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

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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.

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-O_2 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 different 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

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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% CO_2–30% O_2–30% N_2 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 (AACGCGAAGAACCTTAC) 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 $MgCl_2$ 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/DDBJ 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 (CGTCAGCACCGAAAAAGCC), lunF (TGTGGCGATTGCAGGCATT) and carAR (TGATGRCCSAGGCA GATRCC) were used to amplify the *car*A 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 $MgCl_2$ 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.