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Presence of quorum sensing signal molecules in minced beef stored under various temperature and packaging conditions

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ABSTRACT

The presence of acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2)-like activity was observed in meat stored under various temperatures (0, 5, 10 and 15 °C) and packaging (air, modified atmospheres and modified atmospheres with oregano essential oil) conditions, and correlated with the ephemeral spoilage organisms that comprise the microbial community generally associated with this product. Quorum sensing signal molecules were found to be affected by the packaging conditions e.g. temperature and atmosphere used for meat preservation as a consequence of the development of a distinct microbial community. AHL signal molecules were detected at all incubation temperatures in minced beef samples, both stored aerobically and under modified atmospheres, when both pseudomonads and *Enterobacteriaceae* populations ranged from 10^7 to 10^9 CFU/g, but no signal molecules were detected in minced beef stored under modified atmospheres in the presence of volatile compounds of oregano essential oil, where both these groups failed to grow in high numbers. Additionally, no significant AI-2 activity was observed in the tested cell-free meat extracts (CFME), regardless of the indigenous bacterial populations. The presence of *N*-(β-ketocaproyl)-homoserine lactone was confirmed with TLC analysis of CFME.
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1. Introduction

It has been reported that cell-to-cell communication system mainly through Quorum sensing signal molecules (QS) governs bacterial behaviour in food ecosystems among others (Skandamis and Nychas, 2012). Indeed signal molecules, such as acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2), have been found to be either present or to increase their concentration in different food systems (Gram et al., 1999; Bruhn et al., 2004; Lu et al., 2004; Liu et al., 2006b; Pinto et al., 2007).

Although in many products the presence of QS has been indicated (for review see Skandamis and Nychas, 2012) the contribution of microbial association in the whole process is still in the dark. The role of specific spoilage organisms (SSO) has been used to provide explanation for the phenomenon but questions are raised. This is the case with meat (ground beef and chicken) spoilage where AHL production has been detected when the concentrations of both pseudomonads and *Enterobacteriaceae* were adequate to support significant proteolytic activity (Liu et al., 2006b). Under such conditions, *Hafnia alvei* and *Serratia* spp. have been shown to be the dominant species among the *Enterobacteriaceae* community isolated from meat. These organisms are capable of producing AHLs, mainly *N*-(β-ketocaproyl)-homoserine lactone (Gram et al., 1999; Ravn et al., 2001; Bruhn et al., 2004; Blana, 2011), suggesting that they are the main QS signal producers in meat samples. Low AI-2-like activity has been also detected in meat stored under modified atmosphere where the lactic acid bacteria found to dominate and to be the main QS producers (Blana et al., 2011).

It has been suggested that these signal molecules are produced by certain members of a microbial community in food, mainly by the genera of the family *Enterobacteriaceae*, *Pseudomonas* spp. and lactic acid bacteria (LAB), which have been found to be major contributors to muscle and vegetable food spoilage, depending on product type and environmental conditions (Pillai and Jesudhasan, 2006; Nychas et al., 2007). Although a number of studies highlight the possible role of QS signal molecules in microbial spoilage (Nychas et al., 2009; Jamuna Bai and Ravishankar Rai, 2011), very little is known about the influence of food processing and storage conditions (e.g., temperature and packaging) on the qualitative and quantitative development of these signals in foods.

Considering the above, the present study aimed at investigating the possible existence of QS signal molecules in minced beef throughout storage under air, modified atmosphere (40% CO_2/30%O_2/ 30% N_2) and modified atmosphere with the presence of volatile compounds of oregano essential oil (2% v/w) at four different temperatures (0, 5, 10 and 15 °C), and at trying to correlate the findings with the indigenous microbial populations.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The bacterial strains used in this study and their characteristics are listed in Table 1. Stock cultures were maintained in vials of treated

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Table 1
Bacterial strains used in this study and their characteristics.

Bacterial strain	Description	Reference
A. tumefaciens A136	AHL sensor strain; contains *traRG′::lacZ*, β-galactosidase reporter, Sp^r Tc^r; cognate signal: 3OC8-HSL	Fuqua and Winans (1994)
A. tumefaciens KYC6	Bioassay positive control; positive AHL producer (3OC8-HSL)	Fuqua and Winans (1994)
C. violaceum CV026	AHL sensor strain; mini Tn-5 mutant of ATCC31532, violacein reporter, Km^r; cognate signal: C6-HSL	McClean et al. (1997)
C. violaceum ATCC31532	Bioassay positive control; positive AHL producer (C6-HSL)	McClean et al. (1997)
E. coli JM109 (pSB401)	AHL sensor strain; contains *luxRI′::luxCDABE*, bioluminescent reporter, Tc^r; cognate signal: 3OC6-HSL	Winson et al. (1998)
E. coli JM109 (pSB536)	AHL sensor strain; contains *rhlRI′::luxCDABE*, bioluminescent reporter, Ap^r; cognate signal: C4-HSL	Winson et al. (1998)
E. coli JM109 (pSB1075)	AHL sensor strain; contains *lasRI′::luxCDABE*, bioluminescent reporter, Ap^r; cognate signal: 3OC12-HSL	Winson et al. (1998)
Pseud. aeruginosa PAO1	Bioassay positive control; positive AHL producer (C4-HSL, 3OC12-HSL)	Holloway et al. (1979)
V. harveyi BAA-1118	AHL sensor strain; contains *luxP::Tn5*, bioluminescent reporter, cognate signal: 3OC4-HSL	Bassler et al. (1997)
V. harveyi BAA-1120	Bioassay positive control; positive AHL producer (3OC4-HSL)	Bassler et al. (1997)
V. harveyi BAA-1117	Sensor strain; contains *luxN::Tn5*, bioluminescent reporter, cognate signal: borated AI-2	Bassler et al. (1997)
V. harveyi BAA-1119	Bioassay positive control; positive AI-2 producer	Bassler et al. (1997)

Sp, spectinomycin; Tc, tetracycline; Km, kanamycin; Ap, ampicillin.

beads in a cryoprotective fluid (Protect Bacterial Preservers, Technical Service, Lancashire, UK) at −80 °C until use. The cultures were revived by adding one bead of the frozen culture of each strain in 10 mL broth medium and incubated for 24 h. The working cultures were prepared by adding 10 μL of each strain into 10 mL broth medium and incubated for 18 h, unless otherwise stated. All strains, except *Vibrio harveyi*, were grown in Luria–Bertani (LB) medium (Bertani, 1951) supplemented with antibiotics when appropriate (100 μg/mL ampicillin, 25 μg/mL kanamycin, 50 μg/mL spectinomycin and 10 μg/mL tetracycline), while *V. harveyi* strains were grown in autoinducer bioassay (AB) medium (Lu et al., 2004). Among the QS biosensor strains used, *Agrobacterium tumefaciens* A136 (pCF218, pCF372) (Fuqua and Winans, 1994), *Chromobacterium violaceum* CV026 (McClean et al., 1997), *V. harveyi* BAA-1117 (BB-170) and *V. harveyi* BAA-1118 (BB886) (Bassler et al., 1997), as well as the positive controls *A. tumefaciens* KYC6 (pCF28), *C. violaceum* ATCC31532, *V. harveyi* BAA-1119 (BB152) and *V. harveyi* BAA-1120 (MM30) were grown at 30 °C. *Escherichia coli* JM109 (pSB401), *E. coli* JM109 (pSB536) and *E. coli* JM109 (pSB1075) (Winson et al., 1998) biosensor strains, as well as the positive control *Pseudomonas aeruginosa* PAO1 were grown at 37 °C (Holloway et al., 1979).

2.2. Minced beef samples, microbiological analyses and pH measurements

Minced beef sample preparation and the methodology employed for the enumeration of the total viable counts, *Pseudomonas* spp., *Brochothrix thermosphacta*, *Enterobacteriaceae*, LAB, and yeasts and moulds are presented in detail elsewhere (Argyri et al., 2011). Briefly, fresh minced beef was aseptically divided into 75-g portions and packaged either aerobically, under modified atmosphere (40% CO_2/30% O_2/30% N_2) and modified atmosphere with the presence of volatile compounds of oregano essential oil (2% v/w). The samples were stored at 0, 5, 10 and 15 °C for up to 650, 482, 315 and 196 h, respectively. Microbiological analyses were conducted at different time intervals, ranging from 6 to approximately 96 h, based on the storage condition, with duplicate samples analysed at each point. Total viable counts were determined on Tryptic Glucose Yeast Agar (Biolife, Milan, Italy) (aerobic incubation at 30 °C for 48 h), *Pseudomonas* spp. on Pseudomonas Agar Base supplemented with cetrimide fucidin cephalosporin (Oxoid, Basingstoke, UK) (aerobic incubation at 25 °C for 48 h), *Br. thermosphacta* on STA Agar Base (Biolife) (aerobic incubation at 25 °C for 48 h), *Enterobacteriaceae* in Violet Red Bile Glucose Agar (Biolife) (microaerophillic incubation at 37 °C for 24 h), LAB in de Man Rogosa and Sharpe agar (Biolife) (microaerophillic incubation at 30 °C for 72 h), and yeasts and moulds on Rose Bengal Chloramphenicol Agar (LAB M, Lancashire, UK) (aerobic incubation at 25 °C for 72 h). The pH values of the minced beef samples were recorded at the end of every microbiological analysis using a pH metre (Metrohm 691 pH metre, Ion Analysis, Switzerland) with a glass electrode.

2.3. Preparation of cell-free meat extracts

Cell-free meat extracts (CFME) were collected at the same time intervals at which the microbiological analyses were carried out. Minced beef samples (5 g) were aseptically removed and subsequently homogenized with sterile quarter-strength Ringer's solution (10 mL) (Lab M) using a stomacher (Lab Blender, Seward Medical, London, UK) for 60 s at ambient temperature. The CFME were obtained by centrifugation of the homogenate at 5000 ×g for 15 min at 4 °C using a Heraeus Multifuge 1S-R centrifuge (Thermo Electron Corporation, Langenselbold, Germany), followed by filtration through 0.2 μm-pore-size filters (Whatman, Clifton, USA). In addition, meat tissue without the endogenous microflora ("clean meat" Nychas et al., 2009) was obtained as previously described by Tsigarida et al. (2000). Briefly, the surface of a piece from beef tissue was sprayed with 100% alcohol and burned with a gas burner in order to reduce the initial microbial load. The burnt surface tissue was removed aseptically, and the "clean" meat tissue below was excised and used to prepare CFME as described earlier (obtained only at 0 h). The sterility of the meat was confirmed using selective and non-selective media as described previously. All CFME were stored at −20 °C until further use.

2.4. Detection of QS signal molecules

2.4.1. AHLs

2.4.1.1. Well diffusion and microplate bioassays. *A. tumefaciens* A136 and *C. violaceum* CV026 biosensor strains were used for the detection of AHLs in a semi-quantitative well diffusion bioassay as described by Ravn et al. (2001) and a qualitative microplate bioassay as presented for the first time herein, where small volumes of broth media and tested CFME samples were used. Briefly describing the well diffusion bioassay, a preculture was prepared as already reported and 1 mL of the preculture was used to inoculate 50 mL ABT medium (Ravn et al., 2001) for *A. tumefaciens* or 50 mL LB medium for *C. violaceum*. The culture was grown at 30 °C for 24 h with agitation (160 rpm) and was poured into 100 mL ABT-agar (1.5% agar) for *A. tumefaciens* A136 or 100 mL LB-agar (1.5% agar) for *C. violaceum* CV026. The agar–culture solution was immediately poured into 60.0-mm diameter Petri dishes (Greiner Bio-One, Munich, Germany). Sixty microliters of CFME was pipetted into wells (diameter 6.0 mm) punched in the solidified agar using a sterile Pasteur pipette (Normax Lda, Marinha Grande, Portugal). The dishes were incubated at 30 °C for 48 h and 24 h when using the *A. tumefaciens* A136 and *C. violaceum* CV026 biosensor strain, respectively.

The induction diameters (in mm) seen as either a blue circle due to induced β-galactosidase activity or purple circle due to induced violacein formation were measured using a graduated scale in millimetres. Regarding the microplate bioassay, precultures of the biosensor strains were prepared and 10 μL of the preculture was used to

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