds of 2% v/w oregano essential oil.

^c Most common isolate are present in bold font.

the plugs were incubated for 1 h at room temperature in TE solution containing 50 μM thiourea, with gentle agitation, and subsequently washed 3 times with 500 μL of TE solution with gentle agitation for 30 min. The restriction enzyme *SpeI* (10 U) (New England Biolabs) was applied according to the manufacturer's recommendation for 16 h. Restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris–Borate buffer containing 100 μM thiourea on CHEF-DRII (Biorad) equipment with the following running parameters: 6 V cm⁻¹, 5.3 s initial switching time, 34.9 s final switching time and a total run of 20 h at 14 °C (Khan et al., 2007). Gels were then stained with ethidium bromide (0.5 μg mL⁻¹) in water for 1 h and destained for 2 h before being photographed using a GelDoc system.

3. Results

3.1. Identification of isolates from pseudomonas agar base growth medium

A total of 267 isolates from PAB medium were recovered throughout the storage period and subjected to PCR-DGGE. Fifteen different DGGE fingerprints were obtained after PCR – DGGE

analysis; three of them were assigned to *Ps. putida*, *Ps. fragi* and *Ps. fluorescens*; this identification was confirmed by band position analysis using *Ps. putida* KT2440, *Ps. fragi* DSM 3456 and *Ps. fluorescens* GTE 015 as reference strains. The rest of the fingerprints were subjected to 16S rRNA gene sequencing (Table 1), as well as the *carA* multiplex PCR was used to support *Pseudomonas* species differentiation. The DGGE fingerprint (CK2) (migrate the same distance in DGGE gel with strain CK2) was assigned to *Serratia liquefaciens*; fingerprints (CK19) and (CK33) to *Citrobacter freundii*; fingerprint (CK30) to *Serratia grimesii*; fingerprints (CK36), (CK39) and (CK49) to *Hafnia alvei*; fingerprints (CK73) to *Pseudomonas* spp.; (CK119) and (CK148) to *Ps. putida*; fingerprint (CK153) to *Rahnella* spp.; fingerprint (CK265) to *Morganella morganii*. Table 2 summarizes the prevalence of the different bacterial DGGE fingerprints related to the different storage conditions. Those results revealed that members of *Enterobacteriaceae* were able to grow on PAB. It needs to be noted that, *S. liquefaciens* (CK2) was the most common DGGE fingerprint displayed in all conditions of storage of beef samples.

The isolates which have been assigned to pseudomonads were subjected to PFGE to determine the strain diversity. The image analysis of the different PFGE patterns resulted in forty three fingerprints (Fig. 1). A diversity regarding strain occurrence was revealed especially under aerobic storage at chill temperatures (Table 4). From the ten different PFGE fingerprints assigned to *Ps. fragi*, the fingerprint CK4 was detected in the most cases (air, 15 °C under MAP-), while CK5 was only detected under MAP – and MAP+ at 5 °C. Differences in strain diversity also occurred between

the storage temperatures when minced beef was stored aerobically; fingerprints CK79 and CK143 were obtained from 5 °C, CK85 from 10 °C and three fingerprints (CK16, CK25 and CK26) from 10 °C. In the case of *Ps. fluorescens*, different fingerprints were obtained aerobically (CK66, CK67 and CK206), under MAP- (CK149) and MAP+ (CK48A, CK247 and CK248). From the twelve fingerprints assigned to *Pseudomonas* spp. (CK73), fingerprint CK185 and CK197 were detected under aerobic and MAP- and MAP+ conditions, respectively. Fingerprint CK164 and CK186 were detected under MAP- and MAP+ respectively, while the rest of the fingerprints were detected during aerobic storage. Moreover, PCR-DGGE was able to distinguish the *Ps. putida* isolates into 3 subgroups (*Ps. putida*, *Ps. putida* (CK119) and *Ps. putida* (CK148)). The first subgroup (*Ps. putida*), which was detected in all packaging conditions adopted, was consisted of three PFGE fingerprints; CK124 was detected under aerobic and MAP-, CK187 under MAP+ and CK193 under aerobic conditions. On the other hand, the ten PFGE fingerprints were assigned to *Ps. putida* (CK119) obtained from 5 °C under aerobic conditions, but one (CK127) which was also detected at 0 °C. In the case of *Ps. putida* (CK148), PFGE fingerprints CK148 and CK262 were detected at 5 °C under MAP- and 0 °C, respectively.

3.2. Fingerprinting of bulk cells from pseudomonas agar base growth medium

The cultivable fractions harvested from PAB medium were identified by PCR-DGGE of the variable V6–V8 region of the 16S rRNA gene. Twelve different bands occurred throughout the storage

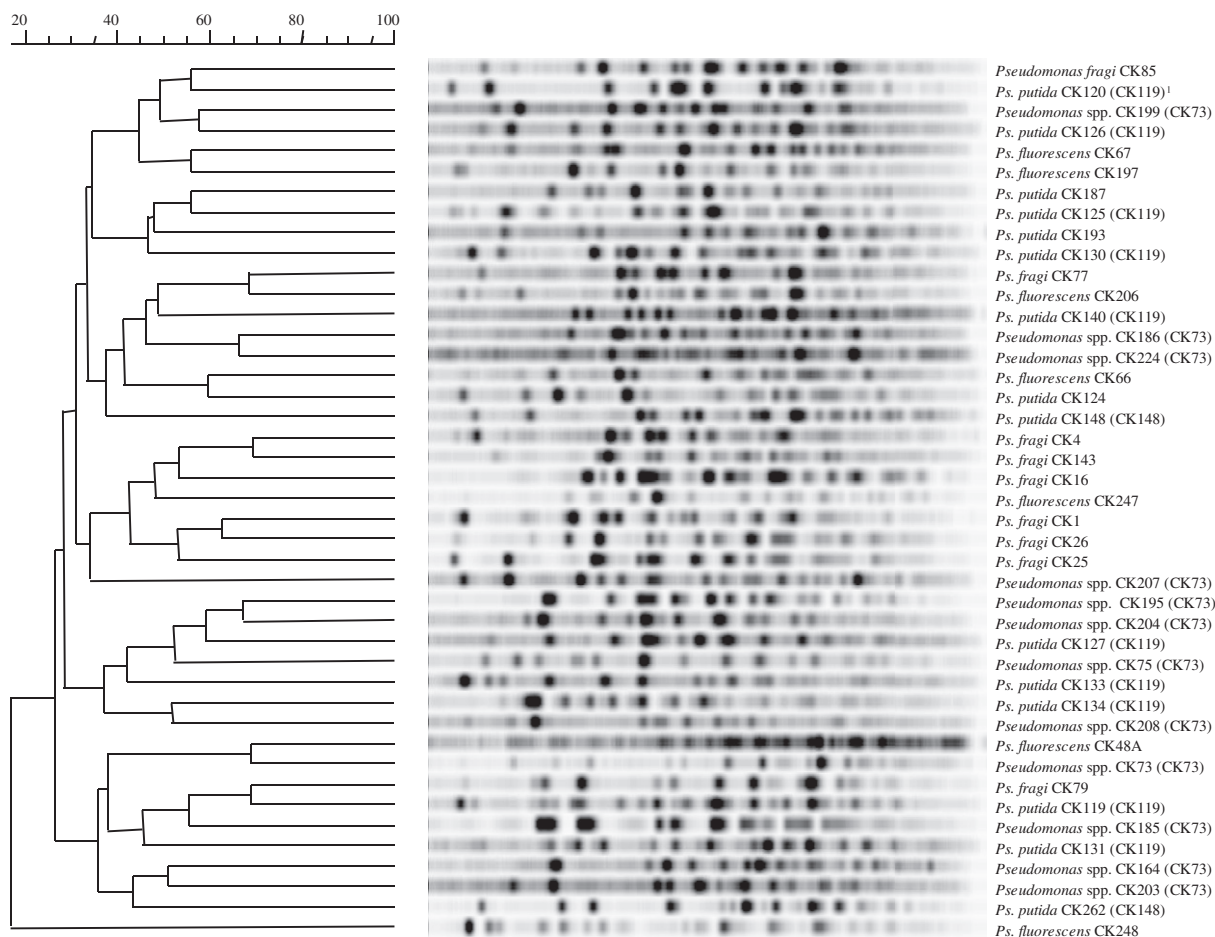


Fig. 1. Cluster analysis of PFGE *SpeI* digestion fragments of the pseudomonads isolates recovered from pseudomonas agar base medium during storage of minced beef under different conditions calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient ($r\%$).

Table 3
Distribution of different bacteria of bulk cells from pseudomonas agar base medium according to PCR-DGGE profiling during storage of minced beef under different packaging (air, MAP– and MAP+) and temperature conditions (0, 5, 10 and 15 °C).

Temperature (°C)	Storage period (h)	Fresh meat	Packaging condition		
			Meat stored aerobically	Meat stored under MAP– ^a	Meat stored under MAP+ ^b
	0	<i>Serratia liquefaciens</i> (CK2)			
0	69	<i>S. liquefaciens</i> (CK2), <i>Pseudomonas fluorescens</i>	<i>S. liquefaciens</i> (CK2), <i>Pseudomonas</i> spp. (CK73), <i>Ps. fragi</i>	<i>Citrobacter freundii</i> (CK33), <i>Ps. fragi</i>	
	196	<i>S. liquefaciens</i> (CK2), <i>Ps. fluorescens</i> , <i>Ps. fragi</i> , <i>Ps. putida</i>	<i>S. liquefaciens</i> (CK2), <i>Morganella morgani</i> (265)	<i>S. liquefaciens</i> (CK2),	
	291	<i>Ps. fragi</i>	<i>Pseudomonas</i> spp. (CK73), <i>M. morgani</i> (265)	<i>S. liquefaciens</i> (CK2), <i>M. morgani</i> (265), <i>S. grimessi</i> (CK30), <i>Pseudomonas</i> spp. (CK73)	
	485	<i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2), <i>Pseudomonas</i> spp. (CK73), <i>Ps. fragi</i>	<i>M. morgani</i> (265), <i>S. liquefaciens</i> (CK2)	
5	48	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>S. grimessi</i> (CK30)	<i>S. liquefaciens</i> (CK2), <i>C. freundii</i> (CK33), <i>Ps. fragi</i>	
	69	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i> , <i>Ps. putida</i> (CK119)	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>	
	114	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i> , <i>Ps. putida</i> (CK119)	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>		
	196	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i> , <i>Ps. putida</i> (CK119), <i>H. alvei</i> (CK39)	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2)	
	244	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i> , <i>Ps. putida</i> (CK119)	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>Pseudomonas</i> spp. (CK73)	
10	18	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2)		
	54	<i>S. liquefaciens</i> (CK2), <i>Ps. putida</i> (CK119), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2), <i>S. grimessi</i> (CK30)	<i>S. liquefaciens</i> (CK2), <i>S. grimessi</i> (CK30)	
	90	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2), <i>S. grimessi</i> (CK30)	<i>S. liquefaciens</i> (CK2), <i>S. grimessi</i> (CK30)	
	162	<i>S. liquefaciens</i> (CK2), <i>Ps. putida</i> (CK119), <i>Ps. fragi</i>	<i>H. alvei</i> (CK39), <i>Pseudomonas</i> spp. (CK73)	<i>S. liquefaciens</i> (CK2)	
15	12	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>H. alvei</i> (CK36)	<i>S. liquefaciens</i> (CK2)	
	36	<i>S. liquefaciens</i> (CK2)			
	69	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2), <i>H. alvei</i> (CK49)	
	110	<i>S. liquefaciens</i> (CK2), <i>Ps. fragi</i>	<i>S. liquefaciens</i> (CK2)	<i>S. liquefaciens</i> (CK2)	

^a Modified atmosphere packaging (40% CO₂/30% O₂/30% N₂).

^b Volatile compounds of 2% v/w oregano essential oil.

period of minced beef under the different conditions adopted. These bands were identified after inclusion in the same DGGE gels three reference strains (*Ps. putida* KT2440, *Ps. fragi* DSM 3456 and *Ps. fluorescens* GTE 015) as well as the different isolates which were recovered from the same medium and exhibited different DGGE fingerprints. In Table 3, the distribution of different bacteria of bulk cells from PAB medium according to PCR-DGGE profiling related to the different storage conditions is summarized.

The analysis of the bulk cells showed that one band occurred at the initial stage of storage; this entity was shown to migrate the same distance in DGGE gel with *S. liquefaciens* (CK2). This band occurred in all conditions tested but at 0 °C under aerobic (291 and 485 h), MAP– (291 h) and MAP+ (69 h) conditions and at 10 °C under MAP– (162 h).

It should be also noted, that the minced beef stored at 15 °C did not show a significant degree of diversity depending on the different packaging conditions based on analysis of bulk cells from PAB medium; this was not the case at lower temperatures.

4. Discussion

Two different strategies were used in the present study to investigate the microbial diversity of the population obtained from a pseudomonas agar medium and detected on minced beef stored under different packaging and temperatures conditions. The traditional method, i.e., pure cultures, were picked randomly from the appropriate dilution of a selective growth medium for pseudomonads, as well as the direct analysis of the whole cultivable

community from the same medium were applied. This approach was used in order to indicate the lack of selectivity.

The outcome of the two strategies was quite different. In the most cases, PCR-DGGE analysis was able to detect only the dominant bacteria based on the results revealed from the pure cultures. Similarly, the lack of selectivity was observed at several time points as a higher diversity or different species based on DGGE bands was observed beside the recovered isolates. It has to be noted that PCR–DGGE was found to be able to distinguish the microbial community at species or subspecies level; two subgroups were detected in the case of *C. freundii* and three subgroups of *Ps. putida* and *H. alvei*. Several researchers reported the ability of the method to differentiate the microbial community at subspecies and strain level (Cocolin et al., 2004; Ercolini et al., 2006).

Based on the results of the recovered/detected pseudomonads from the selective medium for pseudomonas in this study, it was shown that *Ps. fragi* prevailed over the other pseudomonads during the storage minced beef. Under aerobic conditions, *Ps. fragi* dominated the pseudomonads community at 15 °C; equally dominant was *Ps. putida* (CK119) at 5 and 10 °C, while with *Pseudomonas* spp. (CK73) at 0 °C. Furthermore, under modified atmosphere (MAP– and MAP+), *Pseudomonas* spp. (CK73) dominated the pseudomonads community at 0, 5 and 15 °C, while *Ps. fragi* and *Ps. fluorescens* dominated the community at 15 °C under MAP– and MAP+, respectively. It has to be noted that *Ps. putida* was recovered/detected only at chill temperatures (0 and 5 °C) in all packaging conditions adopted. The fact that *Ps. putida* was detected in lower percentage than the other pseudomonads, could be attributed to its

Table 4

Distribution of pseudomonads strains according to their macrorestriction analysis of DNA by PFGE and the specific storage conditions of minced beef.

DGGE fingerprint		PFGE fingerprint	Initial	AIR				MAP ^{-a}				MAP ^{+b}			
Closest relative	Code			0	5	10	15	0	5	10	15	0	5	10	15
<i>Pseudomonas</i> spp.	CK73	CK73													
		CK75													
		CK164													
		CK185		X											
		CK186													
		CK195		X											
		CK199		X											
		CK203		X											
		CK204		X											
		CK207		X											
		CK208		X											
		CK224		X											
		<i>Ps. fluorescens</i>		CK48A											
CK66															
CK67				X			X								
CK197									X						
CK206				X											
CK247											X				X
CK248															X
<i>Ps. fragi</i>		CK1	X												
		CK4	X	X	X	X	X		X		X			X	
		CK16													
		CK25													
		CK26													
		CK77		X			X								
		CK79				X	X								
		CK85					X								
		CK143				X									
		<i>Ps. putida</i>		CK124				X				X			
CK187														X	
CK193				X											
<i>Ps. putida</i>	CK119	CK119				X									
		CK120				X									
		CK125				X									
		CK126				X									
		CK127		X		X									
		CK130				X									
		CK131				X									
		CK133				X									
		CK134				X									
		CK140				X									
<i>Ps. putida</i>	CK148	CK148								X					
		CK262												X	

^a Modified atmosphere packaging (40% CO₂/30% O₂/30% N₂).^b Volatile compounds of 2% v/w oregano essential oil.

inability to dominate. In general, members of the *Ps. fluorescens* group, along with the psychrotrophic *Ps. fragi*, *Ps. lundensis*, and *Ps. putida*, are often isolated from spoiled meat even during storage at low temperatures (Stanbridge and Davies, 1998; Labadie, 1999) in the order *Ps. fragi* > *Ps. lundensis* > *Ps. fluorescens* > *Ps. putida* (Dainty and MacKey, 1992; Garcia-Lopez et al., 1998). A number of studies have indicated that *Ps. fluorescens* is more abundant on fresh meats than *Ps. fragi* but that the latter becomes dominant over time (Lebert et al., 1998). Furthermore, *Ps. fluorescens* was found to grow better at low than at higher temperatures (Olsen and Jezeski, 1963; Liao, 2006). High concentrations of CO₂ (up to 10%) have been found to inhibit the growth of *Ps. fluorescens* and *Ps. fragi* on red meat (Gill and Tan, 1980), whereas *Ps. fragi* was inhibited more than the other pseudomonads like *Ps. fluorescens* and *Ps. lundensis* (Stanbridge and Davies, 1998).

On beef, lamb and pork, studies have shown the predominance of *Ps. fluorescens* from the slaughter line to the chilling process (Gustavsson and Borch, 1993). Additionally, *Ps. fluorescens* is known to be largely present in the environment (floor, water), on animals

(hide, skin) or also in water and surfaces in meat factories (Drosinos and Board, 1995). On cutting lines and during storage and retailing, *Ps. fragi* was found as the dominant biota on meat (Molin and Ternstrom, 1982, 1986). It should be noted that the species *Ps. aeruginosa*, *Ps. maltophilia*, *Ps. fluorescens*, *Ps. putida*, *Ps. cepacia*, *Ps. stutzeri*, and *Ps. putrefaciens*. *Ps. aeruginosa* which are associated with opportunistic infections are probably the most well-known members of the genus (Lerner and Lerner, 2003).

Based on the results for enterobacteria detected on the selective medium for pseudomonads of fresh minced beef, *S. liquefaciens* (CK2) was recovered from fresh meat and prevailed over the other members of *Enterobacteriaceae* community during storage. The aforementioned species dominated the recovered biota especially under MA conditions, while in some cases was the only detected species by PCR-DGGE. The modification of the packaging enhanced the species diversity, as *Rahnella* and *Morganella* were also detected. The detected members of *Enterobacteriaceae* community have been previously associated with the spoilage of meat (Borch et al., 1996; Nychas et al., 1998; Stanbridge and Davies, 1998; Ercolini

et al., 2006, 2010b; Doulgeraki et al., 2011; Pennacchia et al., 2011). Additionally, *Serratia proteamaculans* and *Rahnella* spp. have been previously detected on pseudomonas agar medium (Ercolini et al., 2006). The inability of the medium to select only pseudomonads has been described previously, especially when the number of *Enterobacteriaceae* was high (Stanbridge and Board, 1994; Jeppesen, 1995; Tryfinopoulou et al., 2001; Ramalho et al., 2002). This observation is really important as it seems that the correlation of the counts of pseudomonads selective media with the pseudomonads population is misleading. A number of researchers use the oxidase reaction to distinguish the pseudomonads from the other bacteria that are able to grow on PAB medium (Shaw and Latt, 1982; Stanbridge and Davies, 1998; Tryfinopoulou et al., 2002). Nevertheless, this method can be biased by the fact that pseudomonads show a variety in oxidase reactions (Jay, 2000; Liao, 2006).

In conclusion, the succession of the biota was found to be affected by the different storage conditions. However, the outcome of the two strategies employed in this study was quite different. In the most cases, PCR-DGGE analysis was able to detect only the dominant bacteria based on the pure cultures. Similarly, the lack of selectivity was observed at several time points as a higher diversity or different species based on DGGE bands detected beside the recovered isolates. The above observations are leading to the use of different appropriated molecular approaches in order to widen the knowledge of the bacterial succession of meat.

Acknowledgments

The present study was funded by the European Commission under the 7th Framework Programme for RTD “SYMBIOSIS-EU” Project No. 211638. The information in this document reflects only the authors’ views, and the European Community is not liable for any use that may be made of the information contained therein.

References

- Argyri, A.A., Doulgeraki, A.I., Blana, V.A., Panagou, E.Z., Nychas, G.-J.E., 2011. Potential of a simple HPLC based approach to quantify spoilage of minced beef stored at different temperatures and packaging systems. *International Journal of Food Microbiology* 150, 25–33.
- Borch, E., Kant-Muemans, M.L., Blixt, Y., 1996. Bacterial spoilage of meat and cured meat products. *International Journal of Food Microbiology* 33, 103–120.
- Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C., Comi, G., 2004. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. *Applied and Environmental Microbiology* 70, 1883–1894.
- Dainty, R.H., MacKey, B.M., 1992. The relationship between the phenotypic properties of bacteria from chill-stored meat and spoilage processes. In: Board, R.G., Jones, D., Kroll, R.G., Pettipher, G.L. (Eds.), *Ecosystems: Microbes: Food Society for Applied Bacteriology Symposium Series*, vol. 73, pp. 103–114.
- Doulgeraki, A.I., Paramithiotis, S., Nychas, G.-J.E., 2011. Characterization of the *Enterobacteriaceae* community that developed during storage of minced beef under aerobic or modified atmosphere packaging conditions. *International Journal of Food Microbiology* 145, 77–83.
- Doulgeraki, A.I., Paramithiotis, S., Kaglik, D.M., Nychas, G.-J.E., 2010. Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions. *Food Microbiology* 27, 1028–1034.
- Drosinos, E.H., Board, G., 1995. Microbial and physicochemical attributes of minced lamb: sources of contamination with pseudomonads. *Food Microbiology* 12, 189–197.
- Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. The Potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: bias of culture-dependent and culture-independent analyses. *Systematic and Applied Microbiology* 24, 610–617.
- Ercolini, D., Russo, F., Torrieri, E., Masi, P., Villani, F., 2006. Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Applied and Environmental Microbiology* 72, 4663–4671.
- Ercolini, D., Russo, F., Blaiotta, G., Pepe, I., Mauriello, G., Villani, F., 2007. Simultaneous detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from meat by use of a multiplex PCR assay targeting the *carA* gene. *Applied and Environmental Microbiology* 73, 2354–2359.
- Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Ferranti, P., Mauriello, G., Villani, F., 2010a. Different molecular types of *Pseudomonas fragi* have the overall behaviour as meat spoilers. *International Journal of Food Microbiology* 142, 120–131.
- Ercolini, D., Ferrocino, I., La Stora, A., Mauriello, G., Gigli, S., Masi, P., Villani, F., 2010b. Development of spoilage microbiota in beef stored in nisin activated packaging. *Food Microbiology* 27, 137–143.
- García-Lopez, M.L., Prieto, M., Otero, A., 1998. The physiological attributes of Gram-negative bacteria associated with spoilage of meat and meat products. In: Davies, A., Board, R. (Eds.), *The Microbiology of Meat and Poultry*. Blackie Academic and Professional, London, pp. 1–34.
- Gill, G.O., Newton, K.G., 1977. The development of aerobic spoilage on meat stored at chill temperatures. *Journal of Applied Bacteriology* 43, 189–195.
- Gill, C.O., Tan, K.H., 1980. Effect of carbon dioxide on growth of meat spoilage bacteria. *Applied and Environmental Microbiology* 39, 317–319.
- Gill, C.O., 1982. Microbial interaction with meats. In: Brown, M.H. (Ed.), *Meat Microbiology*. Applied Science Publishers, London and NY, pp. 225–264.
- Gill, C.O., Molin, G., 1991. Modified atmospheres and vacuum packaging. In: Russell, N.J., Gould, G.W. (Eds.), *Food Preservatives*. Blackie Academic and Professional, Glasgow and London, pp. 172–199.
- Gustavsson, P., Borch, E., 1993. Contamination of beef carcasses by psychrotrophic *Pseudomonas* and *Enterobacteriaceae* at different stages along the processing line. *International Journal of Food Microbiology* 20, 67–83.
- Harrigan, W.F., 1998. *Laboratory Methods in Food Microbiology*. Elsevier Science and Technology, California.
- Jay, J.M., 2000. *Modern Food Microbiology*, sixth ed. An Aspen Publication, Gaithersburg.
- Jeppesen, C., 1995. Media for *Aeromonas* spp., *Plesiomonas shigelloides* and *Pseudomonas* spp. from food and environment. *International Journal of Food Microbiology* 26, 25–41.
- Khan, N.H., Ishii, Y., Kimata-Kino, N., Esaki, H., Nishino, T., Nishimura, M., Kogure, K., 2007. Isolation of *Pseudomonas aeruginosa* from open ocean and comparison with freshwater, clinical, and animal isolates. *Microbial Ecology* 53, 173–186.
- Labadie, J., 1999. Consequences of packaging on bacterial growth. Meat is an ecological niche. *Meat Science* 52, 299–305.
- Lebert, I., Begot, C., Lebert, A., 1998. Growth of *Pseudomonas fluorescens* and *Pseudomonas fragi* in a meat medium as affected by pH (5.8–7.0), water activity (0.97–1.00) and temperature (7–25°C). *International Journal of Food Microbiology* 39, 53–60.
- Lerner, K.L., Lerner, B.W., 2003. *World of Microbiology and Immunology*. The Gale Group, Inc, USA.
- Liao, C.H., 2006. *Pseudomonas* and related genera. In: Blackburn, C. de W. (Ed.), *Food Spoilage Microorganisms*. Woodhead Publishing Limited, Abington Hall, Abington, Cambridge, pp. 213–286.
- Molin, G., Ternstrom, A., 1982. Numerical taxonomy of psychrotrophic pseudomonads. *Journal of General Microbiology* 128, 1249–1264.
- Molin, G., Ternstrom, A., 1986. Phenotypically based taxonomy of psychrotrophic *Pseudomonas* isolated from spoiled meat, water, and soil. *International Journal of Systematic Bacteriology* 36, 257–274.
- Muyzer, G., De Weal, E.C., Uitterlinden, A., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59, 695–700.
- Nychas, G.-J.E., Dillon, V.M., Board, R.G., 1988. Glucose, the key substrate in the microbiological changes occurring in meat and certain meat products. *Biotechnology and Applied Biochemistry* 10, 203–231.
- Nychas, G.-J.E., Drosinos, E., Board, R.G., 1998. Chemical changes in stored meat. In: Board, R.G., Davies, A.R. (Eds.), *The Microbiology of Meat and Poultry*. Blackie Academic and Professional, London, pp. 288–326.
- Nychas, G.-J.E., Skandamis, P., 2005. Fresh meat spoilage and modified atmosphere packaging (MAP). In: Sofos, J.N. (Ed.), *Improving the Safety of Fresh Meat*. CRC/Woodhead Publishing Limited, Cambridge, pp. 461–502.
- Nychas, G.-J.E., Marshall, D.L., Sofos, J.N., 2007. Meat, poultry, and seafood. In: Doyle, M.P., Beuchat, L.R. (Eds.), *Food Microbiology: Fundamentals and Frontiers*. ASM Press, Washington, DC, pp. 105–140.
- Odagami, T., Morita, J., Takama, K., Suzuki, S., 1994. Substrate specificities of extracellular proteases produced by marine putrefactive bacteria, *Shewanella putrefaciens* and *Alteromonas haloplanktis*. *Letters in Applied Microbiology* 18, 50–52.
- Olsen, R.H., Jezeski, J.J., 1963. Some effects of carbon source, aeration, and temperature on growth of a psychrophilic strain of *Pseudomonas fluorescens*. *Journal of Bacteriology* 86, 429–433.
- Palleroni, N.J., 1993. *Pseudomonas* classification. A new case history in the taxonomy of Gram-negative bacteria. *Antonie van Leeuwenhoek* 64, 231–251.
- Pennacchia, C., Ercolini, D., Villani, F., 2011. Spoilage-related microbiota associated with chilled beef stored in air or vacuum pack. *Food Microbiology* 28, 84–93.
- Ramalho, R., Cunha, J., Teixeira, P., Gibbs, P.A., 2002. Modified *Pseudomonas* agar: new differential medium for the detection/enumeration of *Pseudomonas aeruginosa* in mineral water. *Journal of Microbiological Methods* 49, 69–74.
- Shaw, B.G., Latt, J.B., 1982. A numerical taxonomic study of *Pseudomonas* strains from spoiled meat. *Journal of Applied Bacteriology* 52, 219–228.
- Skandamis, P.N., Nychas, G.-J.E., 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *Journal of Applied Microbiology* 91, 1011–1022.
- Skandamis, P.N., Nychas, G.-J.E., 2002. Preservation of fresh meat with active and modified atmosphere packaging conditions. *International Journal of Food Microbiology* 79, 35–45.

- Stanbridge, L.H., Board, R.G., 1994. A modification of the *Pseudomonas* selective medium, CFC, that allows differentiation between meat pseudomonads and *Enterobacteriaceae*. *Letters in Applied Microbiology* 10, 327–328.
- Stanbridge, L.H., Davies, A.R., 1998. The microbiology of chill stored meat. In: Davies, A.R., Board, R.G. (Eds.), *The Microbiology of Meat and Poultry*. Blackie Academic and Professional, London, pp. 174–219.
- Tryfinopoulou, P., Drosinos, E.H., Nychas, G.-J.E., 2001. Performance of *Pseudomonas* CFC-selective medium in the fish storage ecosystems. *Journal of Microbiological Methods* 47, 243–247.
- Tryfinopoulou, P., Tsakalidou, E., Nychas, G.-J.E., 2002. Characterization of *Pseudomonas* spp. associated with spoilage of Gilt-Head Sea Bream stored under various conditions. *Applied and Environmental Microbiology* 68, 65–72.
- Tsigarida, E., Skandamis, P.N., Nychas, G.-J.E., 2000. Behaviour of *Listeria monocytogenes* and autochthonous flora on meat stored under aerobic, vacuum and modified atmosphere packaging conditions with or without the presence of oregano essential oil at 5°C. *Journal of Applied Microbiology* 89, 901–909.