CRANFIELD UNIVERSITY

Cranfield Health

PhD THESIS

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QUORUM SENSING: UNDERSTANDING THE ROLE OF BACTERIA IN MEAT SPOILAGE

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2010

ABSTRACT

Quorum sensing is a fundamental process to all of microbiology since it is ubiquitous in the bacterial world, where bacterial cells communicate with each other using low molecular weight signal molecules called autoinducers. Despite the fact that quorum sensing regulates numerous bacterial behaviours, very few studies have addressed the role of this phenomenon in foods.

The microbial association of beef consists mainly of pseudomonads, *Enterobacteriaceae*, *Brochothrix thermosphacta* and lactic acid bacteria as revealed by minced beef samples purchased from retail shops, which fluctuates according to the storage conditions. Certain members of the microbial association, which are considered to produce signal molecules, have been found to be major contributors to meat spoilage. *Pseudomonas fragi* and *Enterobacteriaceae* strains, i.e., *Hafnia alvei* and *Serratia liquefaciens* are among the most common quorum sensing signal producers recovered from various food environments.

N-acyl homoserine lactone (AHL) and autoinducer-2 (AI-2) signal molecules were found to be present in meat stored under different conditions (i.e., temperature and packaging), and correlated with the ephemeral spoilage organisms that comprise the microbial community generally associated with this product. The microbial association was strongly affected by the packaging condition, which consequently had an effect on quorum sensing signals detected throughout storage. The presence of signal molecules was detected in minced beef samples stored aerobically and under modified atmospheres, when pseudomonads and *Enterobacteriaceae* populations ranged from 10^7 to 10^9 CFU g^{-1}, whereas in minced beef stored under modified atmospheres with the presence of volatile compounds of oregano essential oil where

Enterobacteriaceae population was inhibited, no signals were detected. *Enterobacteriaceae* appeared to be the main AHL producers since the *Pseudomonas* spp. did not produce detectable AHL signals with the biosensor strains used.

Members of the microbial association i.e., *Enterobacteriaceae* and lactic acid bacteria, which were detected in high populations in minced meat stored aerobically and under modified atmospheres respectively, were capable of producing quorum sensing signal molecules, either AHLs or AI-2. *H. alvei* and *Ser. liquefaciens* were the most common identified AHL-producing bacteria, and *Leuconostoc* spp. was the most common AI-2-producing strain dominating in minced beef packaged under modified atmospheres. AHL-producing bacteria were isolated from all stages of storage, whereas AI-2 producing ones were recovered from relatively high storage temperatures and the initial stages of beef stored at chill temperatures.

The presence of microbial quorum sensing signal molecules, obtained from AHL-producing *H. alvei* strain 718 and AI-2 producing *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 4/74, affected the growth of the spoilage bacteria *Pseudomonas fluorescens* and *Ser. liquefaciens*. AHL signal molecules encouraged the growth of both spoilage bacteria tested, while bacterial growth was reduced under low amounts of AI-2 molecules.

These data indicate the involvement of quorum sensing signal molecules in modulating the ecology of these bacteria and suggest that they play a role in influencing the rate of spoilage of meat products.

ACKNOWLEDGEMENTS

I would like to express my greatest thanks to my supervisors Prof. *Naresh Magan* and Prof. *George-John Nychas* for their advices and support throughout this study.

I would like to thank my two colleagues and best friends *Anthoula Argyri* and *Agapi Doulgeraki* for working together and supporting me during my study.

Finally, I would like to thank my family, my parents *Anastasios* and *Georgia* and my brother *Nick,* for their support and encouragement all these years.

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ABBREVIATIONS

AB: Autoinducer bioassay medium

AHL: *N*-acyl homoserine lactone

AI: Autoinducer

CFCE: Cell-free culture extract

$CFCE_{AHL}$: Cell-free culture extract derived from AHL-producing strain *Hafnia alvei* 718

$CFCE_{AI2}$: Cell-free culture extract derived from AI-2 producer *Salmonella enterica* serovar Typhimurium 4/74

$CFCE_{E}$: Cell-free culture extracts from *Enterobacteriaceae* strains

$CFCE_{HT}$: Heat treated cell-free culture extract derived from AI-2 producer *Salmonella enterica* serovar Typhimurium 4/74

$CFCE_{LAB}$: Cell-free culture extracts of lactic acid bacteria

$CFCE_{MUT}$: Cell-free culture extract derived from AHL-lacking mutant *Hafnia alvei* 718 *halI*

$CFCE_{ST}$: Cell-free culture extract derived from *Salmonella enterica* serovar Typhimurium 4/74

CFME: Cell-free meat extract

$CFME_{Sterile}$: Cell-free meat extract derived from "sterile" meat tissue

HPLC: High performance liquid chromatography

LAB: Lactic acid bacteria

lag: Lag-phase duration

LB: Luria-Bertani medium

$LB_{glucose}$: Luria-Bertani medium supplemented with 0.5% (w/v) glucose

QS: Quorum sensing

RLU: Relative light units

TLC: Thin layer chromatography

μ_{max}: Maximum specific growth rate

GC: Gas chromatography

Chapter 1

Literature Review and Objectives

1.1 Quorum Sensing

Bacteria communicate with each other by releasing low molecular weight signal molecules comparable to hormones. As in higher organisms, the information supplied by these molecules is essential for coordinating the activities of large groups of cells. In bacteria, communication involves producing, releasing, detecting, and responding to signal molecules called autoinducers (AIs) (Waters & Bassler, 2005). This process, termed quorum sensing (QS) (Fuqua *et al.,* 1994), allows bacteria to monitor the environment for other bacteria and to alter behaviour on a population-wide scale in response to changes in the number and/or species present in a microbial community. Generally QS-controlled processes are unproductive when undertaken by a bacterium acting individually but become beneficial when carried out simultaneously by a large number of cells. Thus, QS confuses the distinction between prokaryotes and eukaryotes because it allows bacteria to behave as multi-cellular organisms. Various bacterial behavioural responses are regulated by QS, including symbiosis, virulence, antibiotic biosynthesis, luminescence, sporulation, motility, plasmid transfer and biofilm formation (Federle & Bassler, 2003; Ammor *et al.*, 2008; Chorianopoulos *et al.,* 2010). Recent studies show that highly specific as well as universal QS languages exist which enable bacteria to communicate within and between species (Schauder & Bassler, 2001; Waters & Bassler, 2005). Quorum sensing was first described in the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi* (Nealson *et al.,* 1970; Nealson & Hastings, 1979), and since then it has been shown to be a widespread mechanism of gene regulation in bacteria.

1.1.1 The languages of bacteria

Quorum sensing in Gram-negative bacteria

In Gram-negative bacteria, the signal molecules are *N*-acyl homoserine lactones (AHLs) used primarily for intra-species communication and known as autoinducer-1 (AI-1) (Miller & Bassler, 2001; Whitehead *et al.,* 2001; Ammor *et al*, 2008). Gram-negative bacteria appear to communicate through the standard signaling circuit LuxI/LuxR (AHL/transcriptional regulator). A general model showing the fundamental components of a Gram-negative QS circuit is presented in Figure 1.1. In brief, the LuxI-like proteins are the autoinducer synthases, and they catalyze the formation of a specific AHL autoinducer molecule (green pentagons). The auto-inducer freely diffuses through the cell membrane and accumulates at high cell density. At high auto-inducer concentration, the LuxR-like proteins bind their cognate autoinducers. The LuxR-auto-inducer complexes bind at target gene promoters and regulate transcription (Schauder & Bassler, 2001; Bassler, 2002; Lazdunski *et al.,* 2004; Gobbetti *et al.,* 2007).

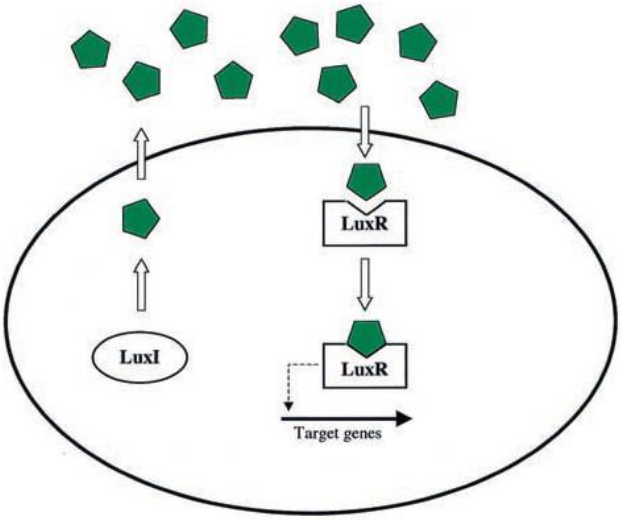

Figure 1.1. The LuxI/LuxR quorum sensing system of Gram-negative bacteria. (Schauder & Bassler, 2001)

Quorum sensing in Gram-positive bacteria

Gram-positive QS bacteria communicate using modified oligopeptides as signals (Miller & Bassler, 2001; Whitehead *et al.,* 2001; Gobbetti *et al.*, 2007) and have evolved a basic communication mechanism that is different from that used by Gram-negative bacteria. A generalized model showing the general components of a Gram-positive QS circuit is presented in Figure 1.2. A specific precursor peptide (large blue bars) is produced. The precursor peptide is modified, processed, and an ATP-binding cassette (ABC) exporter complex secretes the mature oligopeptide autoinducer (short blue bars). The oligopeptide autoinducer accumulates as the cells grow. At high cell density, the autoinducer is detected by a two-component signal transduction system. Specifically, the sensor kinase protein recognizes the autoinducer and subsequently autophosphorylates at a conserved histidine residue (H). The phosphoryl group is transferred to a cognate response regulator protein, and this

protein is phosphorylated on a conserved aspartate residue (D). The phosphorylated response regulator binds to specific target promoters to modulate the expression of QS regulated genes. P denotes that the mechanism of signal transduction is by phosphate transfer between the regulatory elements (Schauder & Bassler, 2001; Bassler, 2002).

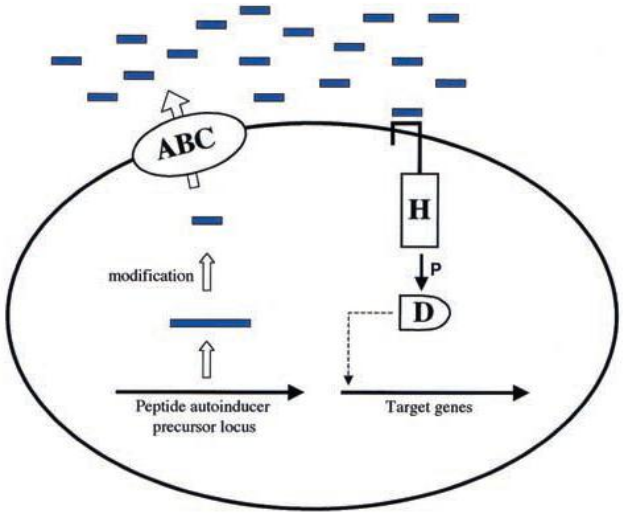

Figure 1.2. Oligopeptide mediated quorum sensing in Gram-positive bacteria. (Schauder & Bassler, 2001)

The universal LuxS language

LuxS protein is responsible for the production of autoinducer-2 (AI-2), which is involved in the quorum-sensing response of the bioluminescent bacterium *V. harveyi*. AI-2 is found in several other gram-negative and gram-positive bacteria and is used for intra-species as well as inter-species communication (Schauder & Bassler, 2001; Chen *et al.,* 2002; Ammor *et al.*, 2008). The structure of *V. harveyi* AI-2 has recently been determined. The AI-2 molecule is a novel furanosyl borate diester with no similarity to other autoinducers (Schauder *et al.,* 2001).

V. harveyi, while closely related to *V. fischeri*, does not live in symbiotic associations with higher organisms. *V. harveyi* is found free-living in the seawater, in

shallow sediments and on the surfaces and in the gut tracts of various marine animals and uses QS to control bioluminescence (Henken & Bassler, 2004). However, unlike *V. fischeri* and all other Gram-negative QS bacteria, *V. harveyi* does not employ a canonical LuxI/LuxR-type QS mechanism. *V. harveyi* has evolved a QS circuit that has characteristics typical of both Gram-negative and Gram-positive bacterial QS systems. Specifically, *V. harveyi* uses an AHL autoinducer similar to other Gram-negative QS bacteria, but the signal detection and relay apparatus consists of two component proteins similar to the QS systems of Gram-positive bacteria. These findings have led to the proposal that AI-2 is a 'universal' signal, which functions in interspecies cell-to-cell communication (Surette & Bassler, 1998; Reading & Sperandio, 2006).

A model showing the hybrid QS circuit employed by *V. harveyi* is presented in Figure 1.3. Briefly, an AHL autoinducer (AI-1, green pentagons) is produced by the activity of LuxLM. A second autoinducer (AI-2, red pentagons) is synthesized by the enzyme LuxS. Both autoinducers accumulate as a function of cell density. The sensor for AI-1 is LuxN, and two proteins, LuxP and LuxQ, function together to detect AI-2. LuxP is homologous to the periplasmic ribose binding protein of *Escherichia coli*. LuxN and LuxQ are hybrid sensor kinase/response regulator proteins that transduce information to a shared integrator protein called LuxU. LuxU sends the signal to the response regulator protein LuxO. The mechanism of signal transduction is phosphorelay (denoted P). LuxO controls the transcription of a putative repressor protein (denoted X), and a transcriptional activator protein called LuxR is also required for expression of the luciferase structural operon (*luxCDABE*). The conserved phosphorylation sites on the two-component proteins are indicated as H (histidine) and D (aspartate) (Schauder & Bassler, 2001; Xavier & Bassler, 2003).

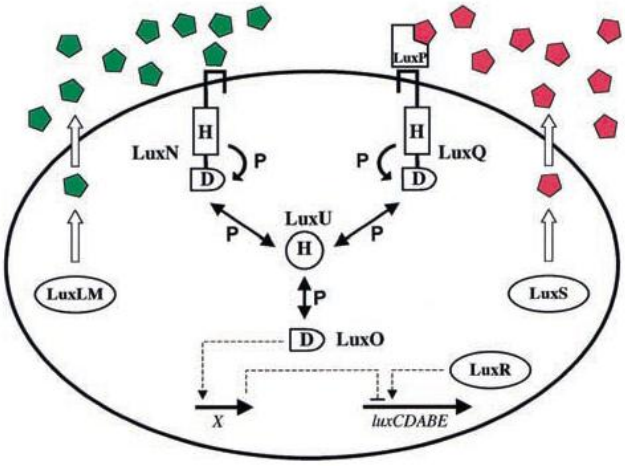

Figure 1.3. The hybrid quorum sensing circuit of *Vibrio harveyi*.
(Schauder & Bassler, 2001)

1.1.2 Quorum sensing signal molecules

Several classes of bacterial signal molecules have until now been identified and can be divided into four broad categories:

(i) fatty acid derivatives, generally *N*-acyl homoserine lactones (AHLs), known as autoinducer-1 (AI-1). They are produced and used by Gram-negative bacteria primarily for intra-species communication (Whitehead *et al.,* 2001; Smith *et al.,* 2006),

(ii) a furanosyl borate diester, which is derived from the recycling of S-adenosyl-homocysteine (SAH) to homocysteine and known as autoinducer-2 (AI-2). It is produced by both Gram-positive and Gram-negative bacteria and thought to serve as a universal signal for inter-species and intra-species communications (Shcauder *et al.,* 2001; Winzer *et al.,* 2003; De Keersmaecker *et al.,* 2006),

(iii) autoinducer-3 (AI-3) of unknown structure, is present in *E. coli* O157:H7 and cross-talk with the mammalian epinephrine host cell signaling system (Sperandio *et al.,* 2003; Reading *et al.,* 2007), and

(iv) autoinducing peptides (AIPs) produced and used by Gram-positive bacteria (Whitehead *et al.,* 2001; Sturme *et al.,* 2002; Lyon & Novick, 2004).

In parallel with the previously mentioned QS signals, other molecules have been also described. The 2-heptyl-3-hydroxy-4-quinolone (PQS) is an intracellular signal molecule that has been found in *Pseudomonas aeruginosa* (Pesci *et al.,* 1999; Wade *et al.,* 2005). Diketopiperazines (DKP), which are small and diffusible molecules, were also found to be involved in QS systems (Holden *et al.,* 1999). They have high biological and pharmacological effects on cells of higher organisms, suggesting their role in bacterial conversation with plant and animal cells rather than with other bacteria. Finally, CAI-1, whose chemical nature is unknown, is proposed to be responsible for *Vibrio*-specific signaling (Henke & Bassler, 2004).

Autoinducer-1

Many Gram-negative bacteria synthesize AHLs with *N*-acyl side chains ranging from 4 to 18 carbons and with an oxo-, hydroxy- or unsubstituent at the C3 position (Figure 1.4) (Whitehead *et al.,* 2001; Reading & Sperandio, 2006; McDougald *et al.,* 2007). The differences in the length of the acyl side chain and of the substitutions on the side chain are specificity determinants for different QS systems (Zhu *et al.,* 2003). AHLs are generally capable of diffusing across the bacterial membrane, but long-chain AHLs seem to be actively transported in and out

of the cells through efflux and influx mechanisms (Pearson *et al.,* 1999, Whitehead *et al.,* 2001).

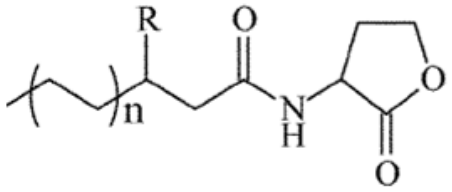

Figure 1.4. *N*-acyl homoserine lactone (AHL) molecule, typical quorum sensing signal in Gram-negative bacteria (R: -H, -OH or =O).

A number of factors such as temperature, pH, NaCl, growth media and growth phase have been reported that might influence AHL amounts, length of the *N*-acyl side chain and of the substitutions on the side chain of carbons of *N*-acyl side chains. Indeed, cultures in minimal media generally displayed one or two more signals, as compared to complex media (Gonzalez *et al.,* 2001; Lithgow *et al.,* 2001). While the addition of casamino acids to autoinducer bioassay (AB) medium reduced the amounts of long chain AHLs produced by *Pseud. aeruginosa* isolates from cystic fibrosis lungs (Geisenberger *et al.,* 2000), lowering the growth temperature from 37 to 28 °C resulted in the disappearance of low molecular weight AHLs produced by *Yersinia pseudotoberculosis* (Atkinson *et al.,* 1999). At alkaline pH, AHLs become unstable and hydrolyze (Ravn *et al.,* 2003). Meanwhile, it has been shown that AHL amounts declined when bacteria entered stationary phase (Byers *et al.,* 2002; Ravn *et al.,* 2003).

Autoinducer-2

The AI-2 is a unique, universal signal that could be used by a variety of bacteria for communication between and among species. It controls a wide variety of phenotypes in many bacterial species including, production of extracellular virulence factors by *Pseud. aeruginosa* (Winzer & Williams, 2001) and *Staphylococcus aureus* (McDowell *et al.,* 2001), levels of an ABC transporter in *Salmonella* Typhimurium (Taga *et al.,* 2001), transcription of a number of genes in *E. coli* (DeLisa *et al.,* 2001), protease production by *Porphyromonas gingivalis* (Burgess *et al.,* 2002), and bioluminescence in *V. fischeri* (Nealson *et al.,* 1970) and *V. harveyi* (Bassler *et al.,* 1994).

It has been revealed that AI-2 production is influenced by temperature and the growth medium (Cloak *et al.,* 2002). Intracellular metabolism and stress conditions can alter the AI-2 production pattern in *E. coli* K-12 (DeLisa *et al.,* 2001). It has also been shown that food additives such as sodium propionate, sodium benzoate, sodium acetate and sodium nitrate may influence AI-2 production (Lu *et al.,* 2004). It has been also demonstrated that fatty acids (medium- and long- chain) isolated from ground beef and poultry can inhibit AI-2 activity (Soni *et al.,* 2008; Widmer *et al.,* 2007) as well as the presence of glucose (Turovskiy & Chikindas, 2006).

1.1.3 Methods for detecting quorum sensing signals in foods and bacteria

Procedures for the detection, analysis, identification, characterization and purification of signal molecules have been previously described (Steindler & Venturi, 2006; Ammor *et al.,* 2008). Generally, the detection of QS signals can be performed

either directly from cell-free supernatants or extracts of food samples, or from spent culture supernatants of bacteria isolated from food products.

Autoinducer-1

Structural elucidation of AHL requires analytical methods including mass spectrometry (MS), gas chromatography (GC)-MS, high-performance liquid chromatography (HPLC)-MS, nuclear magnetic resonance (NMR) spectroscopy and infrared spectroscopy (IR) (Zhang *et al.,* 1993; Throup *et al.,* 1995; Schaefer *et al.,* 2000; Cataldi *et al.,* 2007). However, using bacterial biosensors, it is possible to detect and determine the type of produced AHLs. These biosensors do not produce AHLs either naturally or after inactivation of the *luxI* homologous gene responsible for AHL production. They contain a functional LuxR-family protein cloned with a cognate target promoter, which up-regulates the expression of a reporter gene encoding for a phenotypic response (e.g., bioluminescence, violacein pigment production, ß-galactosidase and green fluorescent protein) only in the presence of exogenous AHLs (Steindler & Venturi, 2007). The biosensor strains that have been developed to detect AHLs are listed in Table 1.1.

Table 1.1. *N*-acyl homoserine lactone (AHL) biosensor strains

Strain/Plasmid sensor	**Host**	**Based on QS system**	**Reporter system**	**Best responds to**	**Good detection**	**Reference**
pCF218 + pCF372	*Agrobacterim tumefaciens* WCF47	TraI/R (*A. tumefaciens*)	ß-galactosidase	3-oxo-C8-HSL	All 3-oxo-HSLs C6-HSL C8-HSL C10-HSL C12-HSL C14-HSL 3-hydroxy-C6-HSL 3-hydroxy-C8-HSL 3-hydroxy-C10-HSL	Zhu *et al.*, 1998
pDCI41E33	*A. tumefaciens* NT1	TraI/R (*A. tumefaciens*)	ß-galactosidase	3-oxo-C8-HSL	As above	Shaw *et al.*, 1997
pZLR4	*A. tumefaciens* NT1	TraI/R (*A. tumefaciens*)	ß-galactosidase	3-oxo-C8-HSL	As above	Farrand *et al.*, 2002
pJZ384 + pJZ410 + pJZ372	*A. tumefaciens* KYC55	TraI/R (*A. tumefaciens*)	ß-galactosidase	3-oxo-C8-HSL	As above	Zhu *et al.*, 2003
pKDT17	*E. coli*	LasI/R (*Pseud. aeruginosa*)	ß-galactosidase	3-oxo-C12-HSL	C12-HSL C10-HSL 3-oxo-C10-HSL	Pearson *et al.*, 1994
M71LZ	*Pseud. aeruginosa lasI⁻*	LasI/R (*Pseud. aeruginosa*)	ß-galactosidase	3-oxo-C12-HSL	3-oxo-C10-HSL	Dong *et al.*, 2005
pJNSinR	*Sinorhizobium meliloti sinI:lacZ*	SinI/R (*S. meliloti*)	ß-galactosidase	3-oxo-C14-HSL	3-oxo-C16:1-HSL C16-HSL C16:1-HSL C14-HSL	Llamas *et al.*, 2004

Table 1.1. Continued

Strain/Plasmid sensor	Host	Based on QS system	Reporter system	Best responds to	Good detection	Reference
S. meliloti sinI:lacZ	*S. meliloti sinI:lacZ*	SinI/R (*S. meliloti*)	ß-galactosidase	3-oxo-C14-HSL	3-oxo-C16:1-HSL C16-HSL C16:1-HSL C14-HSL	Llamas *et al.*, 2004
pSF105 + pSF107	*Pseud. fluorescence* 1855	PhzI/R (*Pseud. fluorescence* 2-79)	ß-glucoronidase ß-galactosidase	3-hydroxy-C6-HSL	3-hydroxy-C8-HSL	Khan *et al.*, 2005
pHV2001	*E. coli*	LuxI/R (*V. fischeri*)	*luxCDABE*	3-oxo-C6-HSL	C6-HSL 3-oxo-C8-HSL C8-HSL	Pearson *et al.*, 1994
pSB315	*E. coli*	LuxI/R (*V. fischeri*)	*luxCDABE*	3-oxo-C6-HSL	C6-HSL 3-oxo-C8-HSL C8-HSL	Swift *et al.*, 1993
pSB401	*E. coli*	LuxI/R (*V. fischeri*)	*luxCDABE*	3-oxo-C6-HSL	C6-HSL 3-oxo-C8-HSL C8-HSL	Winson *et al.*, 1998
pSB403	Broad host range	LuxI/R (*V. fischeri*)	*luxCDABE*	3-oxo-C6-HSL	C6-HSL 3-oxo-C8-HSL C8-HSL	Winson *et al.*, 1998
pSB406	*E. coli*	RhlI/R (*Pseud. aeruginosa*)	*luxCDABE*	3-oxo-C6-HSL	C6-HSL 3-oxo-C8-HSL C8-HSL	Winson *et al.*, 1995
pAL101	*E. coli* (*sdiA* mutant)	RhlI/R (*Pseud. aeruginosa*)	*luxCDABE*	C4-HSL		Lindsay & Ahmer, 2005

Table 1.1. Continued

Strain/Plasmid sensor	**Host**	**Based on QS system**	**Reporter system**	**Best responds to**	**Good detection**	**Reference**
pSB1075	*E. coli*	LasI/R (*Pseud. aeruginosa*)	*luxCDABE*	3-oxo-C12-HSL	3-oxo-C10-HSL C12-HSL	Winson *et al.*, 1998
pSB536	*E. coli*	AhyI/R (*Aeromonas hydrophila*)	*luxCDABE*	C4-HSL		Swift *et al.*, 1997
pJBA-132	Broad host range	LuxI/R (*V. fischeri*)	gfp	3-oxo-C6-HSL	C6-HSL C8-HSL C10-HSL	Andersen *et al.*, 2001
pAHL-GFP	*E. coli* MC4100	LuxI/R (*V. fischeri*)	gfp	C8-HSL		Burmolle *et al.*, 2003
pKR-C12	Broad host range	LasI/R (*Pseud. aeruginosa*)	gfp	3-oxo-C12-HSL	3-oxo-C10-HSL	Riedel *et al.*, 2001
pAS-C8	Broad host range	CepI/R (*B. cepacia*)	gfp	C8-HSL	C10-HSL	Riedel *et al.*, 2001
Chromobacterium violaceum	*C. violaceum*	CviII/R (*C. violaceum*)	Violacein production	C6-HSL	C4-HSL 3-oxo-C6-HSL 3-oxo-C8-HSL C8-HSL	McClean *et al.*, 1997

Modified from Ammor *et al.*, 2008

Autoinducer-2

Chemical detection of AI-2 by HPLC and GC is difficult at present due to the low concentration and instability of the molecule. The detection of AI-2 signal molecule relies on a bioassay that involves the biosensor strain *V. harveyi* BB170 (Bassler *et al.,* 1997). Recently, a LuxP-FRET-based biosensor strain has been developed for the rapid detection and quantification of AI-2, as well as *Salmonella* Typhimurium MET844 through which the nonborated form of AI-2 can be detected (Pillai & Jesudhasan, 2006; Rajamani *et al.,* 2007) (Table 1.2).

Table 1.2. Autoinducer-2 (AI-2) biosensor strains

Strain/Plasmid sensor	**Host**	**Based on QS system**	**Reporter system**	**Responds to**	**Reference**
V. harveyi BB170	*V. harveyi*	*lux*N::Tn5	*luxCDABE*	AI-2	Bassler *et al.,* 1997
FRET-based AI-2 biosensor	*E. coli* BL21	*lux*P::*gfp*	gfp	AI-2	Rasch *et al.,* 2005
MET844	*Salmonella* Typhimurium	*rpsL*, *putRA*::Kan-*lsr-lac-ZYA*, *ΔlsrFGE*::Cm, *luxS*::T-POP	ß-galactosidase	Nonborated AI-2	Taga *et al.,* 2001

Modified from Ammor *et al.,* 2008

1.2 Role of Quorum Sensing in meat spoilage

Quorum sensing has been implicated in microbial food spoilage. Various signal molecules, such as AI-1 and AI-2, have been found to be either present or to increase their concentration in different food systems e.g., fish, milk, meat and vegetables (Gram *et al.,* 1999; Bruhn *et al.,* 2004; Lu *et al.,* 2004; Liu *et al.,* 2006; Pinto *et al.,* 2007). It has been suggested that these signal molecules are produced by certain members of the initial microbial association. This comprises genera from the family of *Enterobacteriaceae, Photobacterium phosphoreum, Shewanella (Alteromonas) putrefaciens, Brochothrix thermosphacta, Pseudomonas* spp., *Aeromonas* 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 conditions surrounding the product (Pillai & Jesudhasan, 2006; Nychas *et al.,* 2007). Although a number of studies highlight the possible role of QS signals in microbial spoilage (Bruhn *et al.,* 2004; Ammor *et al.,* 2008; Nychas *et al.,* 2009), very little is known about the influence of food processing and storage conditions (e.g., temperature, packaging) on the type and amounts of these signals in foods.

1.2.1 Spoilage of meat

Meat spoilage can be considered as an ecological system that encompasses changes in the available substrates during proliferation of bacteria present in the microbial association of the stored meat, called specific spoilage organisms (SSO). In fact, spoilage of meat depends on an even smaller fraction of SSO, called ephemeral spoilage organisms (ESO). The dominance of this particular microbial association in meat depends on factors that persist during processing, storage, transportation and in

the market (Nychas *et al.*, 2007). There is no doubt that microbiological activity is the most important factor influencing the changes that cause spoilage in meat in comparison with the contribution of indigenous meat enzymes to spoilage which is negligible (Tsigarida & Nychas, 2001; Nychas *et αl.,* 1998).

It is generally accepted that bacteria are absent, undetectable, or at extremely low populations in muscle tissues of healthy live food animals. Fresh meat begins to undergo change from the moment of slaughter of the animal (Gill, 2005; Nychas *et αl.,* 2007). As the inherent protective barriers, skin, hides, scales and shells, and natural antimicrobial defense mechanisms (lysozyme and antimicrobial peptides) of the live animal are destroyed at slaughter, the resulting meat is exposed to increasing levels of contamination. Depending on various extrinsic parameters (e.g. temperature, packaging and processing method) the meat may undergo rapid microbial decay. The slaughtering process may lead to extensive contamination of the exposed cut surfaces of muscle tissue with a vast array of Gram-negative and Gram-positive bacteria, as well as molds and yeasts. Sources of these microorganisms include the external surfaces of the animal and the gastrointestinal tract, as well as the environment with which the animal had contact some time before or during slaughter (Nychas *et αl.,* 2007). Studies on the origin of the contaminants have shown that the source of *Enterobacteriaceae* on meat is associated with work surfaces and not with direct faecal contamination. Furthermore, psychrotrophic bacteria are recovered from hides and work surfaces within an abattoir, as well as from carcasses and butchered meat at all stages of processing (Gill & Newton, 1978; Nychas & Drosinos, 2000).

Microorganisms of the spoilage association

Despite the fact that meat may be contaminated with a wide range of microbes, its spoilage is caused by relatively few of these microorganisms that become dominant through selection during storage and develop a microbial association (Nychas & Drosinos, 1999; Nychas *et al.*, 2007). The main bacteria implicated in the spoilage of meat include *Enterobacteriaceae*, *Pseudomonas* spp., *Sh. putrefaciens*, *Br. thermosphacta* and LAB (Lambert *et al.,* 1991; Borch *et al.,* 1996; Ercolini *et al.*, 2006). An association of bacteria, commonly dominated by *Pseudomonas* spp., particularly *Pseud. fragi*, *Pseud. fluorescens* and *Pseud. putida*, is in most cases responsible for spoilage of meat stored aerobically at cold temperatures (Nychas *et al.,* 2007). Psychrotrophic members of the *Enterobacteriaceae*, namely *Hafnia alvei*, *Serratia liquefaciens* and *Enterobacter agglomerans*, also occur on chilled meat and meat products stored aerobically (Jay *et al.,* 2003; Liu *et al.,* 2006) but in terms of population numbers they do not contribute to the microbial associations. Major representatives of this family in ground beef include *Pantoea agglomerans*, *E. coli* and *Ser. liquefaciens* (Nychas *et al.*, 2008).

Br. thermosphacta and LAB represent a significant portion of the spoilage flora of meat stored aerobically at refrigerated conditions, but they are not considered to be essential in spoilage except possibly for lamb (Drosinos, 1994). These organisms have been isolated from beef carcasses during boning, dressing and chilling. Additionally, lairage slurry, cattle hair, rumen contents, soil from the walls of slaughter houses, the hands of workers, air in the chill room, the neck and the skin of the animal, as well as the cut muscle surfaces have been shown to be contaminated with this organism. Both *Br. thermosphacta* and LAB are the main cause of spoilage

recognized as souring rather than putrefaction. The type of spoilage is commonly associated with meat packed under vacuum or modified atmospheres (Nychas *et al.*, 2008).

The type and extent of spoilage are governed by the availability of low-molecular weight compounds (e.g., glucose, lactate and free amino acids) existing in meat (Nychas *et al.,* 1988; Nychas *et al.,* 2007). Only the exhaustion of these compounds affects the activity of extracellular proteolytic enzymes and may influence the development of microbial community in general as well as the microbial domain (e.g., habitat and activity domain) (Boddy *et al.,* 1992; Liu *et al.,* 2006).

1.2.2 Quorum sensing in meat and meat products

The contribution of QS in the spoilage process of fresh meat products stored under aerobic refrigerated conditions, and in the biofilm formation appearing as slime at their surfaces has been already proposed (Jay *et al.,* 2003). AHL production has been detected in such products (e.g., ground beef and chicken) and appears concomitantly with proteolytic activity (Liu *et al.,* 2006). A broader range of AHL signals has been detected in aerobically chill-stored ground beef and chicken, at pseudomonad and *Enterobacteriaceae* concentrations at which significant proteolytic activity was recorded (Liu *et al.,* 2006).

The shelf life of fresh meats stored aerobically at refrigerated temperatures is in the range of days, whereas the shelf life of vacuum-packed meat stored at refrigerated temperatures is extended to weeks or months. In the last case, the microbiota typically consists of *Enterobacteriaceae* and LAB at levels of 10^6 and 10^8

CFU g^{-1}, respectively, thus suggesting that the spoilage is a result of an interaction between *Enterobacteriaceae* and LAB (Bruhn *et al.,* 2004). *H. alvei* and *Serratia* spp. have been shown to be the dominating species among the *Enterobacteriaceae* isolated from vacuum-packed meat. These strains are capable of producing AHLs (Gram *et al.,* 1999; Ravn *et al.,* 2001; Bruhn *et al.,* 2004), while *Pseudomonas* isolates do not produce detectable numbers of AHL signal molecules with the biosensor strains used (Bruhn *et al.,* 2004). However, AHL prevalence in vacuum-packed meats was found to have no significant role in the spoilage of such products (Bruhn *et al.,* 2004). *Pseud. fragi* stains, associated with spoilage, isolated from fresh and spoiled meat produced AI-2 signal molecules when tested using the bioluminescent biosensor *V. harveyi* BB170 (Ferrocino *et al.,* 2009). Thought, the mechanism of AI-2 production and its possible role in spoilage processes needs further study.

Lu *et al.* (2004) reported very low levels of AI-2 activity (less than one fold induction of luminescence compared to the negative control) in meat products although their high indigenous bacterial population loads. On the same study, certain meat matrices were tested for inhibiting AI-2-like activity (Lu *et al.,* 2004). Previous findings suggest that the presence of fatty acids isolated from ground beef and poultry meat can inhibit AI-2-based cell signalling (Widmer *et al.,* 2007; Soni *et al.,* 2008). Additionally, food additives such as sodium propionate, sodium benzoate, sodium acetate and sodium nitrate may influence AI-2 production (Lu *et al.,* 2004).

In a recent study, Nychas *et al.* (2009) found that cell-free meat extract derived from spoiled minced pork meat stored aerobically at 5 and 20 °C contained QS signals. It was also observed, that the addition of cell-free meat extract from spoiled meat (containing QS signal molecules) to cultures of *Pseud. fluorescens* and *Ser.*

marcescens resulted in an extension of the lag phase of *Pseud. fluorescens* but not of *Ser. marcescens* when compared to control samples and in an increase of the metabolic activity for both strains. The observed increase in metabolic activity was suggested to be related to the presence of some compounds in cell-free meat extract, including QS signal molecules (Nychas *et al.*, 2009).

In the following table an overview of QS studies relevant to meat, meat products and strains isolated from these food environments as reported in the literature is summarised (Table 1.3). The classification was based on the biosensor strains used for screening QS signal molecules in these foods and the isolates.

Table 1.3. Overview of quorum sensing (QS) studies relevant to meat, meat products and strains isolated from these food environments based on biosensors used

Strain/Plasmid sensor	**Host**	**Based on QS system**	**Reporter system**	**Best responds to**	**Good detection**	**QS bioassay in**	**Reference**
pSB403	Broad host range	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-HSL	C6-HSL C8-HSL C8-3-oxo-HSL	*Enterobacteriaceae* strains isolated from vacuum-packed chilled meat	Gram *et al.*, 1999
pCF218, pCF372	*A. tumefaciens* WCF47	TraI/R (*A. tumefaciens*)	ß-galactosidase	C8-3-oxo-HSL	All 3-oxo-HSLs C6-HSL C8-HSL C10-HSL C12-HSL C14-HSL 3-hydroxy-C6-HSL 3-hydroxy-C6-HSL 3-hydroxy-C6-HSL	Meat extracts and isolated *Enterobacteriaceae* strains from chill-stored vacuum-packed meat	Bruhn *et al*., 2004
						Pseudomonad and *Enterobacteriaceae* isolates from aerobically chilled-stored proteinaceous raw foods	Liu *et al*., 2006
						Cell-free extracts from minced pork stored aerobically at 5 and 20 °C	Nychas *et al.*, 2009
						Pseud. fragi isolated from fresh and spoiled meat	Ferrocino *et al.*, 2009
C. violaceum CV026	*C. violaceum*	CviI/R (*C. violaceum*)	Violacein production	C6-HSL	C4-HSL C8-HSL C6-3-oxo-HSL C8-3-oxo-HSL	*Enterobacteriaceae* strains isolated from vacuum-packed chilled meat	Gram *et al.*, 1999
						Meat extracts and isolated *Enterobacteriaceae* strains from chill-stored vacuum-packed meat	Bruhn *et al.*, 2004
						Aeromonas hydrophila strains isolated from meat	Medina-Martinez *et al*., 2006
						Y. enterocolitica in fresh foods extracts	Medina-Martinez *et al*., 2006
						Pseudomonad and *Enterobacteriaceae* isolates from aerobically chilled-stored proteinaceous raw foods	Liu *et al*., 2006

Table 1.3. Continued

Strain/Plasmid sensor	Host	Based on QS system	Reporter system	Best responds to	Good detection	QS screening in	Reference
						Cell-free extracts from minced pork stored aerobically at 5 and 20 °C	Nychas *et al.*, 2009
						Pseud. fragi isolated from fresh and spoiled meat	Ferrocino *et al.*, 2009
V. harveyi BB170	*V. harveyi*	*luxN*::Tn5	*luxCDABE*	Borated AI-2		Food samples e.g. beef, chicken, turkey products (AI-2-like activity)	Lu *et al.*, 2004
						Poultry meat-derived fatty acids, as inhibitors to AI-2	Widmer *et al.*, 2007
						Survival and virulence gene expression of *E. coli* O157:H7 in the presence of AI-2 and ground beef extracts	Soni *et al.*, 2008
						Ground beef–derived fatty acids, as inhibitors to AI-2	Soni *et al.*, 2008
						Cell-free extracts from minced pork stored aerobically at 5 and 20 °C	Nychas *et al.*, 2009
						Pseud. fragi isolated from fresh and spoiled meat	Ferrocino *et al.*, 2009
pZLR4	*A. tumefaciens* NT1	TraI/R (*A. tumefaciens*)	ß-galactosidase	C8-3-oxo-HSL	All 3-oxo-HSLs C6-HSL C8-HSL C10-HSL C12-HSL C14-HSL 3-hydroxy-C6-HSL 3-hydroxy-C6-HSL 3-hydroxy-C6-HSL	Meat extracts and isolated *Enterobacteriaceae* strains from chill-stored vacuum-packed meat	Bruhn *et al.*, 2004
						Aeromonas hydrophila strains isolated from meat	Medina-Martinez *et al.*, 2006
						Y. enterocolitica in fresh foods extracts	Medina-Martinez *et al.*, 2006

Table 1.3. Continued

Strain/Plasmid sensor	Host	Based on QS system	Reporter system	Best responds to	Good detection	QS screening in	Reference
pJBA130	Broad host range	LuxI/R (*V. fisheri*)	gfp	C6-3-oxo-HSL		*Y. enterocolitica* in fresh foods extracts	Medina-Martinez *et al*., 2006
pSB401	*E. coli*	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-HSL	C6-HSL C8-HSL C8-3-oxo-HSL	Pseudomonad and *Enterobacteriaceae* isolates from aerobically chilled-stored proteinaceous raw foods	Liu *et al*., 2006
						Pseud. fragi isolated from fresh and spoiled meat	Ferrocino *et al*., 2009
pSB536	*E. coli*	AhyI/R (*A. hydrophyla*)	*luxCDABE*	C4-HSL		Pseudomonad and *Enterobacteriaceae* isolates from aerobically chilled-stored proteinaceous raw foods	Liu *et al*., 2006
						Pseud. fragi isolated from fresh and spoiled meat	Ferrocino *et al*., 2009
pSB1075	*E. coli*	LasI/R (*Pseud. aeruginosa*)	*luxCDABE*	C12-3-oxo-HSL	C10-3-oxo-HSL C12-HSL	Pseudomonad and *Enterobacteriaceae* isolates from aerobically chilled-stored proteinaceous raw foods	Liu *et al*., 2006
						Pseud. fragi isolated from fresh and spoiled meat	Ferrocino *et al*., 2009
MM32	*V. harveyi*	*luxN*::cm *luxS*::Tn5	*luxCDABE*	Borated AI-2		Ground beef–derived fatty acids as inhibitors to AI-2	Soni *et al*., 2008

1.3 Objectives

Quorum sensing is considered as a useful tool controlling numerous functions including food spoilage. The development of a certain microbial association on meat stored under various temperatures and packaging conditions, should be taken into consideration, while trying answer queries concerning the role of QS in spoilage process, the involvement of each group of spoilage microorganisms on the presence of particular QS signal molecules, as well as the contribution of detected QS signals on the growth of each microbial group.

The present study aimed to:

◎ Determine the levels of the microbial contamination of minced beef purchased from retail shops in the Athens area, and to ascertain whether or not weather and type of shop affected the level of contamination.

◎ Monitor the changes of the spoilage-related microbial flora during the storage of beef at various temperatures.

◎ Detect the presence of quorum sensing signals in minced beef throughout storage under air and modified atmospheres with/without the presence of volatile compounds of oregano essential oil, and correlate the findings with the indigenous microbial populations.

◎ Detect the production of *N*-acyl homoserine lactone signals from *Enterobacteriaceae*, which were detected in high loads during minced beef storage.

◎ Detect the production of autoinducer-2 signal molecules from lactic acid bacteria, which are the specific spoilage organisms on meat stored under modified atmospheres.

◎ Evaluate the effect of microbial quorum sensing signals on the growth of two main spoilage bacteria, *Pseudomonas fluorescens* and *Serratia liquefaciens*.

Chapter 2

Materials and Methods

2.1 Survey of minced beef

2.1.1 Minced beef samples

A total of 57 samples of minced beef (approximately 300 g each) were obtained from supermarkets (32) and butcher shops (25) in the Athens area (Greece) and transported under refrigeration to the laboratory within 30 min, where it was held at 1 °C for 1–2 h.

2.1.2 Microbiological analysis

For microbiological analysis 25 g of minced beef were weighed aseptically, added to 225 mL of sterile quarter-strength Ringer's solution (LAB100Z, Lab M, Bury, UK) and homogenized in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at low speed at room temperature. Serial decimal dilutions in quarter-strength Ringer's solution were prepared and 1 or 0.1 mL samples of appropriate dilutions were poured or spread in duplicates on non-selective and selective agar plates. All media used are listed below:

(i) *Tryptic Glucose Yeast Agar* (402145, Biolife, Milan, Italy) for determination of the total viable counts (TVC), incubated at 30 °C for 48 h,

(ii) *MRS Agar* (pH 5.7 and pH 5.2) (401728, Biolife, Milan, Italy) for the enumeration of lactic acid bacteria (LAB), overlaid with the same medium and incubated at 30 °C for 72 h,

(iii) *Pseudomonas Agar Base* (CM559 supplemented with selective supplement SR103, Oxoid, Basingstoke, UK) for the enumeration of *Pseudomonas* spp., incubated at 25 °C for 48 h,

(iv) *STA Agar Base* (402079 supplemented with selective supplement 4240052, Biolife, Milan, Italy) for the enumeration of *Brochothrix thermosphacta*, incubated at 25 °C for 48 h,

(v) *Violet Red Bile Glucose Agar* (402188, Biolife, Milan, Italy) for the enumeration of *Enterobacteriaceae*, incubated at 37 ˚C for 24 h,

(vi) *Iron Agar* (made from basic ingredients in the laboratory, ingredients per liter: 20.0 g bacteriological peptone, 3.0 g beef extract, 3.0 g yeast extract, 0.3 g ferric citrate, 0.3 g sodium thiosulphate, 5.0 g sodium chloride, 0.6 g L-cystein, 12.0 g agar), for the enumeration of hydrogen sulfide-producing bacteria, overlaid with the same medium and incubated at 25 ˚C for 48 h.

2.1.3 pH measurement

The pH value of meat samples was recorded with a digital pH meter (Metrohm 691 pH meter, Ion Analysis, Switzerland). The glass electrode was immersed in the homogenate of minced meat at the end of the microbiological analysis.

2.1.4 Sensory analysis

Sensory analysis of beef samples was performed during storage according to Gill and Jeremiah (1991) by a sensory panel composed of four members (staff from the laboratory). The same trained persons were used in each evaluation, and all were blinded to the sample tested. The sensory evaluation was carried out in artificial light and the temperature of all samples was close to ambient. Colour and odour were assessed before and after cooking for 20 min at 180 ˚C in preheated oven, whereas taste was evaluated only after cooking. Each attribute was scored on a three-point

hedonic scale corresponding to: 1=fresh; 2=marginal; and 3=unacceptable. A score of 1.5 was characterized as semi-fresh and was the first indication of microbial proliferation. Scores above 2 rendered the product spoiled.

2.1.5 Data analysis

Resulting data (CFU) were transformed to $\log_{10}$ values. Mean values and standard deviations were computed, and data analysis was performed using the statistical analysis software XLSTAT® v2006.06 (Addinsoft, Paris, France).

2.2 Microbiological analysis of beef meat

2.2.1 Preparation of beef pieces

Fresh beef muscle (approximately 6 kg) was obtained from a local butcher's shop in the Athens area (Greece) and transported under refrigeration to the laboratory within 30 min, where it was held at 1 °C for 1-2 h. The meat was cut into 240 pieces (20 g each piece, 4.0 x 5.0 cm, thickness 1.0 cm), which were packaged aerobically by placing them individually in sterile Petri dishes and finally stored at 0, 5, 10 15 and 20 ˚C.

2.2.2 Microbiological analysis

Triplicate samples were analyzed at different intervals. For microbiological analysis samples (20 g each piece, 4.0 x 5.0 cm, thickness 1.0 cm) were added to 150 mL of sterile quarter-strength Ringer's solution (LAB100Z, Lab M, Bury, UK) and homogenized in a stomacher (Lab Blender, Seward Medical, London, UK) for 60 s at

low speed at room temperature. Serial decimal dilutions in quarter-strength Ringer's solution were prepared and 1 or 0.1 mL samples of appropriate dilutions were poured or spread on non-selective and selective agar plates.

(i) Total viable counts were determined on *Tryptic Glucose Yeast Agar* (402145, Biolife, Milan, Italy), incubated at 30 ˚C for 48 h,

(ii) LAB in *MRS Agar* (401728, Biolife, Milan, Italy) (pH = 5.7) overlaid with the same medium and incubated at 30 ˚C for 72 h,

(iii) *Br. thermosphacta* on *STA Agar Base* (4020792 supplemented with selective supplement 4240052, Biolife, Milan, Italy), incubated at 25 ˚C for 48 h,

(iv) *Enterobacteriaceae* on *Violet Red Bile Glucose Agar* (402188, Biolife, Milan, Italy) overlaid with the same medium and incubated at 37 ˚C for 24 h,

(v) *Pseudomonas* spp. on *Pseudomonas Agar Base* (CM559 supplemented with selective supplement SR103, Oxoid, Basingstoke, UK), incubated at 25 ˚C for 48 h.

2.2.3 pH measurement

This was determined as detailed earlier (Section 2.1.3).

2.2.4 Sensory analysis

Carried out as detailed in Section 2.1.4. A score of 1.5 was characterized as semi-fresh and was the first indication of meat spoilage (i.e., less vivid red colour, odour and flavour slightly changed from the typical of the fresh meat, but still

acceptable for the consumer). Scores above 2 rendered the product spoiled and indicated the end of shelf life.

2.2.5 Data analysis

Two replicate experiments were conducted, with two samples being evaluated for each replicate. Resulting data (CFU) were transformed to $\log_{10}$ values, before means and standard deviations were computed. The $\log_{10}$ data were fitted using the primary model Baranyi and Roberts' (1994), and the kinetic parameters of maximum specific growth rate and lag phase duration were estimated. For curve fitting, the in-house program DMFit (Institute of Food Research, Norwich, UK) was used, which was kindly provided by Dr. J. Baranyi, available also on the internet (http://www.ifr.ac.uk/safety/DMFit/).

2.3 Detection of quorum sensing signals during minced beef spoilage

2.3.1 Bacterial strains and culture conditions

The strains used in this study, their functions and their antibiotic resistance markers are listed in Table 2.1. Among the QS biosensor strains used, *Agrobacterium tumefaciens* A136 (pCF218, pCF372) (Fuqua & Winans, 1994), *Chromobacterium violaceum* CV026 (McClean *et al.*, 1997), *Vibrio harveyi* BAA-1117 (BB-170) and *V. harveyi* BAA-1118 (BB886) (Bassler *et al.*, 1997) were grown at 30 °C, as well as the positive controls *A. tumefaciens* KYC6, *C. violaceum* ATCC31532, *V. harveyi* BAA-1119 (BB152) and *V. harveyi* BAA-1120 (MM30). *Escherichia coli* JM109 (pSB401), *E. coli* JM109 (pSB536) and *E. coli* JM109 (pSB1075) (Winson *et al.*,

1998) biosensor strains were grown at 37 °C, as well as *Pseudomonas aeruginosa* PAO1 (Holloway *et al.,* 1979).

All strains were grown in Luria-Bertani medium (LB per liter: 10.0 g tryptone, 5.0 g yeast extract, 10.0 g sodium chloride) supplemented with antibiotics when appropriate (100 μg mL^{-1} ampicillin, 25 μg mL^{-1} kanamycin, 50 μg mL^{-1} spectinomycin and 10 μg mL^{-1} tetracycline), and *V. harveyi* were grown in autoinducer bioassay (AB) medium that was prepared as follows. A solution consisting of NaCl (17.5 g L^{-1}), $MgSO_4$ (12.3 g L^{-1}), and casamino acids (2 g L^{-1}) was adjusted to pH 7.5 and autoclave-sterilized. When the solution was cooled down, autoclave-sterilized 1 M potassium phosphate pH 7.0 (10 mL L^{-1}), 50% v/v glycerol (20 mL L^{-1}), and filter-sterilized 0.1M L-arginine (10 mL L^{-1}) were added.

Table 2.1. Strains used in this study, their functions, and their antibiotic resistance markers

Strain	Description	Reference
A. tumefaciens A136	AHL sensor strain; contains *traRG'::lacZ* , ß-galactosidase reporter, Sp^r Tc^r; cognate signal: 3OC8-HSL	Fuqua & Winans, 1994
A. tumefaciens KYC6	Bioassay positive control; positive AHL producer (3OC8-HSL)	Fuqua & 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

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

Table 2.1. Continued

Strain	Description	Reference
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

2.3.2 Minced beef samples

Minced beef samples were obtained as described previously by Argyri *et al.* (2010). Briefly, fresh minced beef was purchased from the central meat market in Athens (Greece) and transported under refrigeration to the laboratory within 30min. The minced beef was divided into portions of 75 g and packaged either aerobically and under modified atmosphere (40% CO_2/ 30% O_2/ 30%N_2) with and without 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, 386 and 220 h respectively.

2.3.3 Microbiological analysis

A detailed description of the methodology employed for the enumeration of the total viable counts, *Pseudomonas* spp., *Br. thermosphacta*, *Enterobacteriaceae*, LAB, yeasts and moulds in this work is presented elsewhere (Argyri *et al.*, 2010).

Briefly, total viable counts were determined on Tryptic Glucose Yeast Agar (402145, Biolife, Milan, Italy), incubated at 30 ˚C for 48 h, *Pseudomonas* spp. on Pseudomonas Agar Base (CM559 supplemented with selective supplement SR103, Oxoid, Basingstoke, UK), incubated at 25 ˚C for 48 h, *Br. thermosphacta* on STA Agar Base (4020792 supplemented with selective supplement 4240052, Biolife, Milan, Italy), incubated at 25 ˚C for 48 h, *Enterobacteriaceae* on Violet Red Bile Glucose Agar (402188, Biolife, Milan, Italy) overlaid with the same medium and incubated at 37 ˚C for 24 h, LAB on MRS agar (401728, Biolife, Milan, Italy) (pH = 5.7) overlaid with the same medium and incubated at 30 ˚C for 72 h, yeasts and moulds on Rose Bengal Chloramphenicol Agar Base (LAB 36 supplemented with selective supplement X009, LAB M, Bury, UK), incubated at 25˚C for 72 h.

2.3.4 pH measurement

This was done as detailed previously in Section 2.1.3.

2.3.5 Preparation of Cell-free Meat Extracts

Cell-free meat extracts (CFME) at the same time intervals as the microbiological assays were collected. Minced beef samples (5 g) were homogenized with sterile quarter-strength Ringer's solution (10 mL) (LAB100Z, Lab M, Bury, UK) in a stomacher for 60 s at ambient temperature. The CFME were obtained by centrifugation at 5.000 *g* for 15 min at 4 °C using a Heraeus Multifuge 1S-R centrifuge (Thermo Electron Corporation, Langenselbold, Germany) and filtration through 0.2 μm-pore-size filters (Whatman, Clifton, USA), as described by Nychas *et al.* (2009). All CFME were stored at -20 °C until the bioassays were performed.

2.3.6 Preparation of Cell-free Meat Extract from "sterile" meat tissue

Cell-free meat extract from meat without the endogenous microflora ($CFME_{Sterile}$) 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 tissue below was excised and used to prepare CFME as described earlier (only time 0 h). The sterility of the meat was examined by using selective and non-selective media as detailed previously (see Section 2.3.3). No viable counts checking the microbial load using any medium were obtained.

2.3.7 Well diffusion assay

A. tumefaciens A136 and *C. violaceum* CV026 biosensor strains were used for the detection of AHLs in a well diffusion assay as described by Ravn *et al.,* (2001). Briefly, a preculture was grown in LB medium at 30 °C for 24 h with agitation (160 rpm) and 1 mL of the preculture was used to inoculate 50 mL ABT medium (ABT per liter: 0.4 g $(NH_4)_2SO_4$, 0.6 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.3 g NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM $FeCl_3$, 2.5 mg thiamine supplemented with 0.5% glucose and 0.5% casamino acids) 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 5.0 cm diameter Petri dishes. Sixty microliters of CFME were pipetted into wells (diameter 6.0 mm) punched in the solidified agar using a sterile Pasteur pipette. The plates were incubated at 30 °C for 48 h when using the *A. tumefaciens* A136, and 24 h

for *C. violaceum* CV026 biosensor strain. All media were supplemented with relevant antibiotics, and the ABT-agar medium for *A. tumefaciens* A136 was supplemented with 25 μg mL^{-1} X-gal (AppliChem GmbH, Darmstadt, Germany). The induction diameters (in mm) seen as either a blue circle due to induced ß-galactosidase activity or purple circle due to induced violacein formation were measured. Cell-free culture extracts (CFCE) of the AHL-producing strains *A. tumefaciens* KYC6 (pCF28) and *C. violaceum* ATCC 31532 were used as positive controls and the biosensor strains as negative control themselves. All bioassays were done in triplicate.

Digital images of the Petri dishes were obtained using a live view digital camera (Olympus, Live View Digital Camera, E-330; Olympus Imaging Corp., Tokyo, Japan).

2.3.8 Spread plating assay

A spread plating assay using *A. tumefaciens* A136 and *C. violaceum* CV026 biosensor strains was based on the method described previously by Nychas *et al.* (2009), following the same monitor principle as in section 2.3.7. Briefly, CFME (120 μL) and 50 μL of the biosensor strains *A. tumefaciens* A136 and *C. violaceum* CV026 were spread on ABT-agar and LB-agar plates, respectively. The plates were incubated at 30 °C for 48 h when using the *A. tumefaciens* A136, and 24 h for *C. violaceum* CV026 biosensor strain. All media were supplemented with relevant antibiotics, and the ABT-agar medium for *A. tumefaciens* A136 was supplemented with 25 μg mL^{-1} X-gal (AppliChem GmbH, Darmstadt, Germany). The development of blue or purple colour in the plates with the CFME and the biosensor strain confirmed the presence of

AHL compounds. Positive and negative controls were used as mentioned previously. All bioassays were done in triplicate.

Digital images of the Petri dishes were obtained using a live view digital camera (Olympus, Live View Digital Camera, E-330; Olympus Imaging Corp., Tokyo, Japan).

2.3.9 Microplate assay

A. tumefaciens A136 and *C. violaceum* CV026 biosensor strains were also used for the detection of AHLs in a microplate assay. Briefly, 180 μL of a culture suspension, which was prepared by diluting a overnight culture of *A. tumefaciens* A136 or *C. violaceum* CV026 in ABT broth for *A. tumefaciens* A136 or LB broth for *C. violaceum* CV026 to give the required number of viable bacteria (~10^6 CFU mL^{-1}), was placed in an individual well of 96-well microplate and inoculated with 20 μL of CFME. The plates were incubated at 30 °C for 48 h when using the *A. tumefaciens* A136, and 24 h for *C. violaceum* CV026 biosensor strain. All media were supplemented with relevant antibiotics, and the ABT broth medium for *A. tumefaciens* A136 was supplemented with 25 μg mL^{-1} X-gal (AppliChem GmbH, Darmstadt, Germany). The development of blue or purple colour in the microplates with the tested CFME and the biosensor strain confirmed the presence of AHL compounds. Positive and negative controls were used as mentioned previously. All bioassays were done in triplicate.

Digital images of the microplates were obtained using a live view digital camera (Olympus, Live View Digital Camera, E-330; Olympus Imaging Corp., Tokyo, Japan).

2.3.10 Luminescence-based broth assays

Luminescence-based broth assays using *E. coli* JM109 (pSB536), *E. coli* JM109 (pSB401) and *E. coli* JM109 (pSB1075) biosensor strains were based on a method described previously (Winson *et al.*, 1998). Briefly, 100 µl of the tested CFME sample were mixed with 100 µl (1:10 dilution of an overnight culture in LB broth) of the *E. coli* biosensor strain in 96-well polystyrene microplates µClear (Greiner Bio-One, Munich, Germany). One hundred microliters of CFME of the 0 h minced beef sample was used as negative control. The plate was incubated at 37 ºC for 7 h, and the luminescence (Relative Light Units – RLU) and turbidity (optical density at 450nm) of the cultures were measured using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA). Values were given in RLU per unit of OD_{450nm} (RLU/OD_{450nm}). Induced bioluminescence was expressed as relative AHL-induction and was calculated as the ratio of RLU/OD_{450nm} of the test sample to the control (negative) sample. Positive controls were used to verify the assays (see Section 2.3.1).

Another bioluminescence based *V. harveyi* BAA-1118 biosensor strain was also used in this study to assess AHL induction in the CFME samples. Bioassay was performed as described by Surette and Bassler (1998). In this case, an overnight culture of *V. harveyi* BAA-1118 was diluted 1:5.000 with fresh AB medium. Ninety microliters of this cell suspension was mixed with 10 µl of the tested CFME sample in a 96-well polystyrene microplate µ-Clear (Greiner Bio-One, Munich, Germany). Ten microliters of CFME of the 0 h minced beef sample was used as negative control. The microplates were incubated at 30 °C and luminescence was measured every 30 min using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA)

until the negative control exhibited an increase in luminescence (De Keersmaecker & Vanderleyden, 2003). Induced bioluminescence was expressed as relative AHL-induction, which was calculated as the ratio of luminescence of the test sample (CFME) to the control (negative) sample.The CFCE (10 µl) of *V. harveyi* BAA-1120 strain was used as positive control to verify the bioassays. All bioassays were done in triplicate.

2.3.11 Autoinducer-2 activity bioassay

The AI-2 activity bioassay was performed as described previously by Surette and Bassler (1998). Briefly, an overnight culture of *V. harveyi* BAA-1117 was diluted 1:5.000 with fresh AB medium. Ninety microliters of this cell suspension was mixed with 10 µl of the tested sample (CFME) in a 96-well polystyrene microplate µ-Clear (Greiner Bio-One, Munich, Germany). Ten microliters of CFME of the 0 h minced beef sample was used as negative control. The CFCE (10 µl) of *V. harveyi* BAA-1119 strain was used as positive control to verify the bioassays.

The microplates were incubated at 30 °C and luminescence was measured every 30 min using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA) until the negative control exhibited an increase in luminescence (De Keersmaecker & Vanderleyden, 2003). AI-2-like activity was expressed as relative AI-2-like activity, which was calculated as the ratio of luminescence of the test sample (CFME) to the control (negative) sample. All bioassays were done in triplicate.

2.3.12 Determination of AHL profiles using Thin Layer Chromatography

The AHL profiles of the CFME samples were determined as described by Liu *et al.* (2006). In brief, 2.5 mL of the CFME prepared as described previously (see Section 2.3.5) were twice extracted with acidified ethyl acetate (containing 0.01% v/v glacial acetic acid). When it was difficult to separate the solvent phase from the aqueous phase, because of the fat content of the meat sample, the inseparable mixture was dissolved in acetone and then separated by adding single drops of methanol while the mixture was shaken. The combined extracts were filtered and evaporated to dryness. Extracted residues were dissolved in ethyl acetate HPLC-grade, an amount was loaded onto thin layer chromatography (TLC) Silica gel RP-18 F_{254S} plates (aluminium sheets 20 x 20 cm, 1.05559, Merck, Darmstadt, Germany). As a reference, various AHL standards were also applied to the plate. The plates were developed in methanol:water (60:40 v/v). The TLC plates were dried and overlaid with a thin layer containing the *A. tumefaciens* A136 biosensor strain prepared as described previously (see Section 2.3.7), and then incubated at 30 °C for optimal signal development (blue spots on the plate).

Digital images of the developed TLC chromatographs were obtained using a live view digital camera (Olympus, Live View Digital Camera, E-330; Olympus Imaging Corp., Tokyo, Japan).

2.3.13 AHL standards

AHL standards were used in all the assays as positive controls. *N*-butyryl-homoserine lactone (C4-HSL) (O9945), *N*-hexanoyl-homoserine lactone (C6-HSL) (O9926), *N*-(ß-ketocaproyl)-homoserine lactone (3OC6-HSL) (K3007), *N*-octanoyl-

homoserine lactone (C8-HSL) (10940), *N*-(ß-ketooctanoyl)-homoserine lactone (3OC8-HSL) (O1764), *N*-decanoyl-homoserine lactone (C10-HSL) (17248), *N*-(3-oxodecanoyl)-homoserine lactone (3OC10-HSL) (O9014) and *N*-dodecanoyl-homoserine lactone (C12-HSL) (17247) (Sigma-Aldrich, St. Louis, USA) stock solutions 10 mM were prepared in acetonitrile and stored at -20 °C.

2.3.14 Data analysis

Microbiological counts of duplicate samples were transformed to $\log_{10}$ values before means and standard deviations were computed. Bioassays were conducted in triplicate, with three samples being evaluated for each replicate. All data were subjected to analysis of variance (ANOVA) using the statistical analysis software XLSTAT® v2006.06 (Addinsoft, Paris, France). Differences among replicates were considered nonsignifacant ($P > 0.05$).

2.4 *N*-acyl homoserine lactone signal production of *Enterobacteriaceae* isolated from minced beef

2.4.1 Bacterial strains and culture conditions

The nineteen different fingerprints (Table 2.2) out of one hundred and four *Enterobacteriaceae* isolates used in the present study were isolated from minced beef stored aerobically and under modified atmosphere (40% CO_2/ 30% O_2/ 30% N_2) with and without the presence of volatile compounds of oregano essential oil (2% v/w) at 0, 5, 10 and 15 °C as presented elsewhere (Argyri *et al.*, 2010). The strains were identified using pulse-field gel electrophoresis (PFGE) and 16S rRNA gene sequence analysis according to Doulgeraki *et al.* (submitted for publication).

Enterobacteriaceae were isolated from the highest dilution from three different time points (initial, middle and final stage of storage), purified by successive subculture in Violet Red Bile Glucose Agar (402188, Biolife, Milano, Italy) and stored at -80 °C in Brain Heart Infusion broth (LAB49, Lab M, Bury, UK) supplemented with 20 % v/v glycerol (Merck, Darmstadt, Germany). All *Enterobacteriaceae* isolates were grown at 37 °C.

Among the biosensor strains used, *A. tumefaciens* A136 (pCF218, pCF372) (Fuqua & Winans, 1994) and *C. violaceum* CV026 (McClean *et al.*, 1997) were grown at 30 °C. *E. coli* JM109 (pSB401), *E. coli* JM109 (pSB536) and *E. coli* JM109 (pSB1075) (Winson *et al.*, 1998) biosensor strains were grown at 37 °C. All strains were grown in LB medium supplemented with antibiotics when appropriate (100 μg mL^{-1} ampicillin, 25 μg mL^{-1} kanamycin, 50 μg mL^{-1} spectinomycin and 10 μg mL^{-1} tetracycline).

Table 2.2. Different *Enterobacteriaceae* fingerprints screened for *N*-acyl homoserine lactone signal production

Strain	Code	Description	Reference
Serratia spp.	VK5	AHL screening; wild type, minced beef isolates	Doulgeraki *et al.*, (submitted for publication)
Serratia proteamaculans	VK6		
Serratia liquefaciens	VK17		
Citrobacter freundii	VK19		
Hafnia alvei	VK20		
Serratia liquefaciens	VK23		
Serratia proteamaculans	VK25		
Hafnia alvei	VK27		
Serratia proteamaculans	VK32		
Serratia liquefaciens	VK40		
Hafnia alvei	VK53		

Table 2.2. Continued

Strain	Code	Description	Reference
Hafnia alvei	VK60	AHL screening; wild type, minced beef isolates	Doulgeraki *et al.*, (submitted for publication)
Serratia liquefaciens	VK74		
Serratia liquefaciens	VK75		
Serratia spp.	VK90		
Proteus vulgaris	VK101		
Proteus vulgaris	VK103		
Serratia spp.	VK108		
Serratia proteamaculans	VK113		

2.4.2 Cross feeding screening for AHL production

Three bacterial monitor principles were used for the detection of AHL-producing *Enterobacteriaceae* strains, i.e., induction of *A. tumefaciens* A136 and *C. violaceum* CV026 and inhibition of the AHL induced *C. violaceum* CV026. The cross feeding screening for AHL detection was based on previous studies (McLean *et al.*, 1997, Ravn *et al.*, 2001). The strains tested for induction of *A. tumefaciens* A136 and *C. violaceum* CV026 were streaked in parallel (1.0 cm apart) to the biosensor strains, and incubated at 30 °C. ABT-agar supplemented with 25 μg mL^{-1} X-gal was used for *A. tumefaciens* A136 and LB-agar was used for *C. violaceum* CV026 biosensor strain. The induction of the biosensor strain *A. tumefaciens* A136 or *C. violaceum* CV026, indicated as either blue due to induced ß-galactosidase activity, or purple due to induced violacein pigment formation. AHL-producing strains *A. tumefaciens* KYC6 (pCF28) and *C. violaceum* ATCC 31532 were used as positive controls and the biosensor strains as negative control themselves.

Presence of long chained AHLs as detected by inhibition of the induced *C. violaceum* CV026 was done by streaking the strain to be tested on LB agar supplemented with 500 nM *N*-(ß-ketocaproyl)-homoserine lactone (3OC6-HSL)

(K3007, Sigma-Aldrich, St. Louis, USA). Plates were incubated for 24 h at 30 °C before *C. violaceum* CV026 was streaked in parallel. Plates were re-incubated at 30 °C and lack of violacein production red as an AHL-positive response. *Pseud. aeruginosa* strain PAO1 was used as positive control and the biosensor strain *C. violaceum* CV026 as negative control.

2.4.3 Preparation of Enterobacteriaceae Cell-free Culture Extracts ($CFCE_E$)

One fresh colony of each *Enterobacteriaceae* isolate was inoculated in 5 mL LB medium and the culture was grown overnight at 37 °C. This culture was then used to inoculate 5 mL LB medium and the suspension was incubated at 37 °C until early stationary phase (about 16 h). $CFCE_E$ were prepared by removing the cells from the growth medium by centrifugation at 5.000 *g* for 15 min at 4 °C using a Heraeus Fresco 21 microcentrifuge (Thermo Electron Corporation, Langenselbold, Germany). The cleared culture supernatants were filtered sterilized using 0.2 µm-pore-size filters (Whatman, Clifton, USA) and stored at -20°C until luminescence-based broth assays were performed.

2.4.4 Luminescence-based broth assays

Luminescence-based broth assays using *E. coli* JM109 (pSB536), *E. coli* JM109 (pSB401) and *E. coli* JM109 (pSB1075) biosensor strains were based on a method described previously (Winson *et al.*, 1998). Briefly, 100 µl of the tested sample ($CFCE_E$) were mixed with 100 µl (1:10 dilution of an overnight culture in LB broth) of the *E. coli* biosensor strain in 96-well polystyrene microplates µClear (Greiner Bio-One, Munich, Germany). One hundred microliters of sterile growth

medium (LB broth) was used as a negative control. The plate was incubated at 37 ºC for 7 h, and the luminescence (RLU) and turbidity (optical density at 450 nm) of the cultures were measured using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA). Values were given in RLU per unit of OD_{450nm} (RLU/OD_{450nm}). Induced bioluminescence was expressed as relative AHL-induction and was calculated as the ratio of RLU/OD_{450nm} of the test sample to the control (negative) sample. Positive controls were used to verify the assays (see Section 2.3.1). All bioassays were done in triplicate.

2.4.5 Determination of AHL profiles using Thin Layer Chromatography

The early stationary growth phase culture extracts (5 mL) of *Enterobacteriaceae* strains were extracted twice with acidified ethyl acetate (containing 0.01% v/v glacial acetic acid). The combined extracts were filtered and evaporated to dryness. Extracted residues were dissolved in ethyl acetate HPLC-grade, an amount was loaded onto TLC Silica gel RP-18 F_{254S} plates (aluminium sheets 20 x 20 cm, 1.05559, Merck, Darmstadt, Germany). As a reference, various AHL standards were also applied to the plate. The plates were developed in methanol:water (60:40 v/v), dried and overlaid with a thin layer containing the *A. tumefaciens* A136 biosensor strain prepared as described previously (see Section 2.3.7). The TLC plates were incubated at 30 °C for optimal signal development, blue spots on the plate.

Digital images of the developed TLC chromatographs were obtained using a live view digital camera (Olympus, Live View Digital Camera, E-330; Olympus Imaging Corp., Tokyo, Japan).

2.4.6 AHL standards

AHL standards were used in the assays as already mentioned in Section 2.3.13.

2.4.7 Data analysis

Each bioassay was conducted in duplicate, with three samples being evaluated for each replicate. Means and standard deviations were computed, and subjected to analysis of variance (ANOVA) using the statistical analysis software XLSTAT® v2006.06 (Addinsoft, Paris, France). Differences among replicates were considered nonsignifacant ($P > 0.05$).

2.5 Autoinducer-2-like activity in lactic acid bacteria isolated from minced beef

2.5.1 Bacterial strains and culture conditions

The fifteen different fingerprints (Table 2.3) out of eighty-nine LAB used in the present study were isolated from minced beef stored under modified atmospheres (40% CO_2/ 30% O_2/ 30% N_2) at 0, 5, 10 and 15 °C as presented elsewhere (Argyri *et al.*, 2010). The strains were identified using pulse-field gel electrophoresis (PFGE) and 16S rRNA gene sequence analysis according to Doulgeraki *et al.* (2010). LAB were isolated from the highest dilution from three different time points (initial, middle and final stage of storage), purified by successive subculture in MRS agar (Biolife, Milano, Italy) and stored at -80 °C in MRS broth (Biolife, Milano, Italy) supplemented with 20 % v/v glycerol (Merck, Darmstadt, Germany). Before

experimental use, each strain was grown twice in quarter strength Brain Heart Infusion broth (BHI) (Lab M, Bury, UK) at 30 °C with agitation (160 rpm).

The *V. harveyi* BAA-1117 (BB-170) biosensor strain was grown at 30 °C (see Section 2.3.1). An exogenous source of AI-2-like molecules was used. The cell-free culture extract from *Salmonella enterica* serovar Typhimurium strain 4/74 ($CFCE_{ST}$) had previously produced AI-2 in our laboratory.

Table 2.3. Different lactic acid bacteria fingerprints screened for their ability to exhibit autoinducer-2-like activity

Strain	Code	Description	Reference
Lactobacillus sakei	B226	AHL screening; wild type, minced beef isolates	Doulgeraki *et al.*, 2010
Lactobacillus sakei	B227		
Leuconostoc spp.	B232		
Leuconostoc spp.	B233		
Weissella viridescens	B234		
Weissella viridescens	B235		
Lactobacillus sakei	B236		
Lactobacillus sakei	B237		
Lactobacillus sakei	B238		
Lactobacillus sakei	B239		
Leuconostoc spp.	B240		
Leuconostoc spp.	B241		
Leuconostoc mesenteroides	B242		
Leuconostoc mesenteroides	B243		
Leuconostoc citreum	B258		

2.5.2 Preparation of lactic acid bacteria Cell-free Culture Extracts ($CFCE_{LAB}$)

LAB isolates were grown in quarter-strength BHI broth (LAB49, LAB M, Bury, UK) in order to avoid the effects of the glucose repression on luminosity of *V. harveyi* BAA-1117 biosensor strain (De Keersmaecker & Vanderleyden, 2003). One

fresh colony of each LAB isolate was inoculated in 5 mL quarter-strength BHI medium and the culture was grown overnight at 30 °C with agitation (160 rpm). This culture was then used to inoculate 5 mL quarter-strength BHI medium and the suspension was incubated at 30 °C with agitation (160 rpm) until early stationary phase (about 20 h). $CFCE_{LAB}$ were prepared by removing the cells from the growth medium by centrifugation at 5.000 *g* for 15 min at 4 °C using a Heraeus Fresco 21 microcentrifuge (Thermo Electron Corporation, Langenselbold, Germany). The cleared culture supernatants were filtered sterilized using 0.2 μm-pore-size filters (Whatman, Clifton, USA) and stored at -20 °C until the AI-2 activity bioassays were performed.

2.5.3 Preparation of Cell-free Meat Extracts

Cell-free meat extracts (CFMEs) were collected at the same time intervals of minced beef spoilage as the LAB isolates recovery (i.e., initial, middle and final stage of storage), which were prepared as detailed earlier in Section 2.3.5.

2.5.4 Autoinducer-2 activity bioassay

The AI-2 activity bioassay was performed as described by Surette and Bassler (1998). Briefly, an overnight culture of *V. harveyi* BAA-1117 was diluted 1:5.000 with fresh AB medium. Ninety microliters of this cell suspension was mixed with 10 μl of the tested sample ($CFCE_{LAB}$) in a 96-well polystyrene microplate μ-Clear (Greiner Bio-One, Munich, Germany). Ten microliters of sterile growth medium (quarter-strength BHI) was used as negative control (Han & Lu, 2009). The CFCE (10

μl) of *V. harveyi* BAA-1119 strain was used as a positive control to verify the bioassays.

In order to identify the inhibition in the induction of luminescence caused by the CFME in the biosensor strain *V. harveyi* BAA-1117, an equal volume (50 μl) of meat extract and $CFCE_{ST}$ of an AI-2 producer (*Salmonella* serovar Typhimurium) were mixed, and AI-2 activity bioassay was performed (Lu *et al.*, 2004). The $CFCE_{ST}$ was used as positive control (50 μl of $CFCE_{ST}$ and 50 μl of AB medium).

The microplates were incubated at 30 °C and luminescence measured every 30 min using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA) until the negative control exhibited an increase in luminescence (De Keersmaecker & Vanderleyden, 2003). AI-2-like activity was expressed as relative AI-2-like activity, which was calculated as the ratio of luminescence of the test sample ($CFCE_{LAB}$) to the control (negative) sample. The inhibition of the AI-2-like activity was expressed as a percentage of luminescence relative to the corresponding positive control i.e., (100 - [(relative light unit of sample/relative light unit of positive control) × 100]) (Lu *et al.*, 2004). All bioassays were done in triplicate.

2.5.5 Data analysis

Each number is the mean of three replicate experiments, with three samples being evaluated for each replicate. Mean values and standard deviations were computed, and subjected to analysis of variance (ANOVA) using the statistical analysis software XLSTAT® v2006.06 (Addinsoft, Paris, France). Differences among replicates were considered nonsignifacant ($P > 0.05$).

2.6 Effect of microbial quorum sensing signals on the growth of spoilage bacteria

2.6.1 Bacterial strains and culture conditions

The *Pseud. fluorescens* strain 395 was originally isolated from raw milk and was grown at 28 °C. The *Ser. liquefaciens* was isolated from minced beef and was grown at 37 °C. The AHL-producing strain *H. alvei* 718, the AHL-lacking mutant of *H. alvei* 718, *H. alvei* 718 *halI*, and the AI-2 producer *Salmonella* Typhimurium were grown also at 37 °C. Among the biosensor strains used, *A. tumefaciens* A136 (Fuqua & Winans, 1994) was grown at 30 °C and the *V. harveyi* BAA-1117 (BB-170) (Bassler *et al.*, 1997) biosensor strain was grown at 30 °C (Table 2.4).

All strains were grown in LB medium supplemented with 0.5% (w/v) glucose ($LB_{glucose}$) or antibiotics when appropriate, and *V. harveyi* was grown in AB medium.

Table 2.4. Bacterial strains used in this study

Strain	Description	Reference
A. tumefaciens A136	AHL sensor strain; contains *traRG'::lacZ* , ß-galactosidase reporter, Sp^r Tc^r; cognate signal: 3OC8-HSL	Fuqua & Winans, 1994
V. harveyi BAA-1117	Sensor strain; contains *luxN::Tn5*, bioluminescent reporter, cognate signal: borated AI-2	Bassler *et al.*, 1997
H. alvei 718	AHL producer (3OC6-HSL)	Bruhn *et al.*, 2004
H. alvei 718 *halI* mutant	AHL-lacking mutant of *H. alvei* 718	Bruhn *et al.*, 2004
Pseud. fluorescens 395	Wild type, raw milk isolate, proteolytic	Liu *et al.*, 2006
Ser. liquefaciens	Wild type, minced beef isolate	Doulgeraki *et al.*, submitted for publication
Salmonella enterica subsp. *enterica* serovar Typhimurium 4/74	AI-2 producer	Hoiseth & Stocker, 1981

Sp, spectinomycin; Tc, tetracycline

2.6.2 Preparation of Cell-free Culture Extracts containing AHLs

The AHL-producing strain *H. alvei* 718 and the AHL-lacking mutant *H. alvei* 718 *halI* were grown in $LB_{glucose}$ medium (see Section 2.6.1). One fresh colony of each strain was inoculated into 10 mL $LB_{glucose}$ and the cultures were grown overnight at 37 °C with agitation (160 rpm). These cultures (1:1000) were used to inoculate 100 mL of the $LB_{glucose}$ contained in 500 mL flasks and the resulting suspensions were incubated at 37 °C with agitation (160 rpm) for 16 h. CFCE were prepared by removing the cells from the growth media by centrifugation at 5.000 *g* for 15 min at 4 °C using a Heraeus Multifuge 1S-R centrifuge (Thermo Electron Corporation, Langenselbold, Germany). The cleared culture supernatants were filtered sterilized using 0.2 µm-pore-size filters (Whatman, Clifton, USA).

2.6.3 Preparation of Cell-free Culture Extracts exhibiting AI-2 activity

The AI-2 producer *Salm.* Typhimurium was grown in $LB_{glucose}$ medium. One fresh colony of the strain was inoculated into 10 mL $LB_{glucose}$ and the culture was grown overnight at 37 °C with agitation (160 rpm). This culture (1:1000) was used to inoculate 100 mL of the $LB_{glucose}$ contained in 500 mL flask and the resulting suspension was incubated at 37 °C with agitation (160 rpm) for 8 h. CFCE was prepared by removing the cells from the growth medium by centrifugation at 5.000 *g* for 15 min at 4 °C. The cleared culture supernatant was filtered sterilized using 0.2 µm-pore-size filters (Whatman, Clifton, USA). CFCE from *Salm.* Typhimurium ($CFCE_{AI2}$) exhibited AI-2 activity as determined by the AI-2 activity bioassay. A quantity of the $CFCE_{AI2}$ was heat inactivated using autoclaving conditions (121 °C for

15 min). Heat treatment has been previously shown to destroy the AI-2 activity (Surette & Bassler, 1998).

2.6.4 Preparation of inocula and inoculation

The growth of *Pseud. fluorescens* and *Ser. liquefaciens* strains was monitored individually in $LB_{glucose}$ broth supplemented with 0, 20 and 50% v/v CFCE from *H. alvei* 718, *H. alvei* 718 *halI* mutant, *Salm.* Typhimurium and heat treated CFCE from *Salm.* Typhimurium. One fresh colony of each strain, *Pseud. fluorescens* and *Ser. liquefaciens*, were grown in 10 mL $LB_{glucose}$ broth and the cultures were grown overnight at relevant temperatures (see Section 2.6.1). These cultures were then used to inoculate 10 mL $LB_{glucose}$ broth and the suspensions were incubated at relevant temperatures until early stationary phase (about 16 h). Cells from cultures were harvested by centrifugation (5.000 *g* for 15 min at 4 °C), washed twice with sterile quarter-strength Ringer's solution and re-suspended in quarter-strength Ringer's solution. Aliquots of inocula (ca. 10^8 CFU mL^{-1}) were serially diluted in quarter strength Ringer's solution to give a suspension that contained the required number of viable bacteria (ca. 10^6 CFU mL^{-1}). Fifty microliters (50 μL) of the cell suspensions were added to duplicate 250 mL flasks containing 50 mL broth to obtain a final cell concentration of 10^3 CFU mL^{-1}. Inoculated flasks were incubated with agitation (160 rpm) at 10 °C.

2.6.5 Microbiological analysis

The growth of *Pseud. fluorescens* and *Ser. liquefaciens* strains was measured by standard plate counting. At each sampling time, 1 mL volume was removed from

flasks. The enumeration of the bacterial population was determined by preparing serial decimal dilutions in quarter-strength Ringer's solution and spreading on $LB_{glucose}$ agar plates, which were incubated at 30 and 37 °C for *Pseud. fluorescens* and *Ser. liquefaciens*, respectively.

2.6.6 pH measurement

The pH value of the sample homogenates was measured with a digital pH meter (Metrohm 691 pH meter, Ion Analysis, Switzerland) at the end of the microbiological analysis.

2.6.7 Well diffusion assay

The well diffusion assay was carried out following the detailed protocol described in Section 2.3.7. Briefly, a preculture of *A. tumefaciens* A136 was grown in LB medium at 30 °C for 24 h with agitation (160 rpm) and 1 mL of the preculture was used to inoculate 50 mL ABT medium. The culture was grown at 30 °C for 24 h with agitation (160 rpm) and was poured into 100 mL ABT-agar. The agar-culture solution, supplemented with relevant antibiotics and 25 μg mL^{-1} X-gal, was immediately poured into 5.0 cm diameter Petri dishes. Sixty microliters of the tested sample were pipetted into wells (diameter 6.0 mm) punched in the solidified agar and using a sterile Pasteur pipette and the plates were incubated at 30 °C for 48 h. The induction of *A. tumefaciens* A136 was seen as a blue circle due to induced ß-galactosidase activity.

Digital images of the Petri dishes were obtained using a live view digital camera (Olympus, Live View Digital Camera, E-330; Olympus Imaging Corp., Tokyo, Japan).

2.6.8 Autoinducer-2 activity bioassay

The AI-2 activity bioassay was performed following the detailed protocol described in Section 2.3.11. Briefly, an overnight culture of *V. harveyi* BAA-1117 was diluted 1:5.000 with fresh AB medium. Ninety microliters of this cell suspension was mixed with 10 μl of the tested sample in a 96-well polystyrene microplate μ-Clear. Ten microliters of sterile growth medium ($LB_{glucose}$) was used as negative control. The CFCE (10 μl) of *V. harveyi* BAA-1119 strain was used as positive control to verify the bioassays. The microplate was incubated at 30 °C and luminescence was measured every 30 min using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA) until the negative control exhibited an increase in luminescence (De Keersmaecker & Vanderleyden, 2003). AI-2-like activity was expressed as relative AI-2 activity, which was calculated as the ratio of luminescence of the test sample to the control (negative) sample. All bioassays were done in triplicate.

2.6.9 Determination of organic acids using high performance liquid chromatography

The metabolic profiles of organic acids from CFCE samples derived from *H. alvei* 718, *H. alvei* 718 *halI* mutant, *Salm.* Typhimurium and heat treated CFCE from *Salm.* Typhimurium were determined using HPLC. One mL of each CFCE was transferred to an eppendorf tube and then 10 μl 1% solution of sodium azide (as a

preservative) and 10 μl of trifluoroacetic acid (TFA) (for protein precipitation) were added. Stirring, centrifugation (9.000 rpm for 5 min at 4 ^{o}C) and filtration of the final supernatant through a 0.22 μm-pore-size filter (Millipore, Billerica, Massachusetts) was followed.

The analysis was performed as described by Skandamis and Nychas (2001) using a Jasco (Japan) HPLC system equipped with a Model PU-980 Intelligent pump, a Model LG-980-02 ternary gradient unit pump and a MD-910 multiwavelength detector. The injection valve was connected with a 20 μl loop, whilst 50 μl of the sample were injected each time. The sample was eluted isocratically with a solution of 0.009 N H_2SO_4 (using HPLC grade solvent and ultra pure water) through an Amminex HPX-87H column (300 x 7.8 mm, Bio-Rad Laboratories, Richmond, CA) at a rate of 0.7 mL/min and oven temperature set at 65 °C. The software used for the collection and the processing of the spectra was the Jasco Chrompass Chromatography Data system v1.7.403.1. Spectral data were collected from 200 to 600 nm, however chromatogram integration was performed at 210 nm.

2.6.10 Data analysis

The experimental procedure was performed twice and duplicate samples for each treatment were taken. Resulting data (CFU) were transformed to $\log_{10}$ values, before means and standard deviations were computed. The $\log_{10}$ data were fitted using the primary model of Baranyi and Roberts' (1994), and the kinetic parameters of maximum specific growth rate (μ_{max}) and lag phase duration (lag) were estimated. For curve fitting, the in-house program DMFit (Institute of Food Research, Norwich, UK)

was used, which was kindly provided by Dr. J. Baranyi, available also in the internet (http://www.ifr.ac.uk/safety/DMFit/).

Chapter 3

Results

3.1 Survey of minced beef

This study was undertaken with the objectives to determine the levels of microbial contamination of minced meat sold in the Athens area and to ascertain whether or not weather and type of shop affected the level of contamination.

Approximately 300 g of minced beef was purchased at around 09:00 hours. The sample was then transported to the laboratory within 1 hour of purchase and held at 5 °C until analyzed. All samples were analyzed microbiologically within 2-3 hours of purchase (see details in CHAPTER 2: Materials and Methods).

A total of 57 samples were obtained during a seven months period (May 2007 - July 2007 and October 2007 - January 2008) from two different types of shops in the Athens area including butcher shops and supermarkets. The total viable counts (TVC), Pseudomonads, *Enterobacteriaceae*, *Brochothrix thermosphacta*, lactic acid bacteria (LAB) and H_2S-producing bacteria counts were included in the survey as well as pH measurements and sensory analysis.

The microbiological analysis of the 57 samples of minced beef (32 from supermarkets and 25 from butcher shops) exhibited a TVC range of 4.18 - 8.17 log CFU g^{-1} whilst most samples were observed to have total microflora of 6.50 - 7.00 log CFU g^{-1}. Analytically, the most variations in contamination of TVCs were between 6.57 - 7.37 and 5.96 - 7.32 log CFU g^{-1}, for supermarkets and bucher shops respectively. The numbers of *Pseudomonas* spp. varied from between 3.30 - 7.79 log CFU g^{-1} and for *Br. thermosphacta* between 2.00 - 7.88 log CFU g^{-1}. In case of LAB the concentration isolated was between 2.85 - 6.76 log CFU g^{-1} (pH 5.2) and between 3.04 – 7.08 log CFU g^{-1} (pH 5.7). The *Enterobacteriaceae* ranged between 2.00 - 6.52

log CFU g^{-1} and H_2S-producing bacteria between 1.00 - 7.18 log CFU g^{-1}. The pH values ranged from 5.40 to 6.09, with most of the samples ranging from 5.49 to 5.78. The sensory analysis revealed mostly samples characterized with scores of 1.5 and 2, without lacking the occurrence of 1, 2.5 and 3 scores. All results are shown in the Table 3.1 as well as in the following charts of frequencies (Figures 3.1, 3.2, 3.3 and 3.4).

The main differences between supermarkets and butcher shops were observed in the numbers of *Enterobacteriaceae* and H_2S-producing bacteria. Counts of *Enterobacteriaceae* and H_2S-producing bacteria appeared lower in butcher shops than supermarkets (Table 3.1).

The season of the year (warm: May - July and cold: October - January) also affected the level of microbial contamination. Microbial counts during the warm period were larger than those obtained during the cold period. Most differences were observed in *Enterobacteriaceae* for butcher shops and both *Enterobacteriaceae* and LAB counts for supermarkets (Table 3.2).

Table 3.1. Microbiological data of minced beef samples sold in Athens (supermarkets and butcher shops)

Microorganisms	Type of shop								
	Supermarkets (32) *			Butcher shops (25) *			Total (57) *		
	N_{min} log CFU g^{-1}	N_{max} log CFU g^{-1}	Mean ± SD log CFU g^{-1}	N_{min} log CFU g^{-1}	N_{max} log CFU g^{-1}	Mean ± SD log CFU g^{-1}	N_{min} log CFU g^{-1}	N_{max} log CFU g^{-1}	Mean ± SD log CFU g^{-1}
Total viable counts	4.18	8.17	6.58 ± 0.83	4.60	8.00	6.30 ± 0.84	4.18	8.17	6.46 ± 0.84
Pseudomonads	4.29	7.61	5.82 ± 0.77	3.30	7.79	5.81 ± 1.18	3.30	7.79	5.82 ± 0.96
Br. thermosphacta	3.52	7.09	5.45 ± 0.88	2.00	7.88	5.01 ± 1.40	2.00	7.88	5.25 ± 1.15
H_2S-producing bacteria	2.11	7.18	4.49 ± 1.26	1.00	5.86	3.62 ± 1.27	1.00	7.18	4.11 ± 1.33
Lactic acid bacteria pH 5.2	3.26	6.76	4.95 ± 0.88	2.85	6.59	4.97 ± 0.94	2.85	6.76	4.96 ± 0.90
Lactic acid bacteria pH 5.7	3.28	7.08	5.18 ± 0.95	3.04	6.58	4.89 ± 0.93	3.04	7.08	5.05 ± 0.94
Enterobacteriaceae	2.08	6.52	4.38 ± 1.02	2.00	4.62	3.42 ± 0.77	2.00	6.52	3.96 ± 1.03
pH	5.43	6.09	5.62 ± 0.15	5.40	5.88	5.60 ± 0.13	5.40	6.09	5.61 ± 0.14

* Number of samples; N_{min}: minimum cell number; N_{max}: maximum cell number; SD: standard deviation

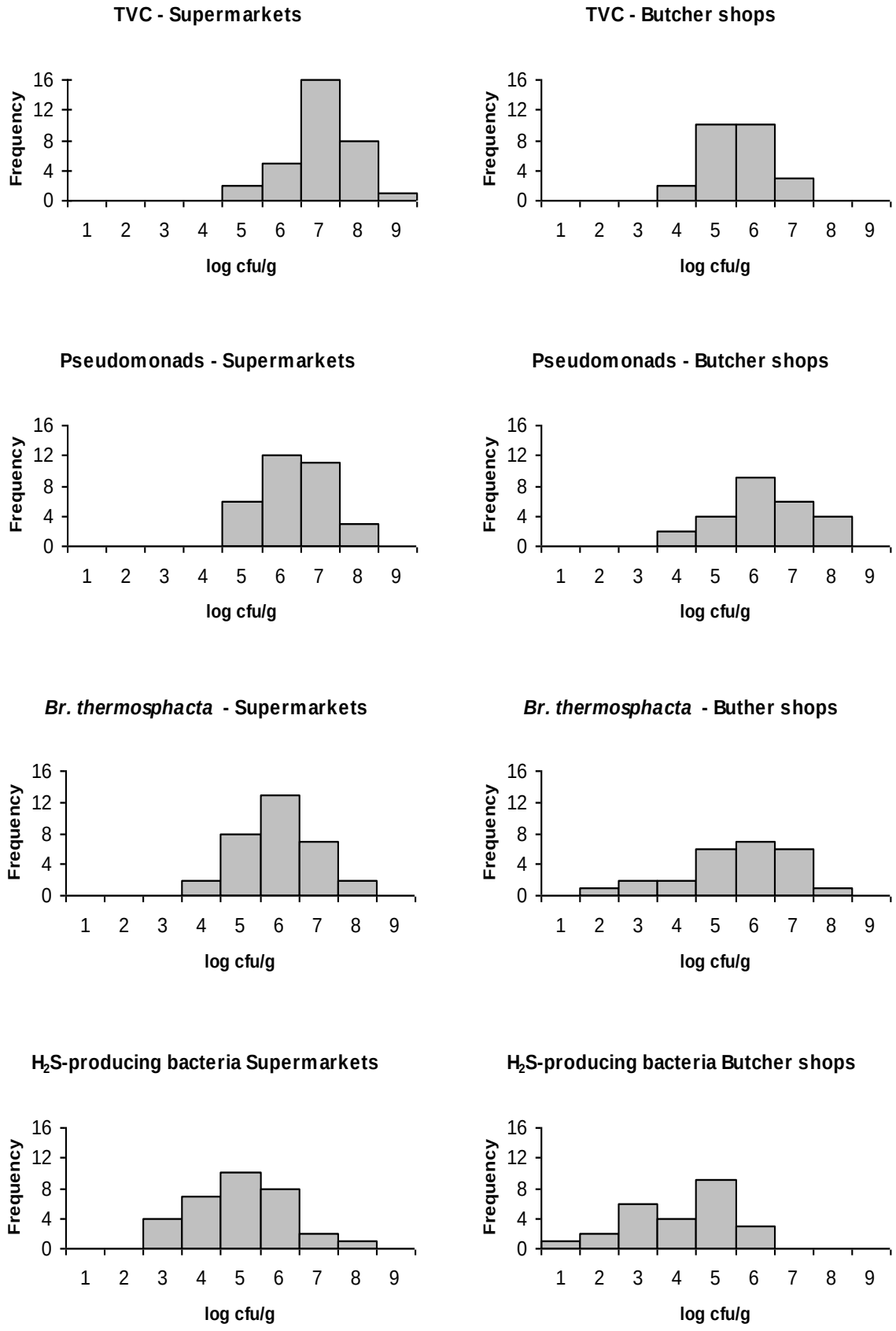

Figure 3.1. Charts of frequency distributions of samples obtained from supermarkets and butcher shops.

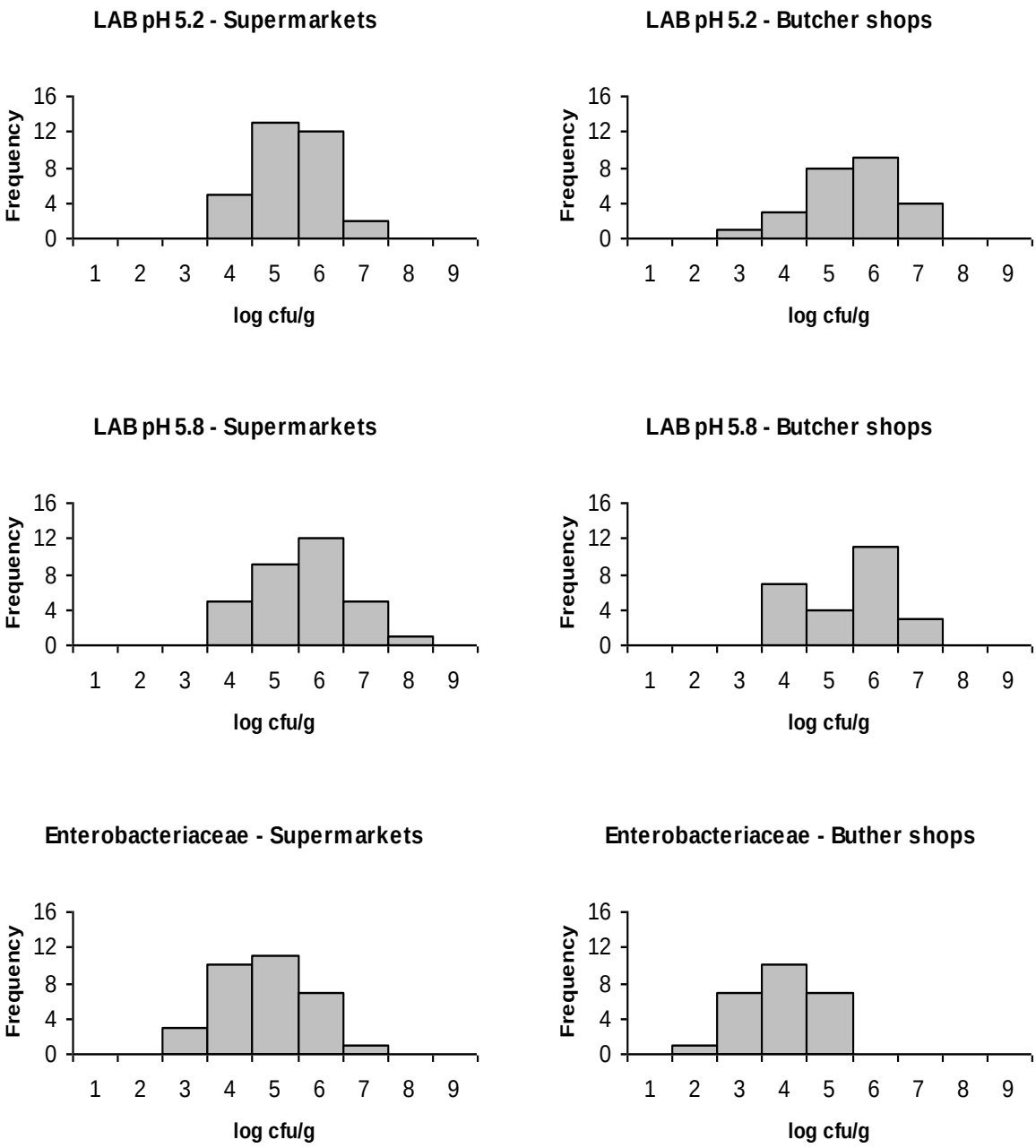

Figure 3.2. Charts of frequency distributions of samples obtained from supermarkets and butcher shops.

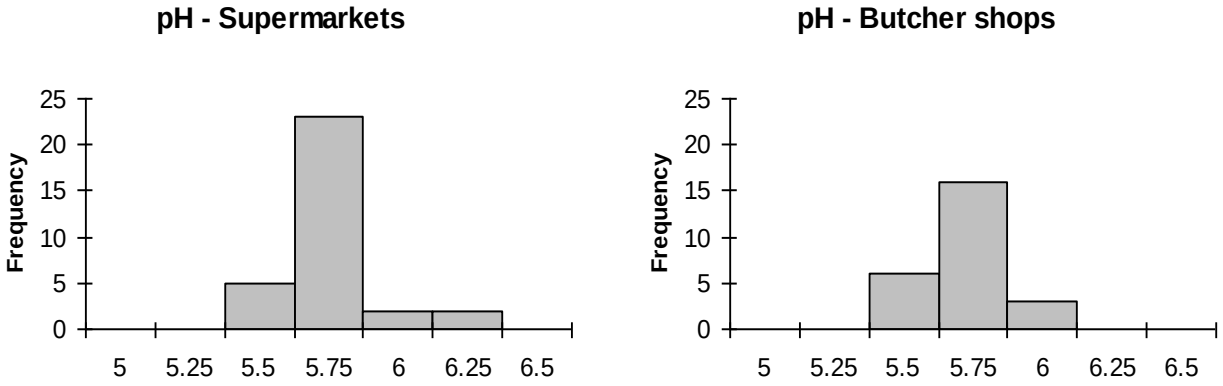

Figure 3.3. Charts of pH frequency distributions of samples obtained from supermarkets and butcher shops.

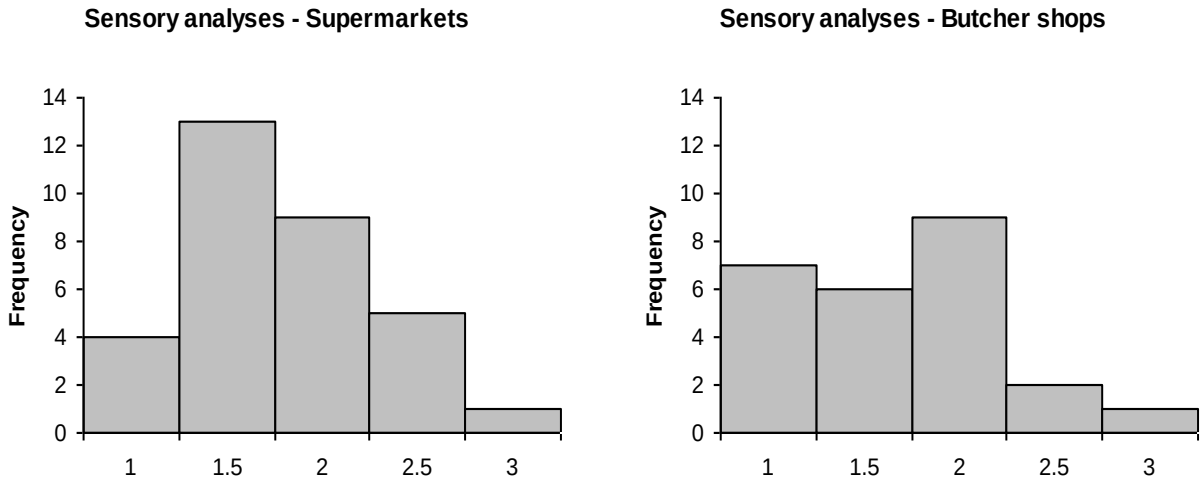

Figure 3.4. Charts of sensory analyses frequency distributions of samples obtained from supermarkets and butcher shops.

Table 3.2. Microbiologacal data of minced beef samples collected at different season (May - July and October - January)

	May - July						**October - January**					
	Supermarkets			Butcher shops			Supermarkets			Butcher shops		
Microorganisms	N_{min} log CFU g^{-1}	N_{max} log CFU g^{-1}	**Mean** log CFU g^{-1}	N_{min} log CFU g^{-1}	N_{max} log CFU g^{-1}	**Mean** log CFU g^{-1}	N_{min} log CFU g^{-1}	N_{max} log CFU g^{-1}	**Mean** log CFU g^{-1}	N_{min} log CFU g^{-1}	N_{max} log CFU g^{-1}	**Mean** log CFU g^{-1}
Total viable counts	5.56	8.17	6.58	5.87	6.90	6.47	4.18	7.84	6.58	4.60	8.00	6.25
Pseudomonads	4.29	7.20	5.70	4.20	7.18	5.61	4.80	7.61	5.86	3.30	7.79	5.88
Br. thermosphacta	3.52	7.09	5.39	3.68	6.49	5.19	3.80	6.58	5.47	2.00	7.88	4.95
H_2S-producing bacteria	3.70	7.18	5.01	4.03	5.86	4.61	2.11	6.88	4.29	1.00	5.78	3.31
Lactic acid bacteria pH 5.2	4.32	6.76	5.50	4.26	6.42	5.38	3.26	6.37	4.74	2.85	6.59	4.84
Lactic acid bacteria pH 5.7	4.45	7.08	5.80	4.00	6.48	5.38	3.28	6.31	4.94	3.04	6.58	4.74
Enterobacteriaceae	3.42	6.52	4.41	2.53	4.62	3.69	2.08	5.99	4.37	2.00	4.56	3.33
pH	5.47	5.81	5.61	5.55	5.88	5.71	5.43	6.09	5.62	5.40	5.76	5.57

N_{min}: minimum cell number; N_{max}: maximum cell number

3.2 Microbiological analysis of beef meat

Fresh beef muscle was obtained from a butcher shop and cut into pieces, which were packaged aerobically and stored at 0, 5, 10, 15 and 20 °C. The microbial fluctuations throughout storage were monitored using selective and non-selective agar media (see details in CHAPTER 2: Material and Methods).

The initial microbial flora present in beef pieces was composed of TVCs (4.65 log CFU cm^{-2}), Pseudomonads (3.54 log CFU cm^{-2}), *Br. thermosphacta* (1.65 log CFU cm^{-2}), LAB (1.39 log CFU cm^{-2}) and *Enterobacteriaceae* (0.41 log CFU cm^{-2}).

Aerobic storage of meat, at all temperatures, allowed total aerobic counts to reach high levels, with *Pseudomonas* spp. being the dominant microorganism, followed by *Br. thermosphacta* and then LAB (Figures 3.5 and 3.6). It needs to be stressed that the growth rate, the lag-phase and the final counts were affected by the storage temperature (Table 3.3).

In all samples pseudomonads predominated with maximum populations of 9.7 log CFU cm^{-2} being isolated, while *Enterobacteriaceae* were the smallest component of the total microbial association for the whole storage period at all temperatures (see Table 3.3). Total viable counts, pseudomonads, *Br. thermosphacta*, LAB and *Enterobacteriaceae* had the highest growth rate at 20 °C and the longest lag-phase at 0 °C. Generally, the growth rate of all microorganisms increased with increasing temperature.

Table 3.3. Growth kinetic parameters (final cell number, lag phase and maximum specific growth rate) of spoilage microorganisms of beef stored aerobically at 0, 5, 10, 15 and 20 °C estimated by the Baranyi model

Temperature (°C)	**Microorganism**	**N_0 ± SD** **log CFU cm^{-2}**	**y_{end} ± SD (N_{end})** **log CFU cm^{-2}**	**lag ± SD** **(hours)**	**μ_{max} ± SD** **($hours^{-1}$)**
0	Total viable counts	4.65 ± 0.12	ND (7.90)	280.03 ± 93.34	0.10 ± 0.07
	Pseudomonads	3.54 ± 0.14	ND (9.22)	195.84 ± 5.09	0.11 ± 0.06
	Br. thermosphacta	1.65 ± 0.34	ND (6.96)	126.20 ± 35.52	0.05 ± 0.01
	Lactic acid bacteria	1.39 ± 0.03	ND (4.65)	219.16 ± 7.13	0.04 ± 0.00
	Enterobacteriaceae	0.41 ± 0.00	ND (1.99)	259.12 ± 18.54	0.02 ± 0.00
5	Total viable counts	4.65 ± 0.12	ND (8.86)	66.22 ± 16.51	0.07 ± 0.02
	Pseudomonads	3.54 ± 0.14	9.46 ± 0.02 (9.68)	35.54 ± 7.32	0.11 ± 0.01
	Br. thermosphacta	1.65 ± 0.34	7.97 ± 0.03 (8.13)	32.78 ± 10.73	0.12 ± 0.01
	Lactic acid bacteria	1.39 ± 0.03	6.69 ± 0.21 (6.99)	57.10 ± 1.64	0.11 ± 0.01
	Enterobacteriaceae	0.41 ± 0.00	5.71 ± 0.51 (5.80)	62.60 ± 6.00	0.07 ± 0.01
10	Total viable counts	4.65 ± 0.12	8.62 ± 0.08 (8.62)	37.86 ± 2.64	0.08 ± 0.01
	Pseudomonads	3.54 ± 0.14	9.20 ± 0.15 (9.56)	26.92 ± 4.02	0.15 ± 0.01
	Br. thermosphacta	1.65 ± 0.34	7.70 ± 0.09 (8.13)	15.21 ± 2.81	0.18 ± 0.01
	Lactic acid bacteria	1.39 ± 0.03	6.21 ± 0.07 (6.56)	22.73 ± 9.19	0.15 ± 0.03
	Enterobacteriaceae	0.41 ± 0.00	ND (6.36)	43.02 ± 2.00	0.16 ± 0.05
15	Total viable counts	4.65 ± 0.12	ND (8.78)	23.85 ± 7.34	0.16 ± 0.07
	Pseudomonads	3.54 ± 0.14	8.73 ± 0.11 (9.22)	0.00 ± 0.00	0.21 ± 0.03
	Br. thermosphacta	1.65 ± 0.34	7.52 ± 0.10 (8.31)	0.00 ± 0.00	0.21 ± 0.05
	Lactic acid bacteria	1.39 ± 0.03	6.45 ± 0.03 (6.94)	11.78 ± 3.44	0.19 ± 0.02
	Enterobacteriaceae	0.41 ± 0.00	ND (6.23)	19.68 ± 9.52	0.17 ± 0.04
20	Total viable counts	4.65 ± 0.12	9.03 ± 0.00 (9.38)	12.73 ± 2.04	0.25 ± 0.02
	Pseudomonads	3.54 ± 0.14	9.21 ± 0.06 (9.50)	6.34 ± 2.58	0.26 ± 0.02
	Br. thermosphacta	1.65 ± 0.34	7.18 ± 0.11 (7.98)	3.97 ± 2.45	0.30 ± 0.00
	Lactic acid bacteria	1.39 ± 0.03	6.59 ± 0.05 (7.09)	5.93 ± 1.00	0.33 ± 0.07
	Enterobacteriaceae	0.41 ± 0.00	7.87 ± 0.00 (8.04)	5.57 ± 1.98	0.28 ± 0.00

No: initial cell number; **y_{end}**: fian cell number estimated by the Baranyi model; **N_{end}**: determined experimentally (values recorded at the end of storage period for each condition); **lag**: lag-phase duration; **μ_{max}**: maximum specific growth rate; **SD**: standard deviation; **ND**: not determined

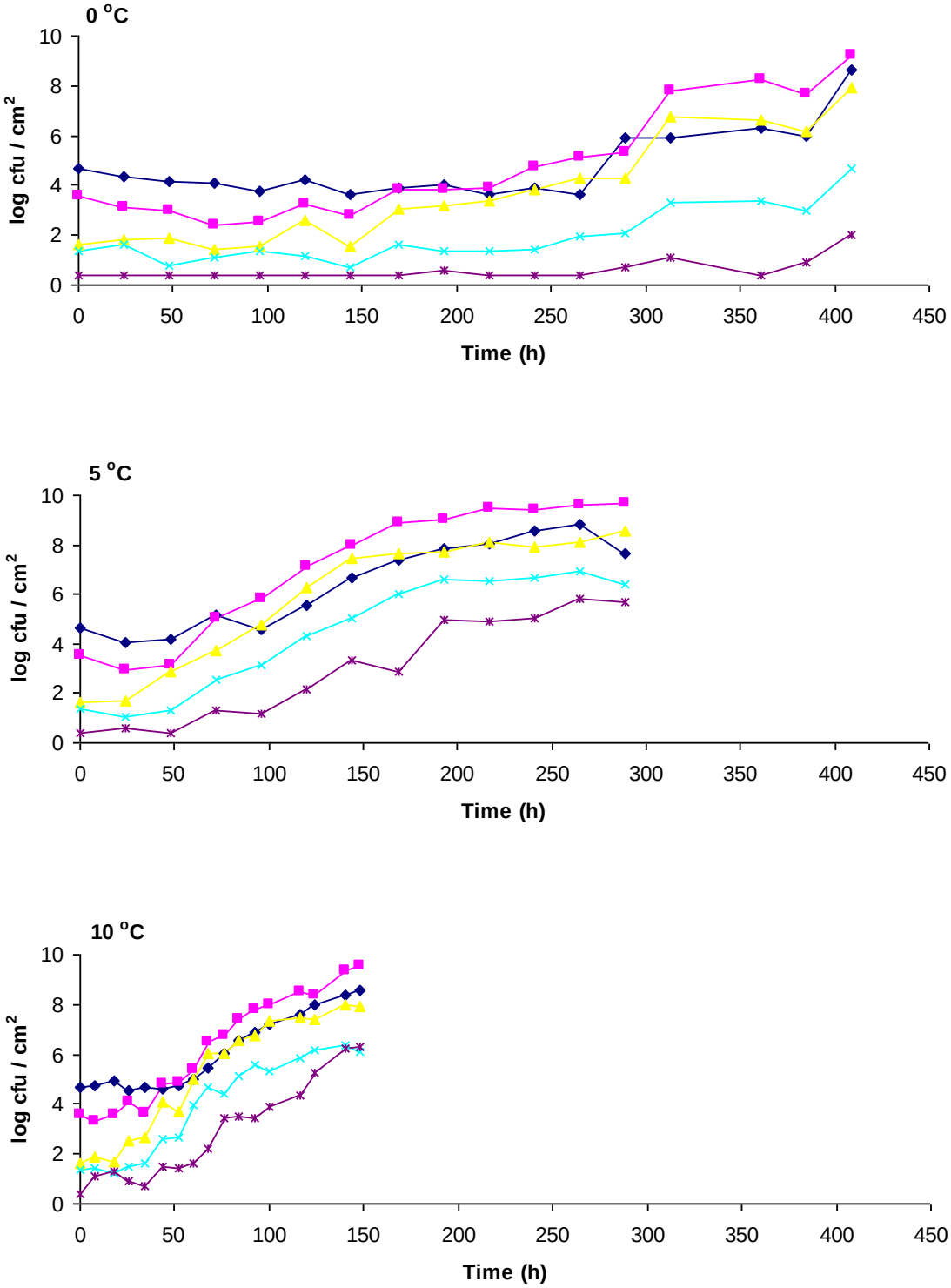

Figure 3.5. Changes in microbial population of beef stored aerobically at 0, 5 and 10 °C. (◆), total viable counts; (▪), pseudomonads; (▲), *Br. thermosphacta*; (×), lactic acid bacteria and (∗), *Enterobacteriaceae*.

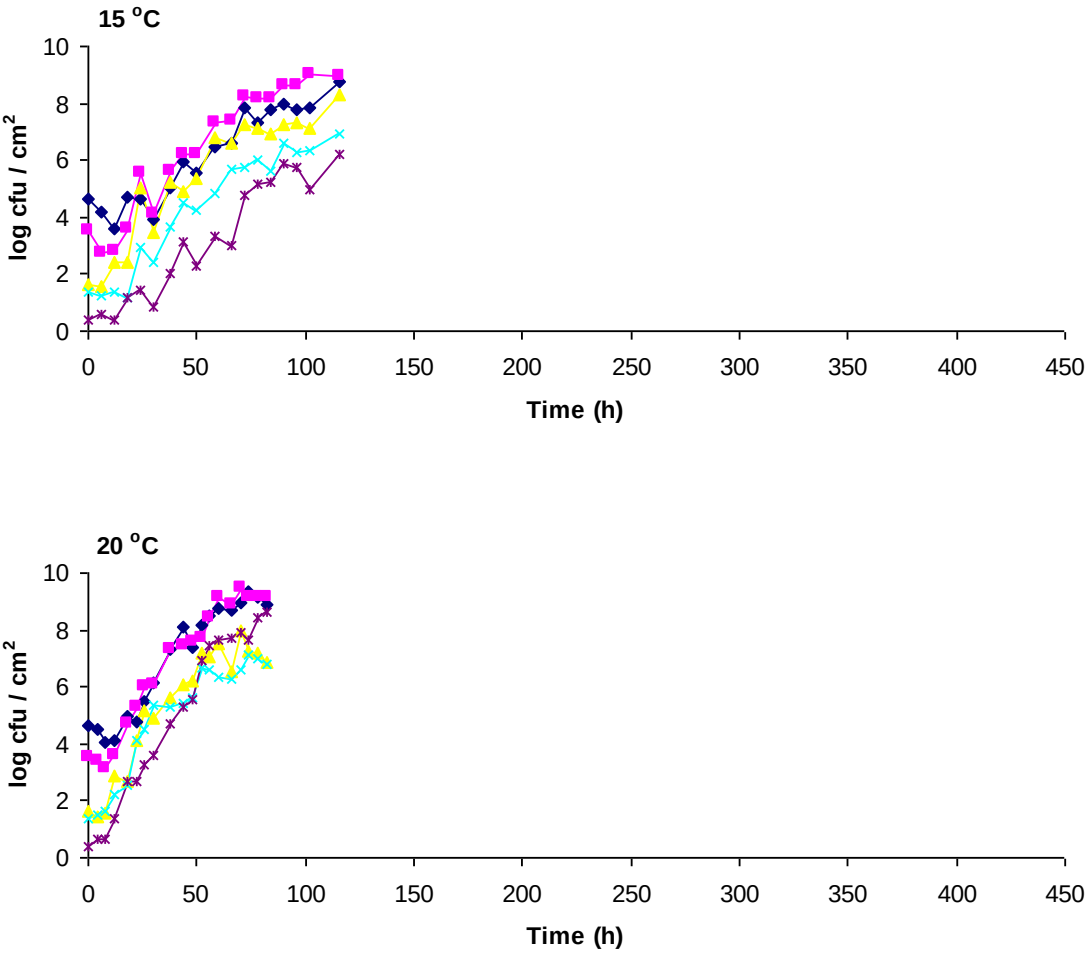

Figure 3.6. Changes in microbial population of beef stored aerobically at 15 and 20 °C. (◆), total viable counts; (▪), pseudomonads; (▲), *Br. thermosphacta*; (×), lactic acid bacteria and (∗), *Enterobacteriaceae*.

3.3 Detection of Quorum Sensing signals during minced beef spoilage

Minced beef samples were stored under three packaging conditions, namely aerobic and under modified atmospheres with/without the presence of volatile compounds of oregano essential oil. The changes in the natural spoilage microflora were monitored by plate counting on selective and non-selective agar media. Cell-free meat extracts (CFME) at the same time intervals as the microbiological assays were collected, and screened for QS signals using various biosensor strains (see details in CHAPTER 2: Materials and Methods).

The microbiological analysis revealed that the initial flora of minced beef consisted of pseudomonads, *Br. thermosphacta*, *Enterobacteriaceae*, LAB, yeasts and moulds. The succession of these groups and their contribution to the final microbiota was influenced by the temperature and type of storage.

Air packaging

Throughout the aerobic storage of minced beef, isolation of all viable microbial groups was higher when compared with other packaging conditions. Pseudomonads were found to be the dominant microorganisms during aerobic storage at all temperatures (0, 5, 10 and 15 °C) (Tables 3.4 and 3.5). In the case of *A. tumefaciens* A136 and *C. violaceum* CV026 biosensor strains various assays (well diffusion, spread plating and microplate assays) led to the same result. The induction of *A. tumefaciens* A136 and *E. coli* JM109 (pSB401) was similar among tested CFCE collected at these conditions. The size of the induction area using *A. tumefaciens* A136 as well as the induced bioluminescence using *E. coli* JM109 (pSB401) began increasing at different time intervals depending on the storage temperature (Figures 3.7 and 3.8). The earliest detectable AHL activity in all samples (CFME) began

increasing when pseudomonads and *Enterobacteriaceae* concentrations reached about 10^8 to 10^9 CFU g^{-1}. None of the samples induced *C. violaceum* CV026, *E. coli* JM109 (pSB1075) or *V. harveyi* BAA-1118 biosensor strains at any time during aerobic storage at all temperatures. Except for the CFME collected at the final storage periods, none of the samples induced the *E. coli* JM109 (pSB536) biosensor strain (Figure 3.9).

Table 3.4. Microbiological data throughout the aerobic storage of minced beef at 0 and 5 °C

Temp. (°C)	Time (h)	Microbial counts log CFU g^{-1}						pH
		TVC	PAB	STAA	MRS	VRBG	RBC	
	0	5.48±0.00	4.30±0.00	4.18±0.10	5.26±0.00	3.95±0.06	4.13±0.07	5.52±0.00
0	**24**	5.63±0.21	4.53±0.05	3.90±0.09	5.17±0.07	3.94±0.14	4.12±0.03	5.56±0.07
	48	5.30±0.03	4.54±0.34	3.92±0.32	5.20±0.04	3.89±0.27	4.38±0.11	5.56±0.05
	69	5.56±0.34	4.85±0.78	4.02±0.14	5.33±0.30	3.94±0.02	4.43±0.25	5.69±0.05
	90	6.18±0.14	5.93±0.07	5.24±0.19	5.79±0.07	4.88±0.28	4.49±0.21	5.60±0.02
	114	6.05±0.20	5.74±0.38	5.48±0.58	5.98±0.01	4.71±0.38	4.71±0.34	5.60±0.01
	196	7.57±0.07	7.80±0.01	7.37±0.04	6.24±0.12	5.48±0.14	6.69±0.01	5.62±0.07
	244	9.12±0.17	9.14±0.17	8.11±0.17	6.96±0.17	5.71±0.99	7.96±0.40	5.83±0.01
	291	9.38±0.01	9.52±0.06	8.04±0.03	7.43±0.06	7.64±0.25	7.77±0.13	6.02±0.02
	338	8.83±0.06	8.90±0.15	7.94±0.24	6.51±0.09	5.39±0.12	7.11±0.58	5.68±0.01
	386	9.39±0.01	9.40±0.03	8.29±0.11	6.89±0.02	6.43±0.18	8.93±0.09	6.04±0.08
	458	9.78±0.36	9.74±0.48	8.49±0.11	7.16±0.08	7.26±0.05	8.05±0.47	6.59±0.11
	554	10.00±0.00	10.11±0.01	8.77±0.05	7.37±0.18	7.83±0.42	8.02±0.19	6.83±0.01
	650	10.04±0.14	10.08±0.15	8.53±0.14	7.66±0.10	8.19±0.05	6.93±0.21	7.18±0.06
5	**24**	5.87±0.24	4.48±0.01	3.78±0.43	5.28±0.53	4.38±0.12	4.27±0.04	5.51±0.05
	48	5.92±0.32	5.18±0.68	4.63±0.28	6.04±0.09	4.99±0.36	4.73±0.35	5.64±0.08
	69	6.82±0.03	6.01±0.22	5.05±0.30	6.53±0.08	4.88±0.04	5.06±0.12	5.84±0.04
	90	6.94±0.34	7.24±0.34	5.79±0.70	6.50±0.46	4.65±0.77	4.90±0.09	5.57±0.08
	114	7.84±0.00	7.70±0.10	7.20±0.29	7.21±0.09	5.99±0.12	5.35±0.48	5.54±0.02
	162	9.07±0.74	9.53±0.01	8.39±0.05	7.27±0.18	7.72±0.13	7.84±0.71	6.00±0.13
	196	9.35±0.01	9.43±0.04	8.12±0.03	7.32±0.11	7.67±0.11	8.00±0.84	5.93±0.10
	220	9.66±0.03	9.70±0.02	8.02±0.25	6.53±0.09	7.47±0.46	9.11±0.07	6.07±0.21
	244	9.74±0.24	9.84±0.17	7.94±0.05	7.66±0.07	8.14±0.21	9.41±0.18	6.11±0.31
	268	9.80±0.35	9.93±0.26	8.23±0.06	7.67±0.25	9.02±0.36	8.95±0.31	6.32±0.04
	291	9.71±0.05	9.75±0.04	8.04±0.15	7.77±0.03	8.47±0.15	8.47±0.18	6.25±0.03
	315	9.96±0.09	9.80±0.04	8.05±0.21	7.69±0.12	8.87±0.10	9.26±0.02	6.82±0.15
	338	9.92±0.10	9.95±0.17	8.30±0.28	7.77±0.02	8.89±0.18	8.43±0.67	6.84±0.04
	386	9.88±0.00	9.89±0.11	8.09±0.18	7.62±0.25	8.72±0.13	8.80±0.09	6.85±0.13
	482	9.90±0.08	9.99±0.08	7.86±0.33	7.70±0.05	9.13±0.17	7.48±0.11	6.93±0.09

TVC: total viable counts; **PAB**: pseudomonads; **STAA**: *Br. thermosphacta*; **MRS**: lactic acid bacteria; **VRBG**: *Enterobacteriaceae*; **RBC**: yeasts and moulds. Microbiological counts and pH values are presented as mean ± standard deviation.

Table 3.5. Microbiological data throughout the aerobic storage of minced beef at 10 and 15 °C

Temp. (°C)	Time (h)	Microbial counts log CFU g^{-1}						pH
		TVC	PAB	STAA	MRS	VRBG	RBC	
	0	5.48±0.00	4.30±0.00	4.18±0.10	5.26±0.00	3.95±0.06	4.13±0.07	5.52±0.00
10	**6**	6.11±0.05	4.93±0.08	4.03±0.31	5.53±0.26	4.75±0.21	4.26±0.10	5.64±0.00
	18	6.54±0.34	5.35±0.21	4.09±0.39	6.01±0.41	4.60±0.05	4.92±0.47	5.46±0.01
	30	6.55±0.33	5.95±0.12	4.09±0.12	6.36±0.09	5.00±0.11	4.59±0.20	5.45±0.04
	42	7.57±0.12	6.75±0.64	5.65±0.05	7.41±0.05	4.60±0.43	5.57±0.29	5.53±0.01
	54	8.20±0.03	6.61±0.25	6.37±0.06	8.15±0.04	6.29±0.13	6.33±0.01	5.65±0.00
	69	8.74±0.09	8.22±0.52	6.93±0.42	8.46±0.03	7.22±0.04	7.50±0.06	5.64±0.01
	78	8.62±0.02	8.18±0.27	7.04±0.03	8.39±0.05	7.68±0.15	7.50±0.15	5.46±0.01
	90	8.89±0.13	8.53±0.19	6.84±0.09	8.50±0.02	7.69±0.70	7.59±0.25	5.42±0.13
	110	9.15±0.07	9.06±0.16	6.73±0.12	8.80±0.70	8.50±0.38	6.74±0.28	5.95±0.11
	134	9.23±0.03	9.17±0.15	6.78±0.43	8.38±0.06	8.79±0.12	8.56±0.03	6.20±0.01
	162	9.64±0.02	9.48±0.08	7.17±0.12	8.50±0.03	9.04±0.17	8.07±0.18	6.49±0.04
	196	9.56±0.03	9.49±0.00	6.70±0.04	8.25±0.00	8.99±0.18	8.44±0.07	6.55±0.11
	220	9.75±0.21	9.77±0.22	6.67±0.62	8.54±0.09	8.99±0.16	8.98±0.10	6.64±0.21
	244	9.68±0.03	9.70±0.02	6.73±0.03	8.55±0.09	9.22±0.02	9.55±0.09	6.76±0.02
	268	9.89±0.04	9.79±0.03	7.30±0.72	8.68±0.08	9.46±0.06	8.63±0.06	6.80±0.02
	315	9.73±0.04	9.45±0.03	7.46±0.06	8.16±0.01	9.27±0.08	7.85±0.11	7.59±0.08
15	**6**	5.60±0.00	4.02±0.34	3.44±0.27	5.62±0.12	3.48±0.10	3.91±0.32	5.56±0.01
	12	5.93±0.21	4.48±0.00	3.86±0.19	5.86±0.09	4.24±0.42	4.25±0.36	5.55±0.05
	18	5.88±0.14	5.88±0.11	3.98±0.39	5.87±0.16	4.13±0.07	4.24±0.04	5.39±0.01
	24	7.08±0.55	6.22±0.62	4.80±0.46	7.32±0.02	5.87±0.44	6.00±0.40	5.59±0.06
	30	7.83±0.04	6.61±0.41	5.53±0.86	7.87±0.02	6.41±0.15	5.77±0.10	5.65±0.04
	36	8.05±0.59	7.16±0.36	6.30±0.16	8.21±0.10	7.03±0.53	6.77±0.17	5.45±0.00
	42	8.39±0.23	7.39±0.12	6.45±0.02	8.29±0.13	7.18±0.10	6.09±0.05	5.44±0.07
	48	8.68±0.10	7.97±0.13	6.52±0.09	8.50±0.10	7.92±0.03	7.52±0.03	5.29±0.07
	54	8.68±0.22	7.89±0.67	6.31±0.26	8.26±0.02	6.93±0.05	7.37±0.05	5.39±0.03
	60	8.79±0.05	8.51±0.23	6.74±0.21	8.42±0.06	8.52±0.06	7.21±0.02	5.63±0.10
	69	9.04±0.16	8.93±0.14	6.62±0.34	8.62±0.02	8.60±0.06	8.01±0.05	5.67±0.13
	78	9.71±0.23	9.04±0.05	6.88±0.20	8.51±0.01	8.66±0.04	8.10±0.03	6.07±0.11
	90	9.41±0.01	9.26±0.12	6.53±0.05	8.49±0.14	8.72±0.08	7.83±0.39	5.86±0.24
	110	9.72±0.07	9.55±0.24	6.80±0.12	8.40±0.01	9.21±0.18	6.90±0.17	6.48±0.21
	134	9.55±0.16	9.62±0.04	6.30±0.21	8.43±0.00	8.78±0.12	8.20±0.09	6.31±0.04
	196	9.60±0.05	9.73±0.04	6.88±0.49	8.32±0.03	9.36±0.03	8.78±0.27	6.87±0.10

TVC: total viable counts; **PAB**: pseudomonads; **STAA**: *Br. thermosphacta*; **MRS**: lactic acid bacteria; **VRBG**: *Enterobacteriaceae*; **RBC**: yeasts and moulds. Microbiological counts and pH values are presented as mean ± standard deviation.

All the tested CFME samples derived from minced beef stored aerobically at 0, 5, 10 and 15 °C, displayed low amounts of AI-2-like activity compared to the control (negative sample) as shown in Table 3.6. AI-2-like activity ranged from 0.22 to 1.94 irrespective to the indigenous bacterial populations (Tables 3.4 and 3.5). CFME from 0 h minced beef sample were used as control samples, in both luminescence-based broth and AI-2 activity bioassays. It should be noted that the control samples exposed similar AHL and AI-2 activity as CFME from "sterile" meat tissue and sterile growth media.

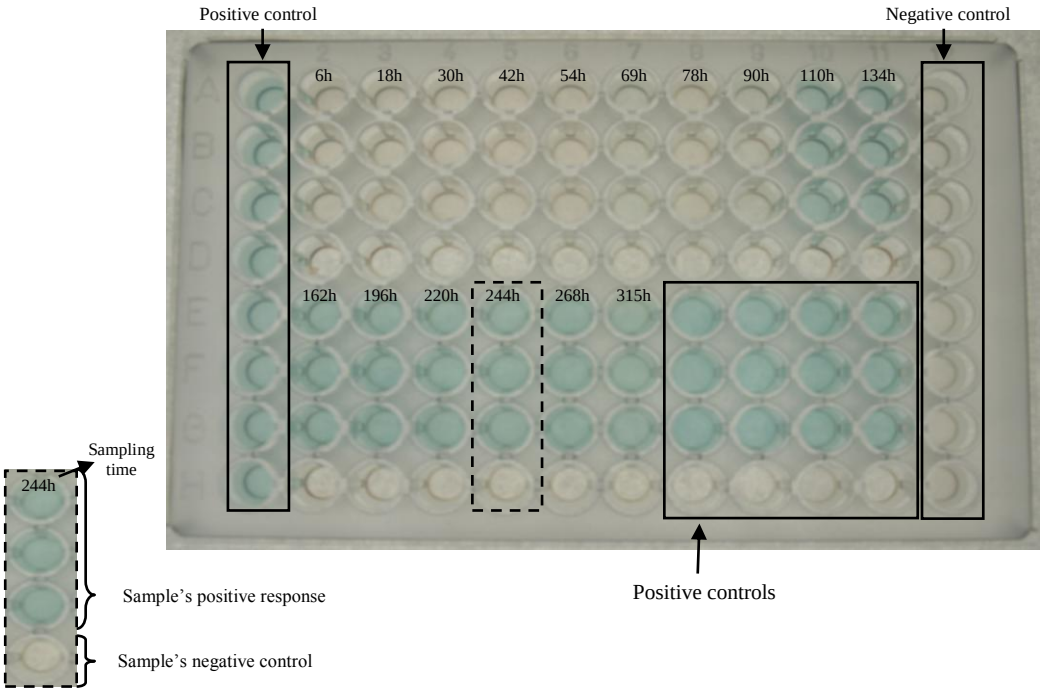

Figure 3.7. AHL induction of *A. tumefaciens* A136 during spoilage of minced beef stored aerobically at 10 °C when microplate bioassay was conducted. Positive and negative controls were used.

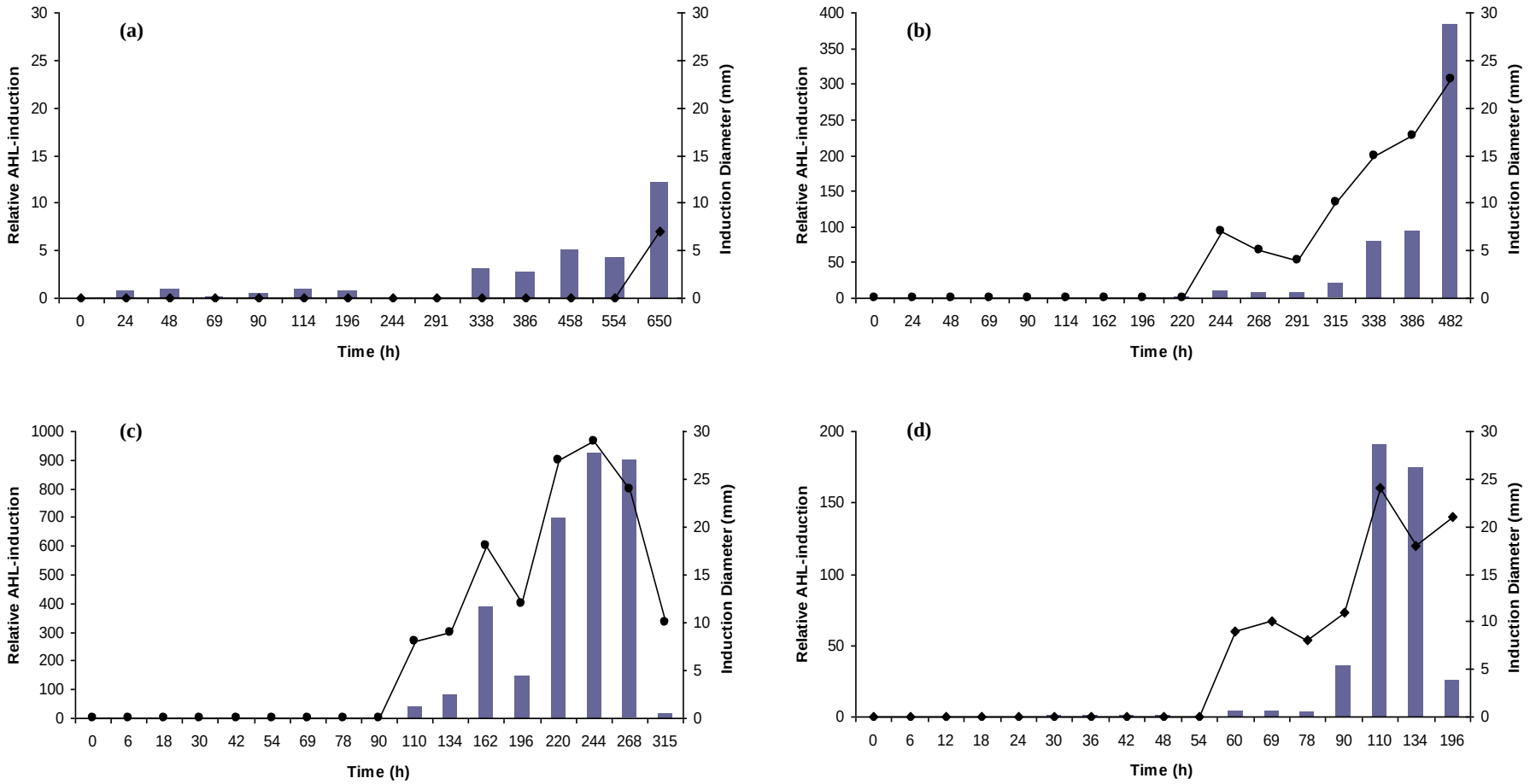

Figure 3.8. AHL induction during spoilage of minced beef stored aerobically at **(a)** 0; **(b)** 5; **(c)** 10 and **(d)** 15 °C determined using *A. tumefaciens* A136 (line) (well diffusion assay) and *E. coli* JM109 (pSB401) (bars) (luminescence-based broth assay) biosensor strains.

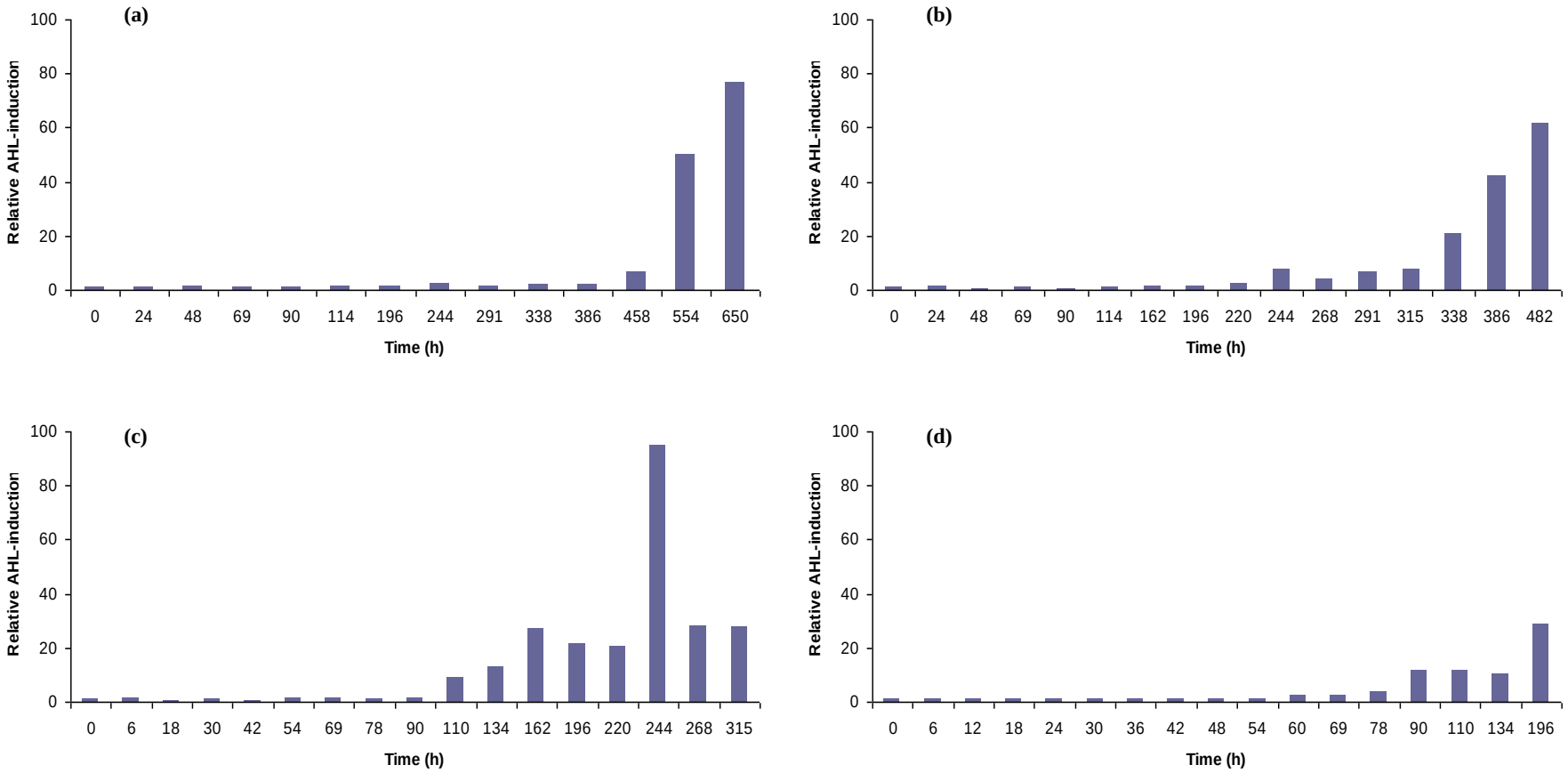

Figure 3.9. AHL induction during spoilage of minced beef stored aerobically at **(a)** 0; **(b)** 5; **(c)** 10 and **(d)** 15 °C determined using *E. coli* JM109 (pSB536) (luminescence-based broth assay) biosensor strain.

Table 3.6. Relative AI-2 activity of CFME derived from minced beef stored aerobically at 0, 5, 10 and 15 °C

	Storage temperature (°C)			
	0	**5**	**10**	**15**
Time (h)	Relative AI-2 activity*	Relative AI-2 activity	Relative AI-2 activity	Relative AI-2 activity
0	1.00 ± 0.00			
6			1.24 ± 0.26	1.37 ± 0.89
12				0.87 ± 0.02
18			0.59 ± 0.01	0.78 ± 0.02
24	0.41 ± 0.29	1.16 ± 0.28		0.42 ± 0.27
30			0.89 ± 0.26	0.95 ± 0.35
36				1.18 ± 0.36
42			0.87 ± 0.14	1.94 ± 0.91
48	0.57 ± 0.08	0.95 ± 0.23		0.66 ± 0.58
54			0.76 ± 0.17	1.57 ± 0.87
60				1.18 ± 0.81
69	1.30 ± 0.05	0.89 ± 0.07	0.52 ± 0.11	0.84 ± 0.32
78			0.74 ± 0.12	0.22 ± 0.08
90	0.82 ± 0.06	0.85 ± 0.06	0.94 ± 0.05	0.54 ± 0.23
110			0.29 ± 0.09	0.96 ± 0.63
114	0.35 ± 0.07	1.19 ± 0.06		
134			0.30 ± 0.32	0.71 ± 0.50
162		1.36 ± 0.03	0.55 ± 0.01	
196	0.60 ± 0.13	1.09 ± 0.32	0.51 ± 0.31	0.43 ± 0.26
220		1.08 ± 0.03	0.42 ± 0.12	
244	0.69 ± 0.38	0.44 ± 0.17	0.43 ± 0.31	
268		0.51 ± 0.03	0.62 ± 0.05	
291	0.82 ± 0.11	0.54 ± 0.48		
315		0.41 ± 0.26	0.41 ± 0.27	
338	0.67 ± 0.21	0.42 ± 0.16		
386	0.58 ± 0.02	0.41 ± 0.09		
458	0.50 ± 0.65			
482		0.59 ± 0.53		
554	0.34 ± 0.04			
650	0.88 ± 0.59			

* Relative AI-2-like activity was calculated as the ratio of luminescence of the test sample (CFME) to the control (negative) sample and is presented as mean ± standard deviation.

Packaging under modified atmospheres

Packaging under modified atmospheres delayed the growth of all members of the microbial association compared with aerobic storage. Moreover, modified atmosphere packaging favoured the dominance of *Br. thermosphacta* and LAB (Tables 3.7 and 3.8). It is worth noting that under modified atmospheres the inhibition of *Enterobacteriaceae* group was observed in samples stored at chill temperatures (0 and 5 °C) in comparison with relatively high temperatures (10 and 15 °C). The induction of *E. coli* JM109 (pSB401) was similar to *A. tumefaciens* A136 (Figure 3.10), whereas none of the samples induced the *C. violaceun* CV026, the *E. coli* JM109 (pSB536) and *V. harveyi* BAA-1118 biosensor strains at any time interval during the spoilage of mince stored under modified atmospheres. Induction of *E. coli* JM109 (pSB1075) was seen on meat extracts taken at the very final storage period of beef stored at relatively high temperatures. More specifically, at 10 °C the AHL induction was 3.36-fold higher when compared with the negative control after 315 h of storage, and at 15 °C the induction was 11.70-fold higher after 196 h of storage. All the tested CFME displayed low amounts of AI-2-like activity using the *V. harveyi* BAA-1117 biosensor, regardless of the indigenous microbial load (Table 3.9).

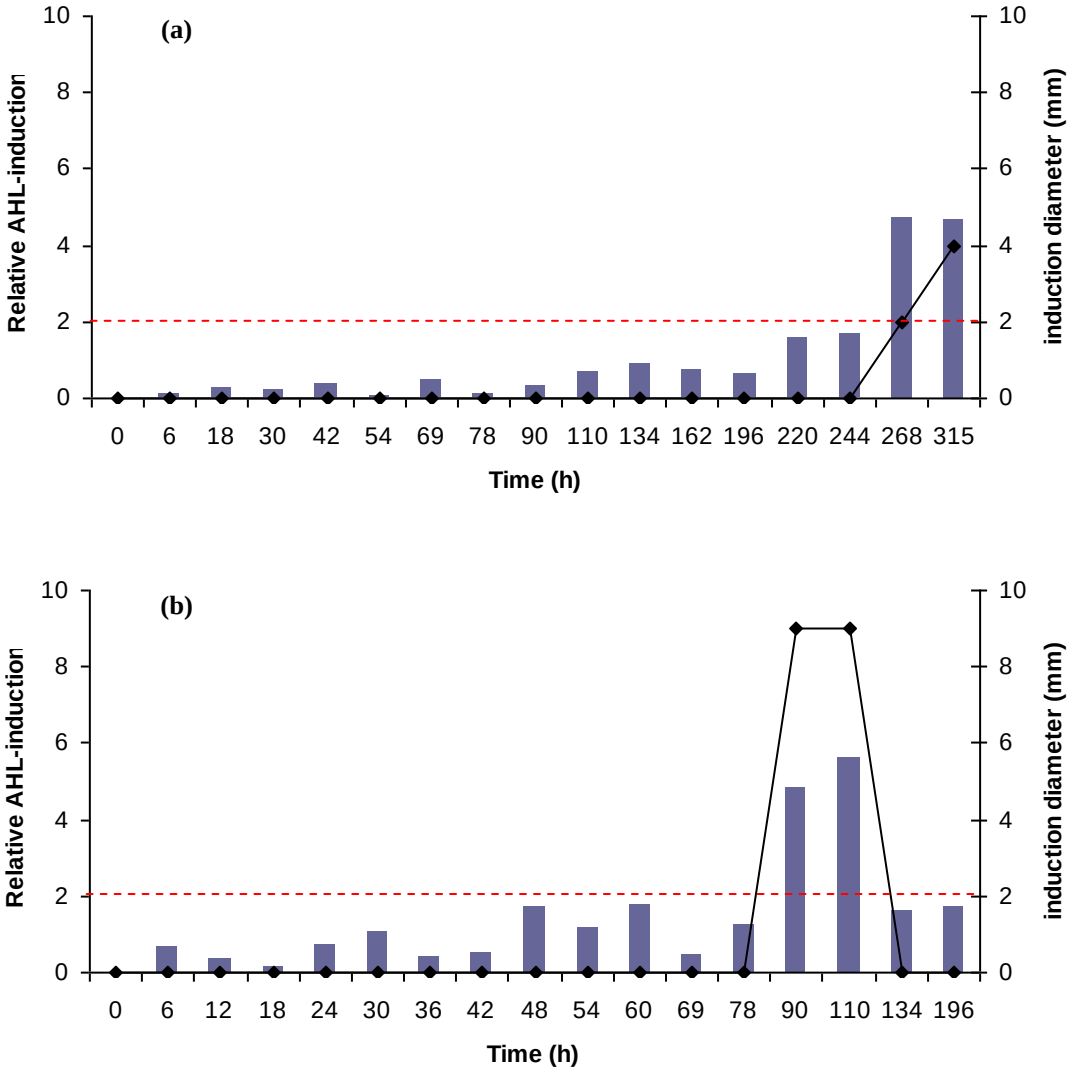

Figure 3.10. AHL induction during spoilage of minced beef stored under modified atmospheres at **(a)** 10 and **(b)** 15 °C determined using *A. tumefaciens* A136 (line) (well diffusion assay) and *E. coli* JM109 (pSB401) (bars) (luminescence-based broth assay) biosensor strains.

Table 3.7. Microbiological data throughout storage of minced beef packaged under modified atmospheres at 0 and 5 °C

Temp. (°C)	Time (h)	Microbial counts log CFU g^{-1}						pH
		TVC	PAB	STAA	MRS	VRBG	RBC	
0	**0**	5.48±0.00	4.30±0.00	4.18±0.10	5.26±0.00	3.95±0.06	4.13±0.07	5.52±0.00
	24	5.42±0.60	4.00±0.11	4.12±0.12	5.18±0.46	4.43±0.02	4.27±0.06	5.46±0.09
	48	5.59±0.16	4.04±0.19	4.07±0.23	5.87±0.46	4.27±0.05	4.25±0.03	5.51±0.03
	69	5.59±0.09	3.04±0.27	3.90±0.32	5.10±0.11	4.17±0.12	3.29±0.30	5.58±0.04
	90	5.05±0.64	3.39±0.55	3.95±0.26	4.55±0.24	4.02±0.22	3.61±0.37	5.38±0.06
	114	5.37±0.10	3.70±0.07	3.18±0.38	5.13±0.07	3.93±0.22	3.72±0.13	5.60±0.06
	196	5.49±0.30	4.08±0.00	4.04±0.37	5.07±0.09	4.24±0.01	4.13±0.03	5.59±0.11
	244	5.90±0.00	4.19±0.02	4.42±0.35	5.41±0.29	3.88±0.57	4.48±0.11	5.59±0.01
	291	6.55±0.24	4.44±0.34	5.01±0.56	6.31±0.24	4.22±0.37	4.07±0.32	5.61±0.08
	338	6.28±0.11	3.71±0.18	4.65±0.62	6.30±0.05	3.76±0.21	3.81±0.20	5.61±0.08
	386	7.00±0.17	4.05±0.10	4.88±0.24	6.99±0.05	4.21±0.01	3.62±0.20	5.46±0.01
	458	7.63±0.18	3.02±0.33	5.88±0.09	7.54±0.11	4.18±0.17	3.74±0.14	5.40±0.01
	554	8.16±0.08	4.73±0.22	6.56±0.38	7.89±0.02	4.67±0.36	5.31±0.03	5.22±0.05
	650	8.27±0.00	4.33±0.04	5.71±0.22	7.95±0.15	4.14±0.20	5.15±0.15	5.16±0.06
5	**24**	5.48±0.00	4.30±0.08	4.19±0.21	5.79±0.18	4.38±0.10	4.20±0.10	5.46±0.03
	48	5.70±0.05	4.14±0.20	4.04±0.19	5.60±0.39	4.22±0.04	3.73±0.70	5.57±0.05
	69	6.32±0.07	4.75±0.21	4.46±0.09	6.19±0.04	4.70±0.31	4.51±0.07	5.78±0.02
	90	6.57±0.18	4.39±0.12	4.63±0.22	6.46±0.16	4.40±0.14	4.43±0.16	5.55±0.02
	114	7.02±0.03	4.69±0.01	5.65±0.05	6.74±0.37	4.44±0.09	4.35±0.17	5.62±0.01
	162	7.26±0.04	4.85±0.07	5.96±0.11	7.10±0.05	4.86±0.26	4.98±0.19	5.56±0.05
	196	7.51±0.05	4.83±0.19	6.21±0.21	7.24±0.08	4.77±0.01	4.38±0.42	5.58±0.03
	220	7.73±0.06	5.25±0.15	6.11±0.42	7.68±0.04	4.92±0.18	5.33±0.07	5.45±0.01
	244	7.86±0.08	5.36±0.03	6.99±0.26	7.47±0.01	5.27±0.00	5.70±0.25	5.48±0.09
	268	8.09±0.03	5.18±0.26	5.99±0.41	7.68±0.01	5.08±0.09	4.96±0.17	5.30±0.06
	291	8.02±0.03	5.20±0.08	6.38±0.61	7.54±0.07	5.15±0.07	5.07±0.23	5.28±0.01
	315	8.15±0.04	5.58±0.33	7.37±0.51	7.74±0.05	5.53±0.35	5.71±0.01	5.49±0.01
	338	7.99±0.03	5.09±0.12	6.37±0.07	7.60±0.12	4.89±0.10	5.29±0.09	5.25±0.04
	386	7.83±0.11	4.84±0.31	6.42±0.39	7.55±0.10	4.91±0.19	5.18±0.47	5.18±0.07
	482	8.00±0.13	4.97±0.08	6.76±0.26	7.54±0.03	4.88±0.09	5.70±0.10	5.11±0.14

TVC: total viable counts; **PAB**: pseudomonads; **STAA**: *Br. thermosphacta*; **MRS**: lactic acid bacteria; **VRBG**: *Enterobacteriaceae*; **RBC**: yeasts and moulds. Microbiological counts and pH values are presented as mean ± standard deviation.

Table 3.8. Microbiological data throughout storage of minced beef packaged under modified atmospheres at 10 and 15 °C

Temp. (°C)	Time (h)	Microbial counts log CFU g^{-1}						pH
		TVC	**PAB**	**STAA**	**MRS**	**VRBG**	**RBC**	
	0	5.48±0.00	4.30±0.00	4.18±0.10	5.26±0.00	3.95±0.06	4.13±0.07	5.52±0.00
10	**6**	5.72±0.17	3.95±0.11	3.77±0.27	5.30±0.42	3.67±0.01	3.83±0.17	5.56±0.04
	18	5.90±0.02	4.48±0.06	4.33±0.14	5.97±0.31	3.78±0.17	4.40±0.02	5.37±0.03
	30	6.82±0.14	4.76±0.21	4.65±0.21	6.66±0.01	4.23±0.10	4.73±0.14	5.48±0.02
	42	6.98±0.03	4.96±0.11	4.72±0.05	7.02±0.17	4.39±0.12	4.54±0.23	5.53±0.01
	54	7.86±0.29	5.10±0.14	5.01±0.18	7.80±0.34	4.94±0.52	5.05±0.35	5.54±0.05
	69	8.36±0.06	5.89±0.30	5.69±0.01	8.30±0.10	6.28±0.31	5.70±0.41	5.62±0.11
	78	8.45±0.15	6.44±0.03	6.24±0.22	8.52±0.15	6.33±0.18	5.41±0.11	5.42±0.07
	90	8.42±0.08	6.35±0.14	6.04±0.24	8.56±0.03	5.96±0.38	5.39±0.02	5.25±0.08
	110	8.15±0.06	5.84±0.24	5.76±0.17	8.18±0.07	5.74±0.13	4.71±0.57	5.19±0.05
	134	8.38±0.00	6.05±0.22	5.63±0.21	8.27±0.09	6.15±0.21	5.89±0.55	5.17±0.05
	162	8.46±0.11	6.72±0.09	6.29±0.32	8.40±0.00	6.79±0.42	6.64±0.03	5.37±0.09
	196	9.56±0.03	6.23±0.26	6.42±0.03	8.45±0.03	6.10±0.02	6.41±0.01	5.20±0.04
	220	9.75±0.21	6.74±0.94	5.42±0.60	8.32±0.09	6.18±0.01	7.13±0.20	5.27±0.23
	244	8.39±0.10	6.32±0.20	5.96±0.69	8.48±0.01	6.29±0.05	9.48±0.01	5.28±0.01
	268	8.65±0.02	6.62±0.30	5.92±0.65	8.55±0.15	6.67±0.65	7.19±0.02	5.34±0.18
	315	8.59±0.13	6.95±0.24	5.49±0.41	8.44±0.00	6.91±0.17	7.58±0.21	5.78±0.06
15	**6**	5.78±0.00	3.70±0.07	3.62±0.19	5.54±0.05	4.52±0.11	3.91±0.32	5.61±0.11
	12	6.68±0.03	3.70±0.09	4.68±0.10	6.86±0.08	4.81±0.08	4.57±0.15	5.56±0.01
	18	7.92±0.22	4.78±0.00	4.94±0.08	7.10±0.02	4.94±0.14	4.60±0.00	5.56±0.04
	24	7.34±0.17	5.00±0.00	4.74±0.65	7.17±0.04	4.38±0.05	5.85±0.15	5.52±0.01
	30	7.58±0.21	5.15±0.21	5.15±0.37	7.57±0.15	5.15±0.21	5.04±0.41	5.49±0.01
	36	7.83±0.00	5.32±0.12	5.50±0.28	7.59±0.02	5.00±0.17	4.73±0.06	5.46±0.01
	42	8.26±0.13	5.52±0.18	5.05±0.22	7.86±0.18	4.30±0.00	5.09±0.20	5.38±0.03
	48	8.21±0.01	5.54±0.09	5.27±0.39	8.17±0.01	5.74±0.19	5.37±0.41	5.11±0.00
	54	7.79±0.51	5.66±0.14	4.62±0.03	8.19±0.05	5.62±0.18	5.05±0.36	5.13±0.01
	60	8.17±0.19	6.10±0.21	5.46±0.09	8.13±0.25	6.56±0.08	5.93±0.09	5.21±0.08
	69	8.35±0.08	6.14±0.25	5.90±0.56	8.44±0.01	6.23±0.04	5.37±0.28	5.34±0.07
	78	8.41±0.00	6.50±0.59	5.47±0.63	8.33±0.04	6.65±0.27	6.38±0.18	5.40±0.02
	90	8.39±0.23	6.94±0.40	5.00±0.43	8.14±0.10	6.95±0.10	6.38±0.65	5.32±0.08
	110	8.48±0.09	6.88±0.40	5.05±0.15	8.43±0.07	7.10±0.00	6.53±0.14	5.34±0.10
	134	8.33±0.04	6.35±0.04	4.35±0.49	7.99±0.13	6.05±0.49	7.17±0.12	5.15±0.08
	196	8.16±0.02	6.94±0.32	4.85±0.25	8.13±0.27	9.30±0.04	8.12±0.33	5.49±0.56

TVC: total viable counts; **PAB**: pseudomonads; **STAA**: *Br. thermosphacta*; **MRS**: lactic acid bacteria; **VRBG**: *Enterobacteriaceae*; **RBC**: yeasts and moulds. Microbiological counts and pH values are presented as mean ± standard deviation.

Table 3.9. Relative AI-2 activity of CFME derived from minced beef stored under modified atmospheres at 0, 5, 10 and 15 °C

	Storage temperature (°C)			
	0	**5**	**10**	**15**
Time (h)	Relative AI-2 activity*	Relative AI-2 activity	Relative AI-2 activity	Relative AI-2 activity
0	1.00 ± 0.00			
6			0.74 ± 0.24	1.97 ± 1.03
12				2.24 ± 1.22
18			0.59 ± 0.12	1.10 ± 0.08
24	1.54 ± 0.07	1.55 ± 0.18		1.01 ± 0.54
30			0.54 ± 0.01	1.72 ± 0.24
36				1.24 ± 0.66
42			0.56 ± 0.18	1.25 ± 0.54
48	1.48 ± 0.13	1.78 ± 1.23		2.37 ± 0.28
54			0.73 ± 0.01	3.07 ± 0.52
60				0.41 ± 0.16
69	1.07 ± 0.43	1.00 ± 0.81	0.41 ± 0.18	1.69 ± 0.91
78			0.88 ± 0.01	1.43 ± 1.23
90	0.71 ± 0.08	0.73 ± 0.49	0.47 ± 0.17	0.67 ± 0.15
110			0.59 ± 0.03	0.34 ± 0.19
114	1.19 ± 0.10	1.49 ± 1.12		
134			0.40 ± 0.19	1.43 ± 0.91
162		1.48 ± 1.21	0.28 ± 0.01	
196	1.40 ± 0.12	1.00 ± 0.53	0.33 ± 0.09	1.22 ± 0.86
220		0.92 ± 0.53	0.47 ± 0.07	
244	1.53 ± 0.25	1.45 ± 0.02	0.37 ± 0.17	
268		0.82 ± 0.07	0.36 ± 0.09	
291	1.21 ± 0.30	0.94 ± 0.57		
315		1.59 ± 0.59	0.37 ± 0.08	
338	1.37 ± 0.21	2.07 ± 0.71		
386	1.07 ± 0.01	0.66 ± 0.23		
458	1.24 ± 0.13			
482		1.52 ± 0.85		
554	0.82 ± 0.14			
650	1.02 ± 0.03			

* Relative AI-2-like activity was calculated as the ratio of luminescence of the test sample (CFME) to the control (negative) sample and is presented as mean ± standard deviation.

Packaging under modified atmospheres with the presence of volatile compounds of oregano essential oil

The most profound changes were evident in samples with the presence of volatile compounds of oregano essential oil that affected the growth of the microbial association of minced beef stored under modified atmospheres. The counts of all

members of the microbial association, with the exception of LAB, were ever lower compared with the samples stored under modified atmospheres (Tables 3.10 and 3.11). None of these samples induced any of the AHL biosensor strains used in this study during the storage period, and no significant AI-2-like activity was detected as well (Table 3.12).

Table 3.10. Microbiological data throughout storage of minced beef packaged under modified atmospheres with the presence of volatile compounds of oregano essential oil at 0 and 5 °C

Temp. (°C)	Time (h)	Microbial counts log CFU g^{-1}						pH
		TVC	PAB	STAA	MRS	VRBG	RBC	
	0	5.48±0.00	4.30±0.00	4.18±0.10	5.26±0.00	3.95±0.06	4.13±0.07	5.52±0.00
0	**24**	5.00±0.00	3.95±0.12	3.98±0.21	5.09±0.08	3.54±0.09	3.99±0.02	5.42±0.00
	48	5.63±0.19	4.09±0.35	3.62±0.87	5.20±0.04	4.05±0.21	5.28±0.13	5.44±0.08
	69	5.3±0.12	3.83±0.18	3.97±0.40	5.07±0.07	3.75±0.06	3.59±0.41	5.59±0.08
	90	4.94±0.12	3.46±0.09	3.68±0.24	4.65±0.12	3.52±0.20	3.18±0.20	5.32±0.01
	114	5.34±0.10	3.77±0.10	4.93±0.12	4.87±0.04	3.35±0.07	3.53±0.00	5.43±0.00
	196	5.25±0.14	3.36±0.40	3.65±0.87	5.04±0.12	3.48±0.01	3.81±0.22	5.41±0.02
	244	5.81±0.05	3.98±0.12	3.60±0.00	5.48±0.00	4.35±0.56	4.40±0.05	5.50±0.02
	291	5.70±0.18	3.51±0.27	5.20±0.77	5.43±0.03	3.69±0.12	3.77±0.11	5.46±0.04
	338	5.85±0.22	3.81±0.06	3.47±0.03	5.27±0.85	3.91±0.12	3.85±0.17	5.46±0.01
	386	6.47±0.07	3.98±0.09	5.02±0.93	6.46±0.15	4.08±0.01	3.76±0.01	5.39±0.03
	458	6.45±0.36	3.58±0.15	3.84±0.73	6.54±0.32	3.48±0.10	3.53±0.02	5.48±0.05
	554	7.34±0.15	3.72±0.34	3.73±0.49	7.29±0.06	3.71±0.15	3.54±0.33	5.45±0.01
	650	7.28±0.01	3.55±0.30	4.07±0.90	7.26±0.03	3.25±0.24	3.40±0.13	5.46±0.01
5	**24**	5.66±0.26	4.30±0.12	4.68±0.31	5.52±0.06	3.80±0.21	3.97±0.12	5.38±0.01
	48	5.59±0.30	3.74±0.37	5.05±0.15	5.60±0.39	3.85±0.15	3.49±0.30	5.61±0.08
	69	6.04±0.11	4.25±0.07	5.02±0.83	5.91±0.19	3.75±0.07	3.85±0.04	5.62±0.02
	90	6.45±0.03	3.78±0.00	4.64±0.11	6.06±0.10	3.39±0.12	3.95±0.13	5.37±0.04
	114	6.01±0.11	3.32±0.09	3.82±0.26	5.95±0.01	3.17±0.08	3.80±0.00	5.47±0.01
	162	5.76±0.01	4.03±0.14	4.87±0.34	6.63±0.10	3.66±0.07	4.39±0.05	5.47±0.04
	196	7.28±0.19	4.21±0.09	5.43±0.68	7.19±0.07	4.31±0.05	4.26±0.35	5.41±0.01
	220	7.36±0.01	4.20±0.31	5.77±0.34	7.30±0.01	4.18±0.38	4.46±0.23	5.39±0.01
	244	7.43±0.01	4.05±0.50	5.68±0.24	7.47±0.00	4.44±0.07	5.48±0.17	5.37±0.01
	268	7.69±0.01	4.33±0.23	6.20±0.01	7.53±0.15	4.39±0.13	4.96±0.35	5.31±0.01
	291	7.55±0.17	4.02±0.17	5.80±0.28	7.41±0.09	4.25±0.63	5.10±0.02	5.30±0.01
	315	7.73±0.07	4.36±0.18	6.69±0.03	7.58±0.06	4.32±0.40	5.28±0.20	5.62±0.02
	338	7.84±0.06	5.25±0.29	5.65±0.66	7.67±0.05	4.85±0.79	5.40±0.06	5.23±0.01
	386	7.59±0.08	3.95±0.00	5.44±0.57	7.42±0.11	3.95±0.07	5.53±0.21	5.16±0.04
	482	7.85±0.16	4.00±0.00	5.93±0.08	7.64±0.03	3.66±0.35	5.53±0.04	5.06±0.08

TVC: total viable counts; **PAB**: pseudomonads; **STAA**: *Br. thermosphacta*; **MRS**: lactic acid bacteria; **VRBG**: *Enterobacteriaceae*; **RBC**: yeasts and moulds. Microbiological counts and pH values are presented as mean ± standard deviation.

Table 3.11. Microbiological data throughout storage of minced beef packaged under modified atmospheres with the presence of volatile compounds of oregano essential oil at 10 and 15 °C

Temp. (°C)	Time (h)	Microbial counts log CFU g^{-1}						pH
		TVC	PAB	STAA	MRS	VRBG	RBC	
	0	5.48±0.00	4.30±0.00	4.18±0.10	5.26±0.00	3.95±0.06	4.13±0.07	5.52±0.00
10	**6**	5.99±0.30	4.08±0.05	4.38±0.39	5.66±0.28	4.48±0.01	3.95±0.06	5.41±0.04
	18	6.02±0.45	4.36±0.15	4.54±0.18	5.85±0.07	3.70±0.00	4.01±0.05	5.44±0.09
	30	6.27±0.10	4.32±0.09	4.53±0.32	6.24±0.28	3.85±0.05	4.51±0.03	5.38±0.01
	42	6.69±0.12	4.38±0.12	4.83±0.06	6.52±0.74	4.00±0.10	3.80±0.71	5.34±0.00
	54	7.22±0.14	4.46±0.09	5.22±0.05	7.12±0.26	3.48±0.07	4.34±0.32	5.44±0.01
	69	7.81±0.16	4.46±0.12	4.48±0.10	7.76±0.03	3.99±0.12	4.78±0.06	5.54±0.02
	78	7.95±0.27	4.57±0.35	5.43±0.08	7.89±0.21	4.72±0.34	4.90±0.04	5.39±0.00
	90	7.77±0.03	4.50±0.16	4.78±0.64	7.85±0.31	3.72±0.17	4.59±0.18	5.30±0.01
	110	7.46±0.79	4.94±0.16	4.80±0.13	7.67±0.36	4.82±0.18	5.29±0.39	5.23±0.02
	134	7.27±0.47	5.10±0.20	4.57±0.35	7.53±0.16	5.16±0.13	5.92±0.14	5.11±0.02
	162	7.42±0.96	5.27±0.18	5.65±0.87	7.74±0.11	5.37±0.33	5.98±0.28	5.07±0.05
	196	7.88±0.06	5.34±0.20	6.16±0.05	7.13±0.75	5.14±0.14	6.44±0.23	4.97±0.00
	220	7.72±0.01	5.01±0.04	4.33±0.10	8.03±0.92	5.10±0.18	6.57±0.34	5.02±0.01
	244	7.71±0.15	5.26±0.26	4.10±0.21	7.63±0.41	5.36±0.06	8.56±0.14	4.96±0.03
	268	7.75±0.16	5.25±0.49	4.90±0.47	7.56±0.12	5.29±0.12	6.63±0.17	4.96±0.00
	315	7.95±0.10	5.44±0.01	4.48±0.15	7.91±0.06	5.28±0.11	7.69±0.06	5.33±0.05
15	**6**	6.52±0.00	4.84±0.71	5.29±0.05	6.62±0.08	4.41±0.17	4.12±0.03	5.53±0.00
	12	5.62±0.13	4.39±0.12	4.73±0.09	6.38±0.10	4.30±0.00	3.98±0.24	5.41±0.04
	18	7.29±0.49	4.30±0.31	4.44±0.29	6.42±0.21	4.63±0.04	4.20±0.20	5.34±0.04
	24	6.86±0.13	4.63±0.54	4.72±0.15	6.70±0.12	4.11±0.21	3.87±0.16	5.42±0.03
	30	6.81±0.69	4.86±0.09	4.69±0.01	7.30±0.14	4.28±0.19	4.41±0.10	5.45±0.00
	36	7.38±0.11	4.85±0.15	5.87±0.00	7.44±0.03	4.34±0.05	4.25±0.19	5.38±0.02
	42	8.14±0.13	5.84±0.24	5.36±0.06	8.09±0.01	4.28±0.35	5.13±0.02	5.31±0.02
	48	7.82±0.06	5.46±0.66	4.45±0.57	7.65±0.03	4.60±0.14	5.06±0.07	5.13±0.01
	54	7.80±0.15	4.60±0.43	4.88±0.18	7.75±0.21	4.91±0.19	4.90±0.15	5.17±0.05
	60	7.88±0.14	4.71±0.31	4.29±0.05	7.91±0.12	5.13±0.07	5.24±0.02	5.00±0.00
	69	7.59±0.18	4.72±0.17	4.97±0.91	7.62±0.15	4.87±0.04	5.10±0.17	5.00±0.03
	78	7.64±0.21	4.93±0.36	4.08±0.43	7.57±0.06	5.22±0.11	5.96±0.49	4.94±0.02
	90	7.59±0.20	5.05±0.52	4.32±000	7.76±0.27	4.96±0.01	5.85±0.00	4.87±0.01
	110	7.60±0.00	5.09±0.10	2.59±0.16	7.84±0.15	5.18±0.05	6.23±0.54	4.87±0.00
	134	7.94±0.06	6.26±0.15	4.08±0.54	7.41±0.55	6.35±0.23	7.03±0.06	5.27±0.01
	196	7.93±0.04	6.45±0.90	2.84±0.53	8.23±0.04	6.88±0.29	7.29±0.00	5.37±0.01

TVC: total viable counts; **PAB**: pseudomonads; **STAA**: *Br. thermosphacta*; **MRS**: lactic acid bacteria; **VRBG**: *Enterobacteriaceae*; **RBC**: yeasts and moulds. Microbiological counts and pH values are presented as mean ± standard deviation.

Table 3.12. Relative AI-2 activity of CFME derived from minced beef stored under modified atmospheres with the presence of volatile compounds of oregano essential oil at 0, 5, 10 and 15 °C

	Storage temperature (°C)			
	0	**5**	**10**	**15**
Time (h)	Relative AI-2 activity*	Relative AI-2 activity	Relative AI-2 activity	Relative AI-2 activity
0	1.00 ± 0.00			
6			0.48 ± 0.24	0.43 ± 0.31
12				0.29 ± 0.25
18			0.34 ± 0.16	0.46 ± 0.30
24	0.41 ± 0.28	0.42 ± 0.28		0.29 ± 0.34
30			0.25 ± 0.09	0.52 ± 0.29
36				0.31 ± 0.24
42			0.29 ± 0.11	0.91 ± 0.36
48	0.30 ± 0.42	0.41 ± 0.20		0.89 ± 0.42
54			0.31 ± 0.07	0.77 ± 0.23
60				0.67 ± 0.17
69	0.37 ± 0.32	0.27 ± 0.26	0.36 ± 0.12	0.90 ± 0.17
78			0.58 ± 0.19	0.80 ± 0.04
90	0.23 ± 0.16	0.56 ± 0.46	2.67 ± 1.47	0.78 ± 0.22
110			0.52 ± 0.39	1.68 ± 0.63
114	0.28 ± 0.39	0.32 ± 0.28		
134			0.29 ± 0.21	2.93 ± 0.74
162		0.35 ± 0.30	0.39 ± 0.16	
196	0.46 ± 0.35	0.48 ± 0.36	0.43 ± 0.13	2.63 ± 0.81
220		0.49 ± 0.34	0.34 ± 0.13	
244	0.24 ± 0.25	0.65 ± 0.44	0.63 ± 0.16	
268		0.30 ± 0.20	0.71 ± 0.36	
291	0.41 ± 0.08	0.55 ± 0.35		
315		0.29 ± 0.12	2.53 ± 0.68	
338	0.50 ± 0.26	0.42 ± 0.06		
386	0.35 ± 0.47	0.47 ± 0.36		
458	0.51 ± 0.41			
482		0.47 ± 0.36		
554	0.33 ± 0.13			
650	0.36 ± 0.30			

* Relative AI-2-like activity was calculated as the ratio of luminescence of the test sample (CFME) to the control (negative) sample and is presented as mean ± standard deviation.

AHL profiles

The AHL profiles of selected CFME samples were determined using thin layer chromatography (TLC). The TLC analysis indicated the presence of *N*-(ß-ketocaproyl)-homoserine lactone (3OC6-HSL) in various samples collected mainly at the final storage periods, where *A. tumefaciens* A136 and *E. coli* JM109 (pSB401) biosensor strains were induced (Figure 3.11).

Figure 3.11. AHL profiles of CFME samples collected from minced beef stored aerobically, visualized using *A. tumefaciens* A136 biosensor strain. **(1)** *N*-(ß-ketocaproyl)-homoserine lactone (3OC6-HSL) standard; **(2)** meat stored for 110 h at 10 °C; **(3)** 220 h at 10 °C; **(4)** 110 h at 15 °C and **(5)** 386 h at 5 °C.

3.4 *N*-acyl homoserine lactone signal production of *Enterobacteriaceae* isolated from minced beef

Nineteen different fingerprints (assigned to *Serratia* spp., *Ser. proteomaculans*, *Ser. liquefaciens*, *Citrobacter freundii*, *H. alvei* and *Proteus vulgaris*) out of one hundred and four *Enterobacteriaceae* isolates recovered from minced beef stored under various conditions (temperature and packaging), were screened for their ability to produce *N*-acyl homoserine lactone (AHL) signals (see details in CHAPTER 2: Materials and Methods).

The *Enterobacteriaceae* strains elicited a diversity of response in the three AHL monitor systems (induction of *A. tumefaciens* A136 and *C. violaceum* CV026 and inhibition of the AHL induced *C. violaceum* CV026), as well as the luminescence-based broth assays using *E. coli* JM109 (pSB401, pSB536 and pSB1075) biosensor strains. The responses in the AHL monitoring systems were evaluated as positive or negative according to the controls used (Figure 3.12).

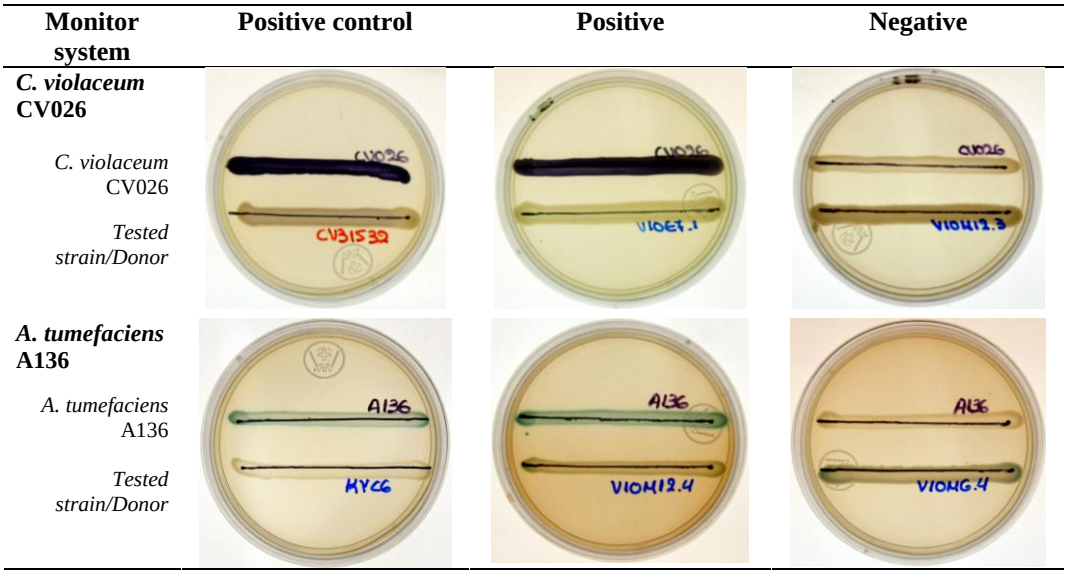

Figure 3.12. Cross feeding screening for AHL production of *Enterobacteriaceae* strains using *C. violaceum* CV026 and *A. tumefaciens* A136 biosensor strains.

Seventeen of the 19 *Enterobacteriaceae* strains were identified as AHL producers (Table 3.13). The strains *Citro. freundii*, *Serratia* spp., *Ser. liquefaciens* (VK23, VK40, VK74 and VK75) and *Ser. proteomaculans* (VK25, VK32 and VK113) inhibited violacein production in the induced *C. violaceum* CV026 strain. Inhibition of violacein production can be exploited as an assay for the detection of longer side-chain AHLs. All the *H. alvei* strains and the *Ser. liquefaciens* VK17 elicited the same response to the biosensor strains used. These strains induced *A. tumefaciens* A136, *C. violaceum* CV026 and *E. coli* JM109 (pSB401). The biosensor strain *E. coli* JM109 (pSB401) has similar detection specificities to *A. tumefaciens* A136 detecting a relatively broad range of medium-side-chain AHLs, whereas *C. violaceum* CV026 is induced by AHLs evaluated with *N*-acyl side chains from C4 to C8 in length. Two strains of *Ser. proteomaculans*, VK5 and VK6, induced all the biosensor strains used, except for *E. coli* JM109 (pSB536) and (pSB1075), which detect small acyl side-chain and long acyl side-chain AHLs respectively. Finally, *Pr. vulgaris* strains did not induce either of the biosensor strains used in this study.

Results from all *Enterobacteriaceae* culture supernatants able to induce biosensor strain *E. coli* JM109 (pSB401) are reported in Figure 3.13. Induced bioluminescence was expressed as relative AHL-induction and was calculated as the ratio of RLU/OD_{450nm} of the test sample to the control (negative) sample.

Table 3.13. Response of *Enterobacteriaceae* strains in AHL biosensor strains

Strain	Code	A136[a]	CV026[a]	CV026[b] inhibited	pSB401[a]	pSB536[a]	pSB1075[a]
Citrobacter freundii	VK19	-	-	+	-	-	-
Hafnia alvei	VK20	+	+	-	+	-	-
	VK27	+	+	-	+	-	-
	VK53	+	+	-	+	-	-
	VK60	+	+	-	+	-	-
Proteus vulgaris	VK101	-	-	-	-	-	-
	VK103	-	-	-	-	-	-
Serratia spp.	VK90	-	-	+	-	-	-
	VK108	-	-	+	-	-	-
Serratia liquefaciens	VK17	+	+	-	+	-	-
	VK23	-	-	+	-	-	-
	VK40	-	-	+	-	-	-
	VK74	-	-	+	-	-	-
	VK75	-	-	+	-	-	-
Ser. proteomaculans	VK5	+	+	+	+	-	-
	VK6	+	+	+	+	-	-
	VK25	-	-	+	-	-	-
	VK32	-	-	+	-	-	-
	VK113	-	-	+	-	-	-

[a] +, biosensor strain induced; -, biosensor strain not induced
[b] +, biosensor strain not induced is red as AHL-positive response; -, biosensor strain induced is red as AHL-negative response

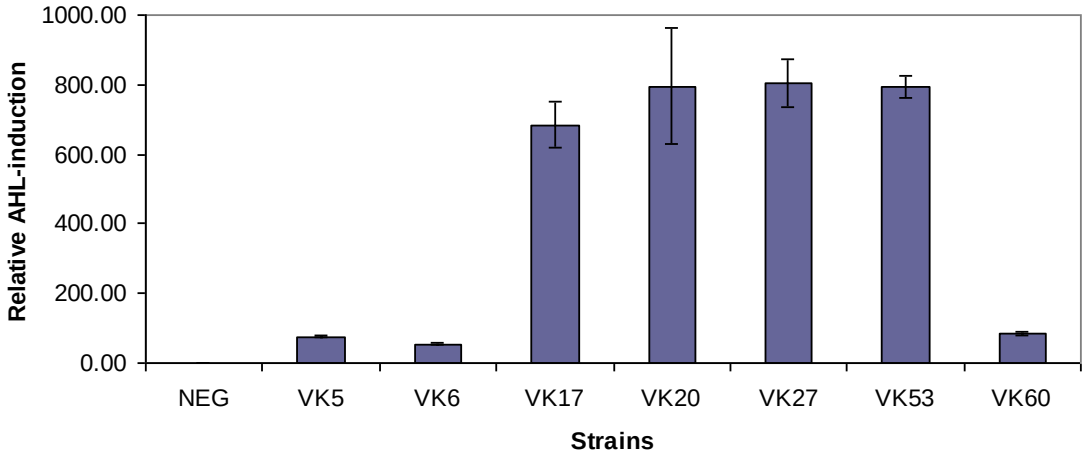

Figure 3.13. Relative AHL-induction of the *Enterobacteriaceae* culture supernatants determined with *E. coli* JM109 (pSB401) biosensor strain. *Serratia proteomaculans* (VK5 and VK6), *Ser. liquefaciens* (VK17) and *Hafnia alvei* (VK20, VK27, VK53 and VK60). Values were calculated as the ratio of RLU/OD_{450nm} of the test sample to the control (negative-NEG) sample. Error bars indicate standard deviation of three sample measurements.

The AHL profiles of the *Enterobacteriaceae* strains were determined using thin layer chromatography. The TLC analysis performed on ethyl acetate extracts obtained from the 19 different strains indicated the presence of *N*-(ß-ketocaproyl)-homoserine lactone (3OC6-HSL) in *Ser. proteomaculans* (VK5 and VK6), *Ser. liquefaciens* (VK17) and all *H. alvei* strains (VK20, VK27, VK53 and VK60) (Figure 3.14).

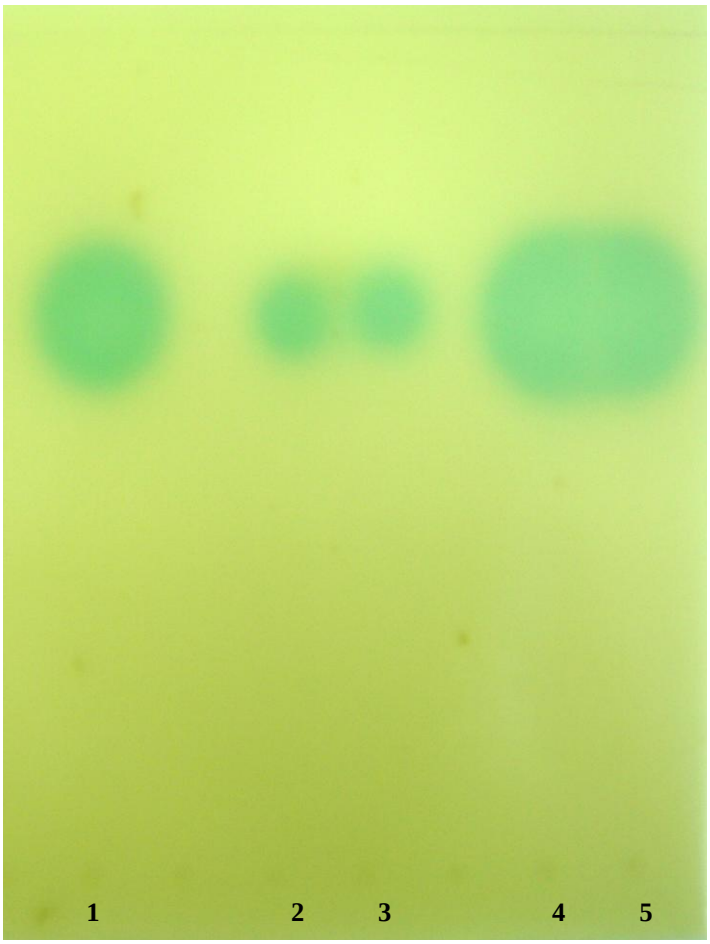

Figure 3.14. AHL profiles of *Enterobacteriaceae* strains, isolated from minced beef, developed with *A. tumefaciens* A136 biosensor strain. **(1)** *N*-(ß-ketocaproyl)-homoserine lactone (3OC6-HSL) standard; **(2)** *Serratia proteomaculans* VK5; **(3)** *Ser. liquefaciens* VK17; **(4)** *Hafnia alvei* VK20 and **(5)** *H. alvei* VK53.

3.5 Autoinducer-2-like activity in lactic acid bacteria isolated from minced beef

Fifteen different fingerprints out of 89 LAB isolated from minced beef stored under modified atmospheres at various temperatures (0, 5, 10 and 15 °C) were screened for their ability to exhibit AI-2-like activity, under certain growth conditions. At the same time intervals as the microbiological analysis and the isolates recovery, cell-free meat extracts (CFME) were collected and tested for the presence of AI-2-like signals.

Eighty-nine $CFCE_{LAB}$ and thirteen CFME samples were tested for the production of AI-2-like activity and the presence of the AI-2-like signal, respectively. The AI-2 activity bioassay was used, which relies on the ability of the *V. harveyi* BAA-1117 biosensor strain to produce light in response to AI-2. The tested $CFCE_{LAB}$ were collected from equal number of isolates (assigned to *Leuconostoc* spp., *Leuc. mesenteroides*, *Weissella viridescens*, *Leuc. citreum* and *Lactobacillus sakei*), recovered from initial, middle and final stage of minced beef storage. From those isolates fifteen different fingerprints were obtained. Identical isolates were tested and verified for presence or absence of relative AI-2-like activity. The isolates exhibiting AI-2-like activity are shown in Table 3.14. The $CFCE_{LAB}$ extracted from the *Leuconostoc* sp. type B233 isolate expressed AI-2-like activity ranging from 12.41 to 26.84-fold, compared with the negative control. No significant differences ($P > 0.05$) in AI-2-like activity were found among these identical strains regardless of the stage of storage (initial, middle and final) and storage temperature of minced meat. The *Leuconostoc* spp. (B232 and B240) and *Leuc. mesenteroides* (B243) strains also expressed AI-2-like activity (Table 3.14). Eleven fingerprints assigned to *Lact. sakei* (B222, B227, B236, B237, B238 and B239), *W. viridescens* (B234 and B235),

Leuconostoc sp. (B241), *Leuc. citreum* (B258) and *Leuc. mesenteroides* (B242) did not express detectable AI-2-lke activity under standard growth conditions.

Table 3.14. Representative lactic acid bacteria exhibiting AI-2-like activity at initial, middle and final stages of minced beef storage. Total number of isolates recovered from each storage period in accordance with those exhibiting AI-2

Temp. (°C)	Storage period	No. of isolates	Strains exhibiting AI-2 / No. of identical isolates exhibiting AI-2		AI-2-like activity of strains [a]
	Day 0/ initial flora	6	*Leuconostoc* spp. (B233)[a]	/ 5	25.90 ± 11.60
			Leuconostoc spp. (B232)[b]	/ 1	2.23 ± 0.32
0	Initial	5	*Leuconostoc* spp. (B233)[a]	/ 2	13.28 ± 1.79
	Middle	6	*Leuconostoc* spp. (B233)[a]	/ 5	14.81 ± 1.32
	Final	5		/ 0	
5	Initial	6	*Leuconostoc* spp. (B233)[a]	/ 4	22.11 ± 2.13
	Middle	6	*Leuconostoc* spp. (B233)[a]	/ 5	18.03 ± 0.85
	Final	9	*Leuconostoc* spp. (B233)[a]	/ 2	13.86 ± 1.89
10	Initial	6	*Leuconostoc* spp. (B233)[a]	/ 6	13.97 ± 4.73
	Middle	8	*Leuconostoc* spp. (B233)[a]	/ 8	13.41 ± 1.58
	Final	10	*Leuconostoc* spp. (B233)[a]	/ 8	12.41 ± 0.53
			Leuc. mesenteroides (B243)[b]	/ 1	03.24 ± 0.74
15	Initial	6	*Leuconostoc* spp. (B233)[a]	/ 6	25.73 ± 10.73
	Middle	8	*Leuconostoc* spp. (B233)[a]	/ 8	24.71 ± 09.41
	Final	8	*Leuconostoc* spp. (B233)[a]	/ 6	26.84 ± 13.12
			Leuconostoc spp. (B240)[b]	/ 1	03.01 ± 01.14
Total		89		/ 68	

[a] AI-2-like activity was calculated as the ratio of luminescence of the test sample ($CFCE_{LAB}$) to the control (negative) sample and is presented as mean ± standard deviation. Values with the same letter are not significantly different ($P > 0.05$).

All the tested CFME samples displayed low amounts of AI-2-like activity ranging between 0.47 and 2.24 compared to the control (negative) sample as shown in Table 3.15. The CFME from 0 h minced beef sample was used as control sample, which should be noted that expressed AI-2-like activity similar to that of CFME from "sterile" meat tissue and sterile growth medium (data not shown). No significant correlation was observed between the occurrence of AI-2-like activity and the

enumerated bacterial population, which ranged between 5.10 and 8.56 log CFU g^{-1}. The low values of AI-2-like activity led us to evaluate the possible inhibitory effect caused to the biosensor strain's activity by the CFME, which was determined by mixing equal volumes from the $CFCE_{ST}$ of the AI-2-producing *Salmonella* serovar Typhimurium strain with the CFME and performing the AI-2 activity bioassay. The inhibitory effect ranged between 51.11 and 91.09 % (Table 3.15).

Table 3.15. Relative CFME AI-2-like activity, inhibition of AI-2 activity by CFME and bacterial counts at initial, middle and final stages of mined beef storage

Temp. (°C)	Storage period	Relative AI-2-like activity of CFME [a]	% Inhibition of AI-2-like activity [b]	Bacterial counts (log CFU g^{-1})
	Day 0/initial flora	-	89.50 ± 0.37	5.26 ± 0.13
0	Initial	1.07 ± 0.43	84.70 ± 0.04	5.10 ± 0.11
	Middle	1.21 ± 0.30	82.92 ± 4.47	6.31 ± 0.24
	Final	1.24 ± 0.13	85.35 ± 3.30	7.54 ± 0.11
5	Initial	1.78 ± 1.23	75.76 ± 2.03	5.60 ± 0.39
	Middle	1.49 ± 0.12	81.30 ± 2.88	6.74 ± 0.37
	Final	1.00 ± 0.53	91.09 ± 0.49	7.24 ± 0.08
10	Initial	0.59 ± 0.12	83.87 ± 4.31	5.97 ± 0.42
	Middle	0.56 ± 0.18	81.62 ± 4.89	7.02 ± 0.17
	Final	0.47 ± 0.17	51.11 ± 4.89	8.56 ± 0.15
15	Initial	2.24 ± 1.22	83.55 ± 1.48	6.86 ± 0.08
	Middle	1.01 ± 0.54	85.61 ± 2.98	7.17 ± 0.04
	Final	1.69 ± 0.91	78.45 ± 1.07	8.44 ± 0.01

[a] Relative AI-2-like activity was calculated as the ratio of luminescence of the test sample (CFME) to the control (negative) sample and is presented as mean ± standard deviation.

[b] Inhibition of AI-2-like activity was expressed as a percentage relative to the corresponding positive control.

Taking into account the above, a correlation between the LAB displaying AI-2-like activity and the storage temperatures was performed. Concisely, among the 89 isolated LAB (fifteen different fingerprints were obtained after the analysis of the PFGE patterns), 68 (76.4%) of the isolates were exhibiting AI-2-like activity. At chill

temperatures (0 and 5 °C) eleven different fingerprints were recovered, whereas at relatively high temperatures (10 and 15 °C) strain diversity was reduced, since five different fingerprints were detected. It needs to be stressed that the initial flora (isolates at day 0), two different *Leuconostoc* spp. strains (B 232 and B 233) were recovered, and both exhibited AI-2-like activity (Table 3.14). At 10 and 15 °C *Leuconostoc* sp. (B233) represented the dominant biota, whilst at 0 and 5 °C was prevalent in the initial and middle stage of storage. Indeed 44 (95.7%) of the tested LAB isolated from 10 and 15 °C exhibited AI-2-like activity, whereas only 18 (48.6%) of LAB isolated from 0 and 5 °C displayed AI-2-like activity. Twenty-three (95.8%) and twenty-one (95.5%) isolates recovered from 10 and 15 °C were positive for AI-2-like activity, respectively. The isolates that exhibited positive response in the AI-2 activity bioassay were characterized as *Leuconostoc* spp. (B233 and B240) and *Leuc. mesenteroides* (B243), and those that did not exhibit AI-2-like activity were characterized as *W. viridescens* (B234) and *Lact. sakei* (B237). Seven (43.8 %) and eleven (52.4%) of the LAB isolates recovered from meat stored at 0 and 5 °C, respectively, exhibited AI-2-like activity. These isolates were identified as *Leuconostoc* sp. (B233). The isolates that did not exhibit any light induction at chill temperatures belong to ten different fingerprints, i.e., *Lact. sakei* (B226, B227, B236, B237, B238, B239 and B241), *Leuc. mesenteroides* (B242), *Leuc. citreum* (B258) and *W. viridescens* (B235). These isolates were recovered mainly from the final stages of meat storage as depicted in Table 3.14, where only a small fraction of isolates recovered from storage at 5 °C displayed induction of luminescence.

3.6 Effect of microbial quorum sensing signals on the growth of spoilage bacteria

This set of experiments was undertaken in order to examine the effect of microbial cell-free culture extract (CFCE), in which QS signal molecules (AHLs and AI-2) were present, on the growth kinetics (lag phase and maximum specific growth rate) of two spoilage bacteria, *Pseudomonas fluorescens* and *Serratia liquefaciens*, held at 10 °C.

AHL signal molecules

Aliquots of CFCE (20% and 50% v/v) from the AHL-producing strain *H. alvei* 718 ($CFCE_{AHL}$) and the AHL-lacking mutant *H. alvei* 718 *halI* ($CFCE_{MUT}$) were transferred to $LB_{glucose}$ broth inoculated with 10^3 CFU mL^{-1} of 16 h cultures of *Pseud. fluorescens* and *Ser. liquefaciens*. $LB_{glucose}$ (0% v/v CFCE) and $CFCE_{MUT}$ served as controls. Moreover, both $CFCE_{AHL}$ and $CFCE_{MUT}$ were tested for presence of AHL signal molecules using the well diffusion bioassay. The AHL signal molecules in the $CFCE_{AHL}$ were also checked for its stability over 72 h at 10 °C, and the $CFCE_{AHL}$ and $CFCE_{MUT}$ metabolic profiles (organic acids) were determined using the HPLC method presented below.

The viable count growth curves for each treatment of *Pseud. fluorescens* and *Ser. liquefaciens*, enumerated by standard plate counting, are presented in Figures 3.15 and 3.16, respectively. Experimental data fitted well with the Baranyi model, as judged by the small standard error of fit and high coefficients of determination ($R^2 > 0.97$). Maximum specific growth rates (μ_{max}), lag-phase durations (lag), initial and final counts of bacteria are presented in Tables 3.16 and 3.17.

The growth rate of *Pseud. fluorescens* in 0% (v/v) CFCE treatment was 0.3486 h^{-1}. The presence of $CFCE_{AHL}$ and $CFCE_{MUT}$ affected the growth of *Pseud. fluorescens*. The addition of 20 and 50% v/v $CFCE_{AHL}$ resulted in growth rate increase of *Pseud. fluorescens*, compared to the negative controls, 20 and 50% (v/v) $CFCE_{MUT}$, respectively. Similar trend has been occurred in the lag-phase durations (Table 3.16). On the other hand, the growth rate of *Ser. liquefaciens* in 0% (v/v) CFCE treatment was 0.2405 h^{-1}. The addition of $CFCE_{AHL}$ (20 and 50% v/v) did not affect the growth rate of *Ser. liquefaciens*, compared to the negative controls (20 and 50% v/v $CFCE_{MUT}$) ($P > 0.05$). It is worth noting, however, that the addition in both treatments of $CFCE_{AHL}$ (20 and 50% v/v) resulted in lag-phase reduction (Table 3.17).

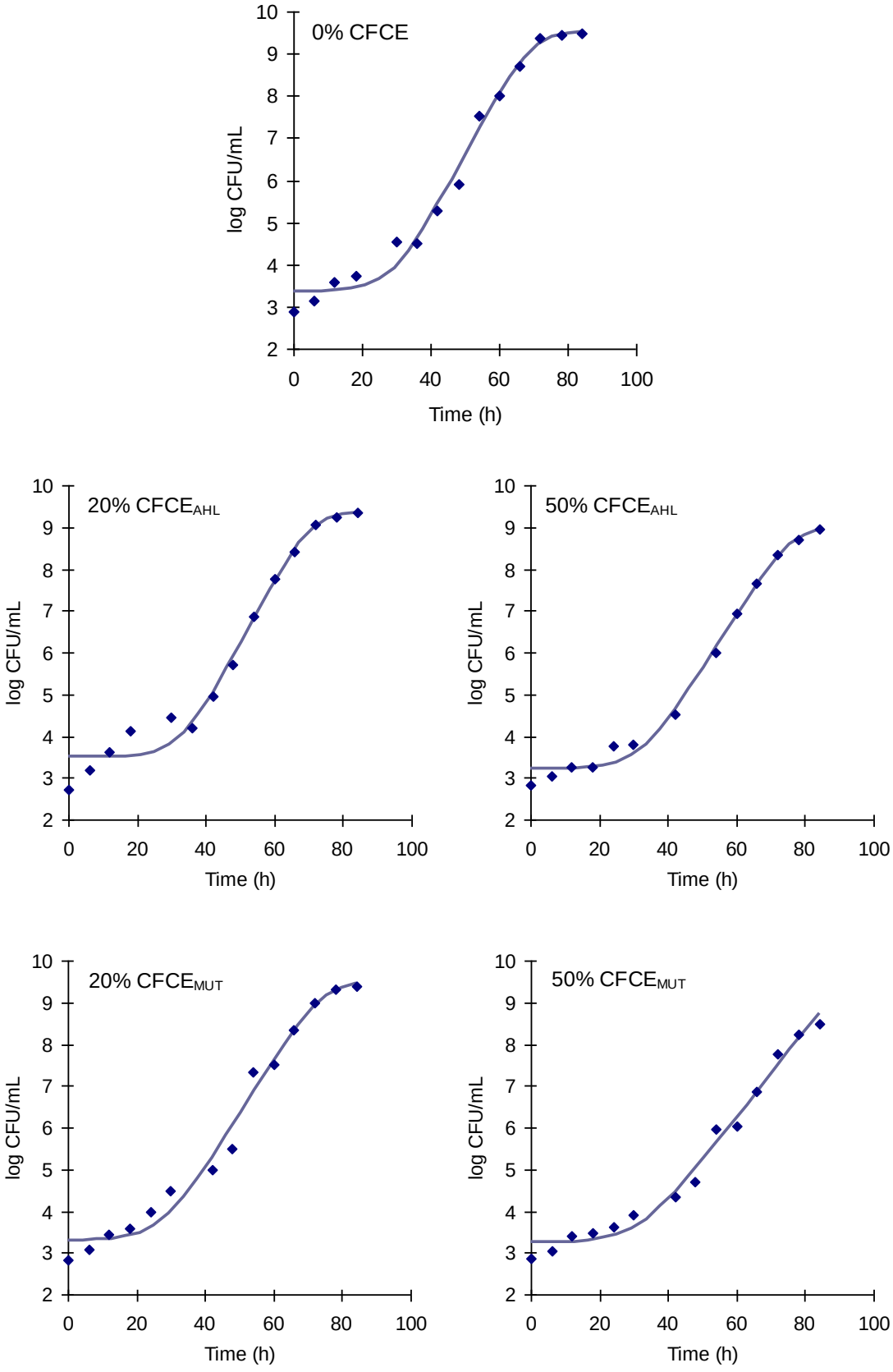

Figure 3.15. Growth of *Pseudomonas fluorescens* in 0, 20 and 50% (v/v) CFCE derived from the AHL-producing strain *Hafnia alvei* 718 ($CFCE_{AHL}$), and the AHL-lacking mutant *H. alvei* 718 *halI* ($CFCE_{MUT}$). Growth curves are fitted with the Baranyi model.

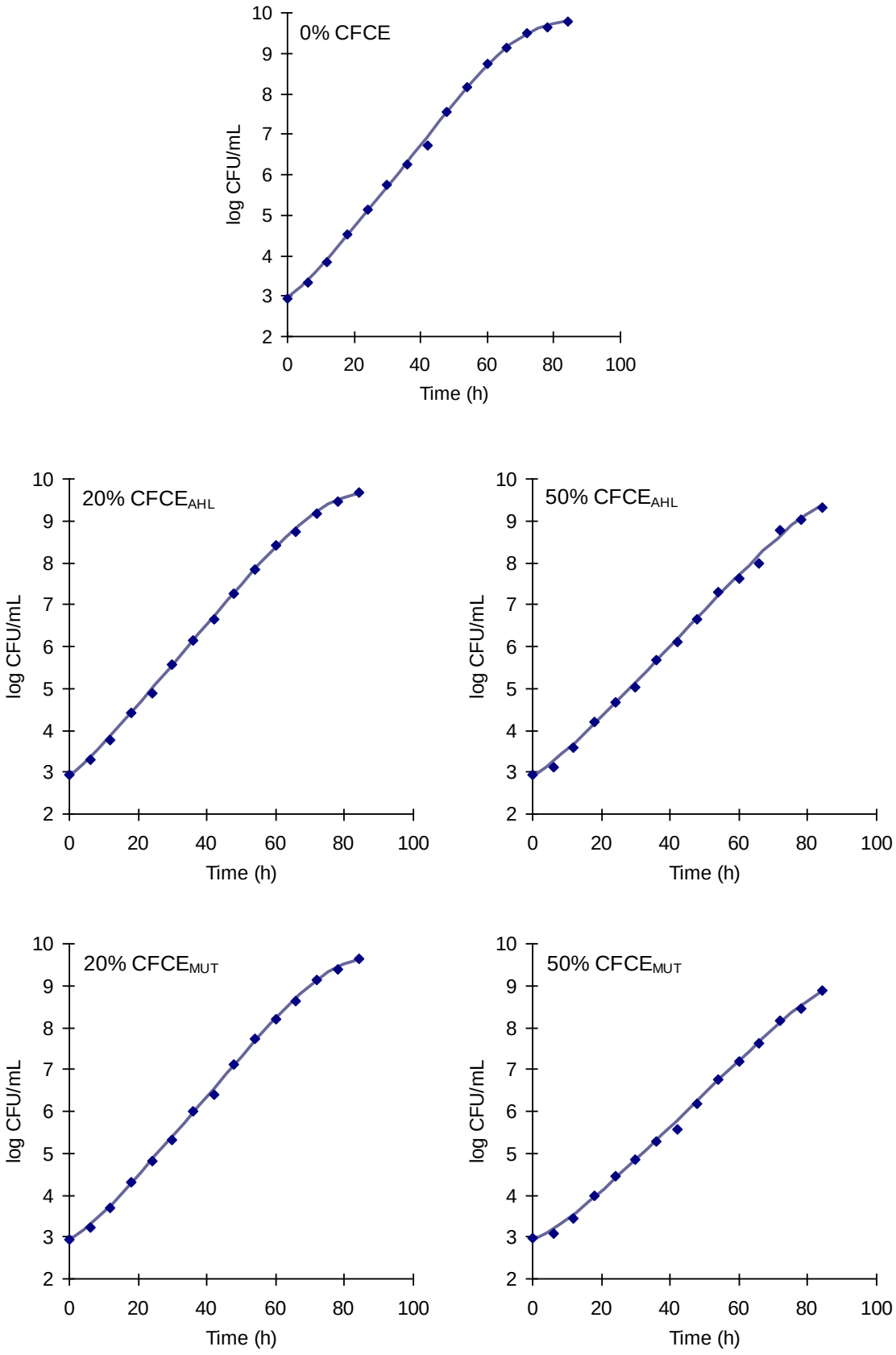

Figure 3.16. Growth of *Serratia liquefaciens* in 0, 20 and 50% (v/v) CFCE derived from the AHL-producing strain *Hafnia alvei* 718 ($CFCE_{AHL}$), and the AHL-lacking mutant *H. alvei* 718 *halI* ($CFCE_{MUT}$). Growth curves are fitted with the Baranyi model.

Table 3.16. The effect of CFCE derived from the AHL-producing strain *Hafnia alvei* 718 and the AHL-lacking mutant *H. alvei* 718 *halI* on the growth kinetic parameters of *Pseudomonas fluorescens* estimated by the Baranyi model

Treatment	$\mathbf{N_0}$ **log CFU mL^{-1}**	$\mathbf{y_{end}}$ **($\mathbf{N_{end}}$) log CFU mL^{-1}**	**lag (hours)**	μ_{max} **(hours^{-1})**	$\mathbf{R^2}$
0% CFCE	3.37±0.05	9.53±0.03 (9.48)	28.80±1.42	0.3486±0.0132	0.9832±0.0071
20% $CFCE_{AHL}$	3.50±0.14	9.36±0.01 (9.36)	32.65±1.01	0.3542±0.388	0.9741±0.0169
50% $CFCE_{AHL}$	3.22±0.01	9.03±0.03 (8.95)	32.13±1.11	0.3034±0.0029	0.9909±0.0000
20% $CFCE_{MUT}$	3.30±0.03	9.52±0.02 (9.40)	27.34±2.69	0.3053±0.0652	0.9784±0.0102
50% $CFCE_{MUT}$	3.25±0.04	ND (8.48)	31.23±0.56	0.2392±0.0011	0.9797±0.0026

$CFCE_{AHL}$: Cell-free culture extract obtained from the AHL-producing strain *H. alvei* 718
$CFCE_{MUT}$: Cell-free culture extract obtained from the AHL-lacking mutant *H. alvei* 718 *halI*
$\mathbf{N_0}$: initial cell number; $\mathbf{y_{end}}$: final cell number estimated by the Baranyi model; $\mathbf{N_{end}}$: estimated experimentally; **lag**: lag-phase duration; μ_{max}: maximum specific growth rate; **ND**: not determined
Values are presented as mean ± standard deviation (n=4).

Table 3.17. The effect of CFCE derived from the AHL-producing strain *Hafnia alvei* 718 and the AHL-lacking mutant *H. alvei* 718 *halI* on the growth kinetic parameters of *Serratia liquefaciens* estimated by the Baranyi model

Treatment	$\mathbf{N_0}$ **log CFU mL^{-1}**	$\mathbf{y_{end}}$ **($\mathbf{N_{end}}$) log CFU mL^{-1}**	**lag (hours)**	μ_{max} **(hours^{-1})**	$\mathbf{R^2}$
0% CFCE	2.93±0.04	9.85±0.01 (9.78)	3.70±1.37	0.2405±0.0021	0.9989±0.0008
20% $CFCE_{AHL}$	2.86±0.02	9.79±0.01 (9.69)	2.39±0.73	0.2224±0.0009	0.9993±0.0004
50% $CFCE_{AHL}$	2.87±0.01	9.92±0.05 (9.33)	4.05±0.30	0.1991±0.0008	0.9977±0.0005
20% $CFCE_{MUT}$	2.91±0.04	9.80±0.09 (9.63)	4.89±2.19	0.2236±0.0104	0.9993±0.0008
50% $CFCE_{MUT}$	2.90±0.02	9.64±0.02 (8.87)	6.47±0.46	0.1844±0.050	0.9982±0.0000

$CFCE_{AHL}$: Cell-free culture extract obtained from the AHL-producing strain *H. alvei* 718
$CFCE_{MUT}$: Cell-free culture extract obtained from the AHL-lacking mutant *H. alvei* 718 *halI*
$\mathbf{N_0}$: initial cell number; $\mathbf{y_{end}}$: final cell number estimated by the Baranyi model; $\mathbf{N_{end}}$: estimated experimentally; **lag**: lag-phase duration; μ_{max}: maximum specific growth rate
Values are presented as mean ± standard deviation (n=4).

AI-2 signal molecules

Cell-free culture extract (20% and 50% v/v) from the AI-2 producer *Salmonella* Typhimurium 4/74 ($CFCE_{AI2}$) and heat treated CFCE from the same strain ($CFCE_{HT}$) were transferred to $LB_{glucose}$ broth inoculated with 10^3 CFU mL^{-1} of 16 h cultures of *Pseud. fluorescens* and *Ser. liquefaciens*. Heat treatment has been previously shown to destroy the AI-2 activity (Surette & Bassler, 1998). A portion of $CFCE_{AI2}$ was heat inactivated using autoclaving conditions (121 °C and 15 min) and this preparation was termed as $CFCE_{HT}$. $LB_{glucose}$ (0% v/v CFCE) and $CFCE_{HT}$ were served as controls. AI-2 activity in both the $CFCE_{AI2}$ and $CFCE_{HT}$ was checked using the AI-2 activity bioassay. AI-2 activity presented in the $CFCE_{AI2}$ and $CFCE_{HT}$ was checked for its stability over 96 h at 10 °C. The organic acid metabolic profile of the $CFCE_{AI2}$ and $CFCE_{HT}$ was also determined using HPLC, as presented below.

The viable count growth curves of *Pseud. fluorescens* and *Ser. liquefaciens*, enumerated by standard plate counting are presented in Figures 3.17 and 3.18, respectively. Viable counts (transformed to $\log_{10}$ values) were fitted to the Baranyi model in order to estimate the maximum specific growth rates (μ_{max}), the lag-phase durations (lag) and the final counts of bacteria.

The growth rates of *Pseud. fluorescens* and *Ser. liquefaciens* in 0% (v/v) CFCE were 0.2598 and 0.2317 h^{-1}, respectively. Addition of 20% (v/v) $CFCE_{AI2}$ and $CFCE_{HT}$ affected the growth of both *Pseud. fluorescens* and *Ser. liquefaciens*. Presence of 20% (v/v) $CFCE_{AI2}$ resulted in growth rate and lag-phase reductions of both examined bacteria (compared to controls $CFCE_{HT}$), whereas no significant growth was observed using 50% (v/v) $CFCE_{AI2}$ (Table 3.18 and 3.19).

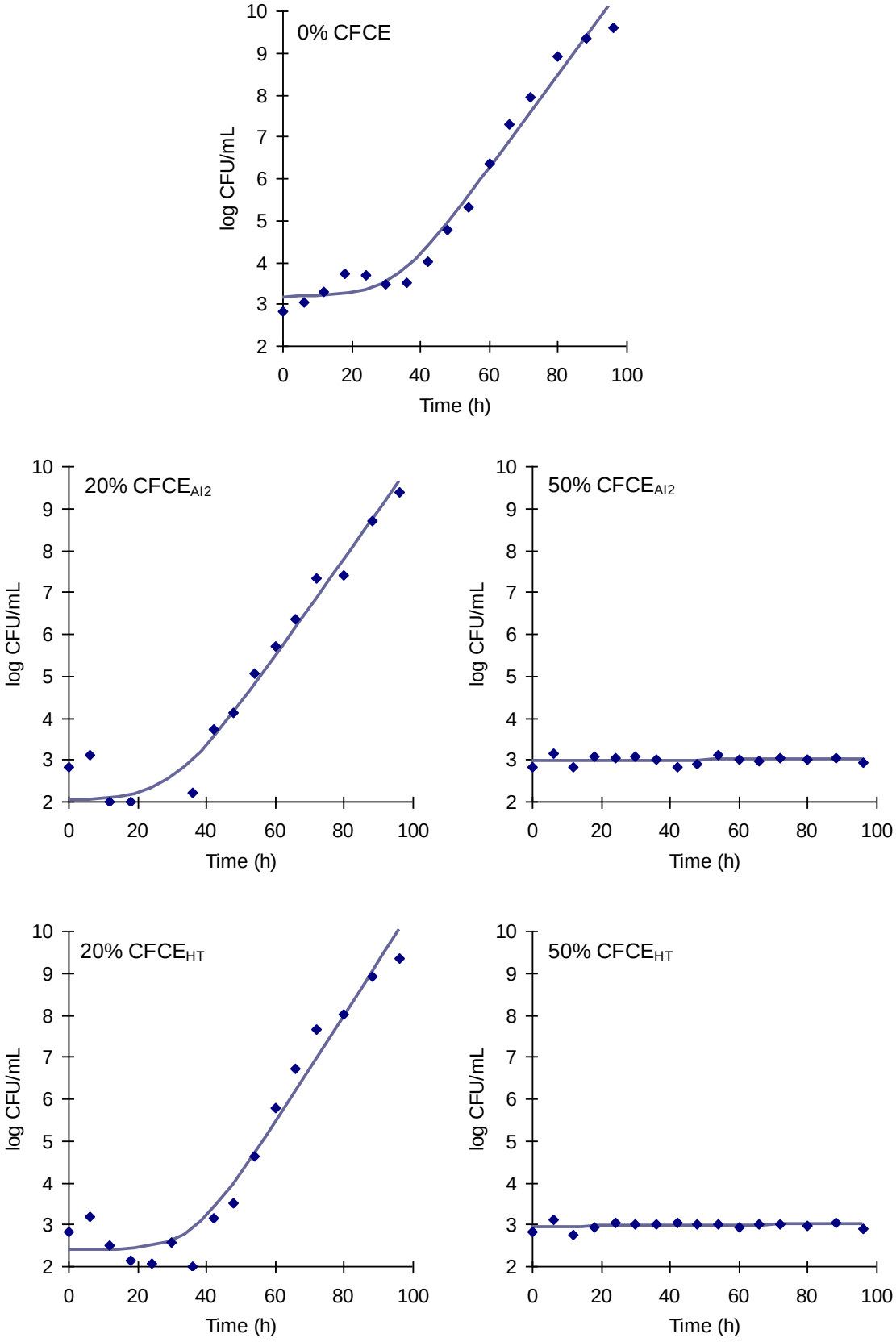

Figure 3.17. Growth of *Pseudomonas fluorescens* in 0, 20 and 50% (v/v) CFCE derived from the AI-2 producer *Salmonella* Typhimurium 4/74 ($CFCE_{AI2}$), and heat treated CFCE from the same *Salm.* Typhimurium strain ($CFCE_{HT}$). Growth curves are fitted with the Baranyi model.

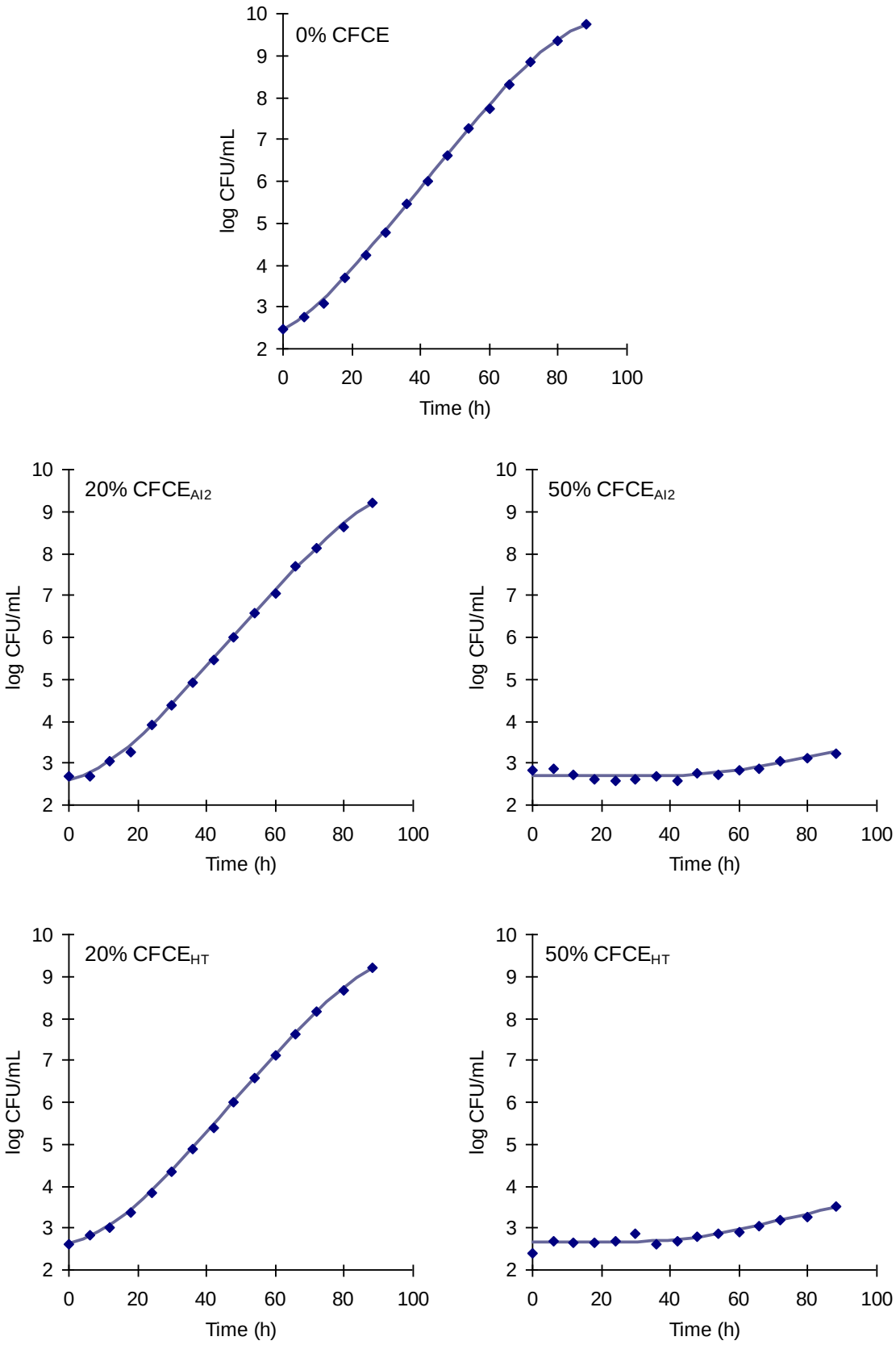

Figure 3.18. Growth of *Serratia liquefaciens* in 0, 20 and 50% (v/v) CFCE derived from the AI-2 producer *Salmonella* Typhimurium 4/74 ($CFCE_{AI2}$), and heat treated CFCE from the same *Salm.* Typhimurium strain ($CFCE_{HT}$). Growth curves are fitted with the Baranyi model.

Table 3.18. The effect of CFCE derived from the AI-2 producer *Salmonella* Typhimurium 4/74 and heat treated CFCE from the same *Salm.* Typhimurium strain on the growth kinetic parameters of *Pseudomonas fluorescens* estimated by the Baranyi model

Treatment	N_0 log CFU mL^{-1}	y_{end} (N_{end}) log CFU mL^{-1}	lag (hours)	μ_{max} ($hours^{-1}$)	R^2
0% CFCE	2.82±0.05	ND (9.61)	33.10±2.03	0.2598±0.0046	0.9744±0.0080
20% $CFCE_{AI2}$	2.83±0.04	ND (9.40)	31.03±0.08	0.2694±0.0012	0.9452±0.0114
50% $CFCE_{AI2}$	2.84±0.10	ND (2.92)	ND	0.0005±0.0002	ND
20% $CFCE_{HT}$	2.84±0.06	ND (9.36)	36.78±1.35	0.2969±0.0139	0.9569±0.0024
50% $CFCE_{HT}$	2.82±0.01	ND (2.90)	ND	0.0015±0.0004	ND

$CFCE_{AI2}$: Cell-free culture extract obtained from the AI-2-producing strain *Salm.* Typhimurium 4/74
$CFCE_{HT}$: Heat treated cell-free culture extract obtained from the AI-2-producing strain *Salm.* Typhimurium. Heat treatment (autoclaving) inactivate AI-2 activity
N_0: initial cell number; **y_{end}**: final cell number estimated by the Baranyi model; **N_{end}**: estimated experimentally; **lag**: lag-phase duration; **μ_{max}**: maximum specific growth rate; **ND**: not determined
Values are presented as mean ± standard deviation (n=4).

Table 3.19. The effect of CFCE derived from the AI-2 producer *Salmonella* Typhimurium 4/74 and heat treated CFCE from the same *Salm.* Typhimurium strain on the growth kinetic parameters of *Serretia liquefaciens* estimated by the Baranyi model

Treatment	N_0 log CFU mL^{-1}	y_{end} (N_{end}) log CFU mL^{-1}	lag (hours)	μ_{max} ($hours^{-1}$)	R^2
0% CFCE	2.48±0.00	9.99±0.07 (9.74)	6.49±1.38	0.2317±0.0063	0.9996±0.0002
20% $CFCE_{AI2}$	2.70±0.07	9.63±0.03 (9.22)	11.06±0.69	0.2134±0.0018	0.9988±0.0002
50% $CFCE_{AI2}$	2.81±0.09	ND (3.23)	52.66±0.05	0.0364±0.0003	0.8023±0.0022
20% $CFCE_{HT}$	2.60±0.00	9.56±0.05 (9.20)	12.21±1.32	0.2162±0.0085	0.9997±0.0001
50% $CFCE_{HT}$	2.39±0.13	ND (3.51)	45.01±0.13	0.0444±0.0018	0.8796±0.0399

$CFCE_{AI2}$: Cell-free culture extract obtained from the AI-2-producing strain *Salm.* Typhimurium 4/74
$CFCE_{HT}$: Heat treated cell-free culture extract obtained from the AI-2-producing strain *Salm.* Typhimurium. Heat treatment (autoclaving) inactivate AI-2 activity
N_0: initial cell number; **y_{end}**: final cell number estimated by the Baranyi model; **N_{end}**: estimated experimentally; **lag**: lag-phase duration; **μ_{max}**: maximum specific growth rate; **ND**: not determined
Values are presented as mean ± standard deviation (n=4).

AHL induction

Aliquots of CFCE derived from the AHL-producing strain *H. alvei* 718 ($CFCE_{AHL}$) and the AHL-lacking mutant *H. alvei* 718 *halI* ($CFCE_{MUT}$) were tested for AHL-production using the *A. tumefaciens* A136 biosensor strain, which detects a broad range of AHLs (see details in CHAPTER 2: Materials and Methods). $CFCE_{AHL}$ induced *A. tumefaciens* A136, and a blue circle was observed around the well (Figure 3.19). Growth medium ($LB_{glucose}$) supplemented with 0, 20 and 50% (v/v) $CFCE_{AHL}$ were also tested for the presence of AHLs (Figure 3.20). Sample with 0% (v/v) CFCE was used as control and did not induce *A. tumefaciens* A136, whereas samples with 20 and 50% (v/v) $CFCE_{AHL}$ induced the biosensor strain exhibiting different induction area (diameter) (Figure 3.20).

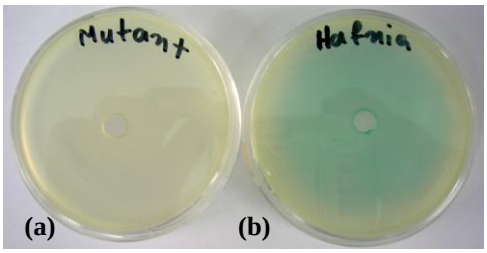

Figure 3.19. Detection of AHLs in CFCE derived from **(a)** the AHL-lacking mutant *Hafnia alvei* 718 *halI*, and **(b)** the AHL-producing strain *H. alvei* 718, using the *A. tumefaciens* A136 biosensor strain.

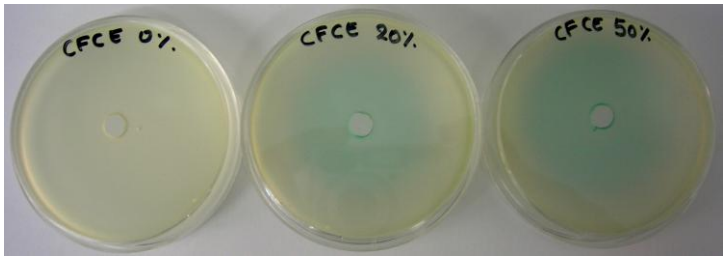

Figure 3.20. Detection of AHLs in growth medium ($LB_{glucose}$) supplemented with 0, 20 and 50% (v/v) CFCE derived from the AHL-producing strain *Hafnia alvei* 718, using the *A. tumefaciens* A136 biosensor strain.

The $CFCE_{AHL}$ was stored at 10 °C and tested at periodic time intervals (0, 24, 48 and 72 h) to check its ability to induce *A. tumefaciens* A136 biosensor strain (presence of AHLs). $CFCE_{AHL}$ exhibited the same level of induction (induction area) at all time points, ensuring that AHL signal molecules were stable at the tested temperature (Figure 3.21).

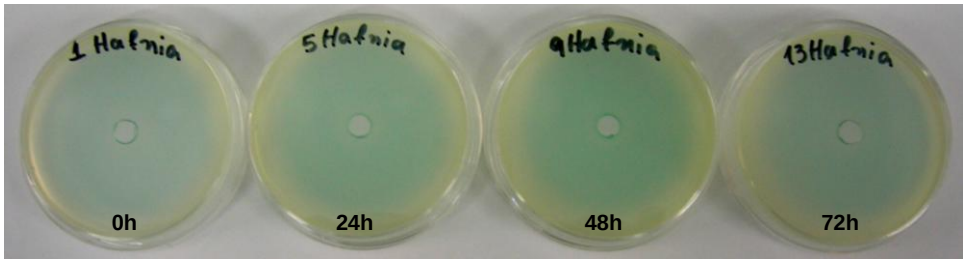

Figure 3.21. Detection of AHLs during storage of CFCE derived from the AHL-producing strain *Hafnia alvei* 718 at 10°C for 72 h, using the *A. tumefaciens* A136 biosensor strain.

During the growth of *Pseud. fluorescens* and *Ser. liquefaciens*, in the presence of $CFCE_{AHL}$ and $CFCE_{MUT}$, samples were taken at periodic time intervals and tested for AHL induction. The induction level (induction area) was similar among the same treatments and during all time points as presented in Figures 3.22 and 3.23.

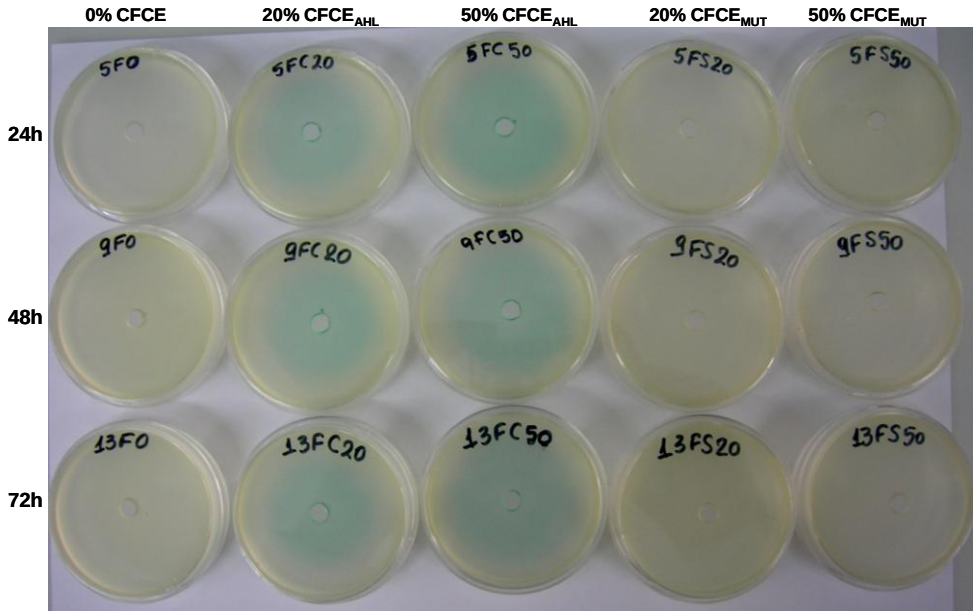

Figure 3.22. AHL induction of samples collected during the growth of *Pseudomonas fluorescens*, when supplemented with 0, 20 and 50% (v/v) CFCE derived from the AHL-producing strain *Hafnia alvei* 718 ($CFCE_{AHL}$), and the AHL-lacking mutant *H. alvei* 718 *halI* ($CFCE_{MUT}$), using the *A. tumefaciens* A136 biosensor strain.

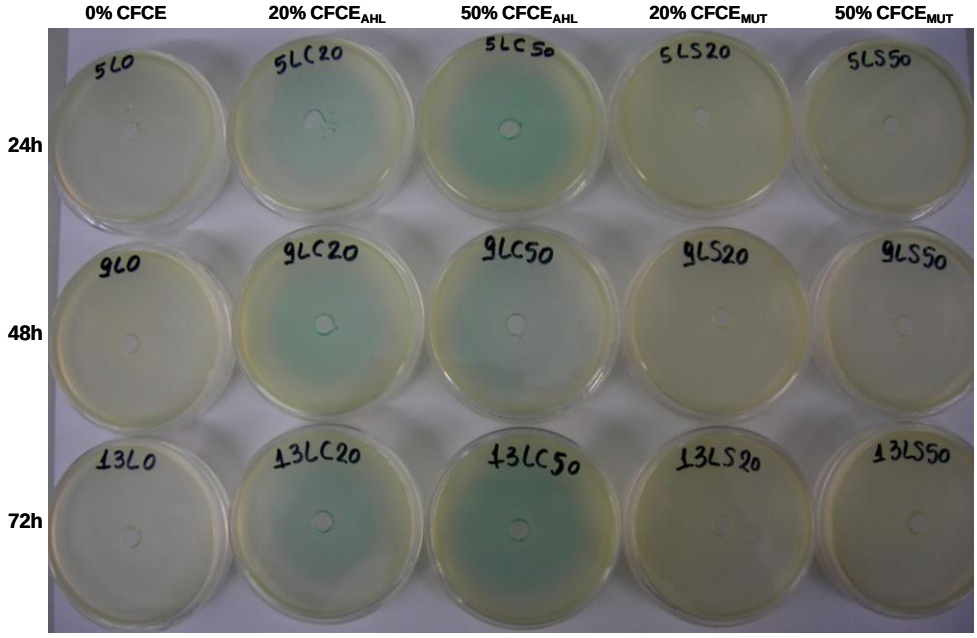

Figure 3.23. AHL induction of samples collected during the growth of *Serratia liquefaciens*, when supplemented with 0, 20 and 50% (v/v) CFCE derived from the AHL-producing strain *Hafnia alvei* 718 ($CFCE_{AHL}$), and the AHL-lacking mutant *H. alvei* 718 *halI* ($CFCE_{MUT}$), using the *A. tumefaciens* A136 biosensor strain.

AI-2 activity

AI-2 activity in the CFCE derived from the AI-2 producer *Salm.* Typhimurium 4/74 ($CFCE_{AI2}$) and the heat treated CFCE derived from the same *Salm.* Typhimurium strain ($CFCE_{HT}$) was checked using the AI-2 activity bioassay. In Figure 3.24 the relative AI-2 activity, which was calculated as the ratio of luminescence of the test sample to the control (negative) sample, of the tested $CFCE_{AI2}$ and $CFCE_{HT}$ is presented. Sterile growth medium ($LB_{glucose}$) and CFCE derived from the AI-2-producing strain *V. harveyi* BAA-1119 were used as negative and positive controls respectively. The $CFCE_{AI2}$ induced luminescence, whereas $CFCE_{HT}$ did not exhibit light induction in *V. harveyi* BAA-1117 biosensor strain.

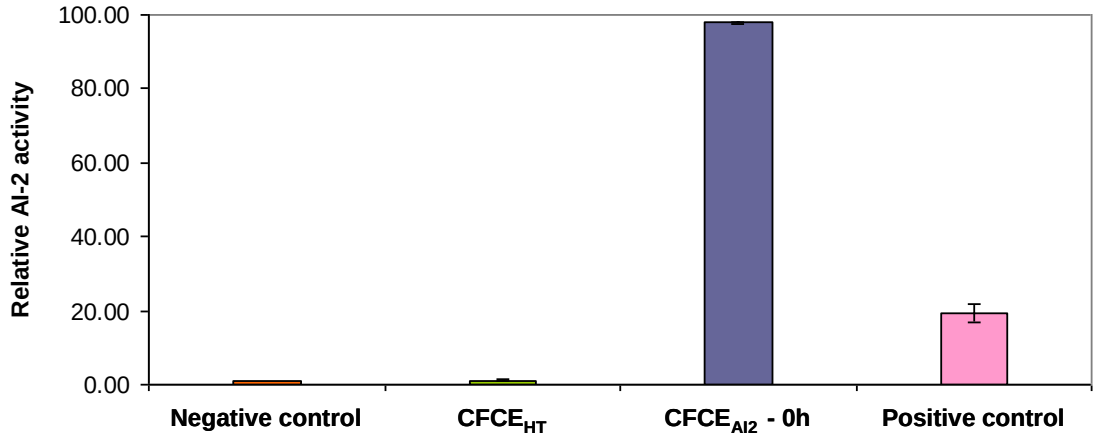

Figure 3.24. Relative AI-2 activity of CFCE derived from the AI-2 producer *Salmonella* Typhimurium 4/74 ($CFCE_{AI2}$), and the heat treated CFCE derived from the same *Salm.* Typhimurium strain ($CFCE_{HT}$). Negative (sterile growth medium) and positive (CFCE from AI-2 producing strain *V. harveyi* BAA-1119) controls were also used.

The $CFCE_{AI2}$ and $CFCE_{HT}$ were stored at 10 °C and tested at periodic time intervals (0, 24, 48, 72 and 96 h) to check their ability to induce luminescence response in *V. harveyi* BAA-1117 biosensor strain (measure of AI-2 activity).

$CFCE_{AI2}$ exhibited the same level of light induction in *V. harveyi* BAA-1117 biosensor during all time points, ensuring that AI-2 signal molecules were stable at the tested temperature ($P > 0.05$). On the other hand, when $CFCE_{HT}$ was used, no induction of luminescence was observed at any time point. AI-2 activity expressed in relation to that of the negative control is presented in Figure 3.25.

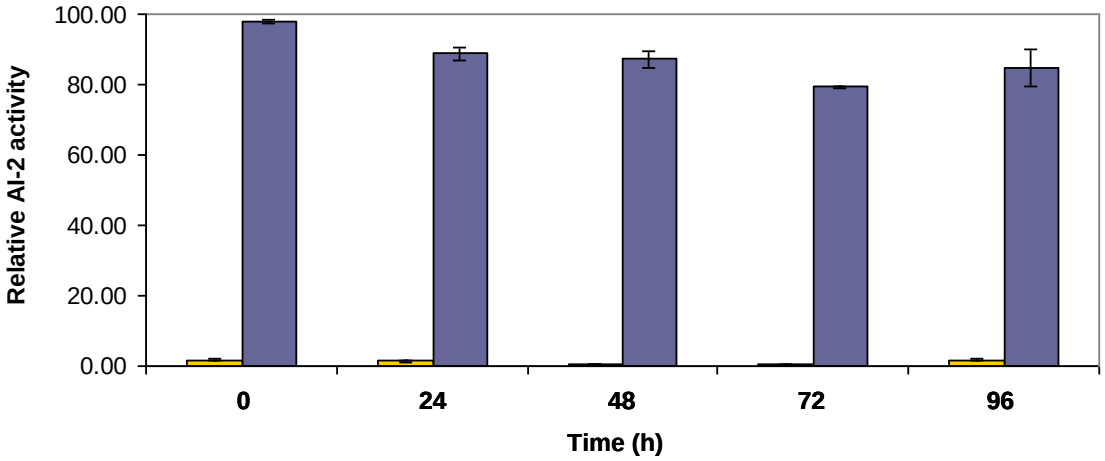

Figure 3.25. Relative AI-2 activity of (■) CFCE derived from the AI-2 producer *Salmonella* Typhimurium 4/74, and (■) heat treated CFCE derived from the same *Salm.* Typhimurium strain, stored at 10 °C for 96 h.

During the growth of *Pseud. fluorescens* and *Ser. liquefaciens*, in the presence of 0, 20 and 50% (v/v) $CFCE_{AI2}$, samples were taken at periodic time intervals and tested for AI-2 activity. The level of light induction in *V. harveyi* BAA-1117 biosensor strain, expressed as relative AI-2 activity, was similar among the same treatments and during all time points. Concerning *Pseud. fluorescens*, samples supplemented with 0% (v/v) CFCE did not exhibit AI-2 activity when compared to the negative control, whereas the relevant samples of *Ser. liquefaciens* exhibited AI-2 activity after 72 h of incubation at 10 °C (Figures 3.26 and 3.27).

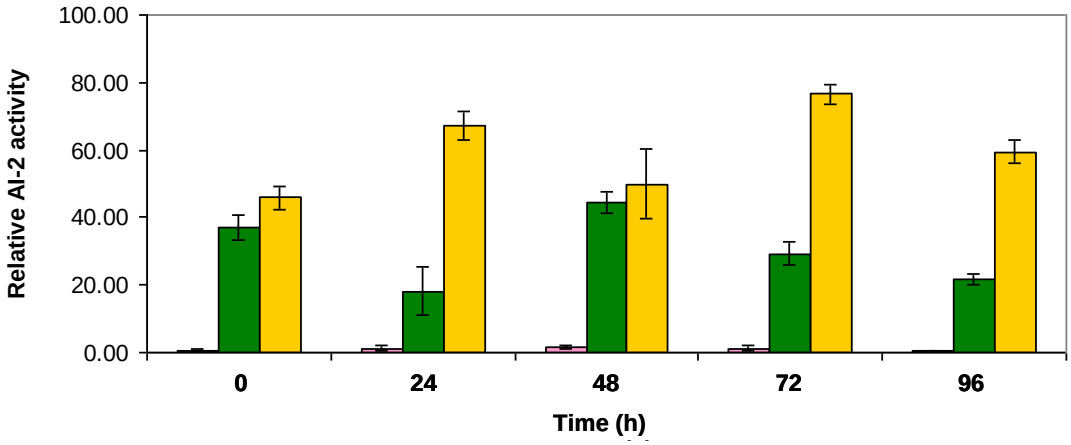

Figure 3.26. Relative AI-2 activity of samples collected during the growth of *Pseudomonas fluorescens* when supplemented with (■) 0, (■) 20 and (■) 50% (v/v) $CFCE_{AI2}$ derived from the AI-2 producer *Salmonella* Typhimurium 4/74.

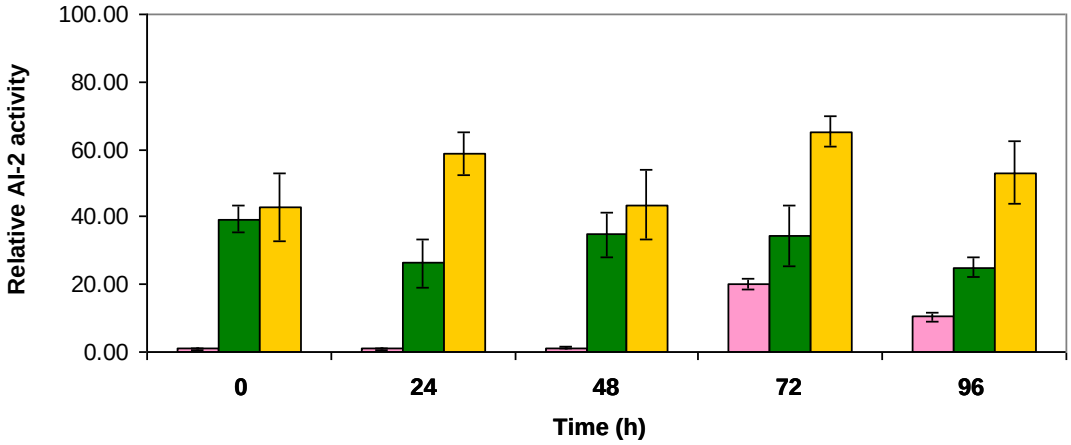

Figure 3.27. Relative AI-2 activity of samples collected during the growth of *Serratia liquefaciens* when supplemented with (■) 0, (■) 20 and (■) 50% (v/v) $CFCE_{AI2}$ derived from the AI-2 producer *Salmonella* Typhimurium 4/74.

HPLC metabolic profiles

Cell-free culture extract derived from the AHL-producing strain *H. alvei* 718 and the control AHL-lacking mutant *H. alvei* 718 *halI* revealed to exhibited the same metabolic profile of organic acids as determined using the HPLC method and presented in Figure 3.28.

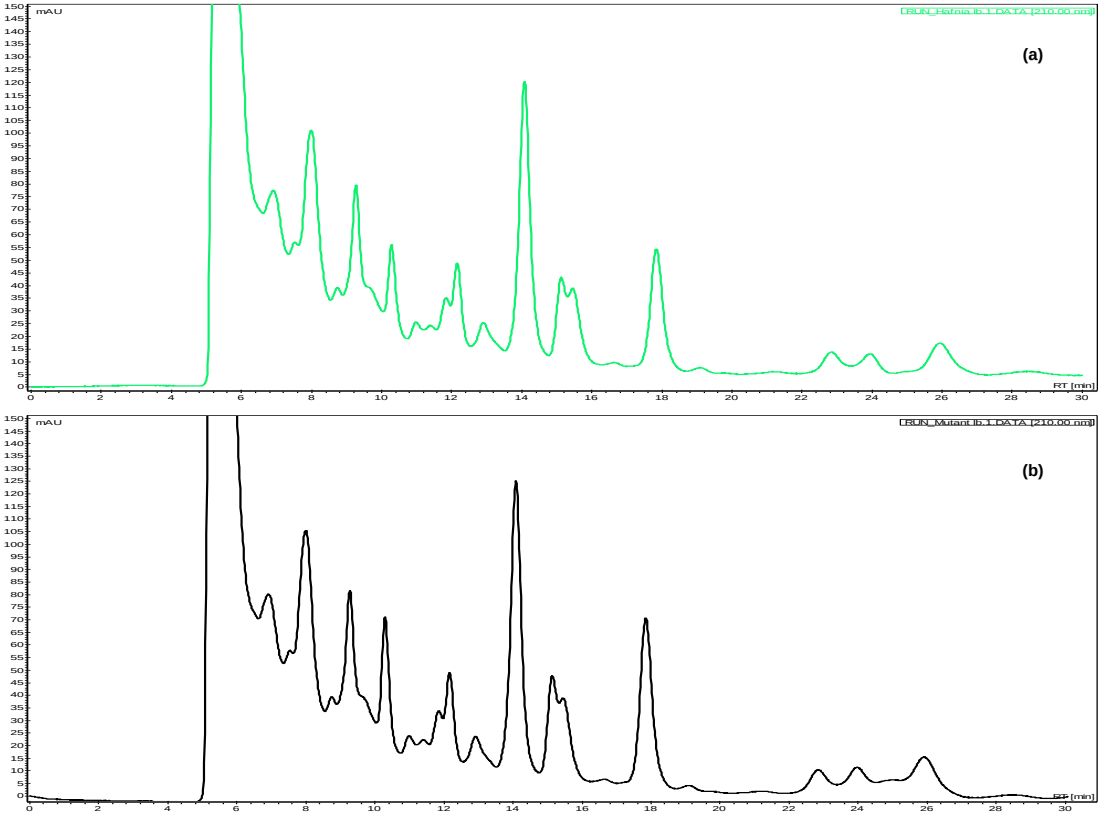

Figure 3.28. HPLC metabolic profiles of **(a)** CFCE derived from the AHL-producing strain *Hafnia alvei* 718, and **(b)** CFCE derived from the AHL-lacking mutant *H. alvei* 718 *halI*.

Cell-free culture extract derived from the AI-2 producer *Salmonella* Typhimurium 4/74 and heat treated CFCE from the same *Salm.* Typhimurium strain exhibited the same metabolic profile of organic acids as determined using the HPLC method and presented in Figure 3.29.

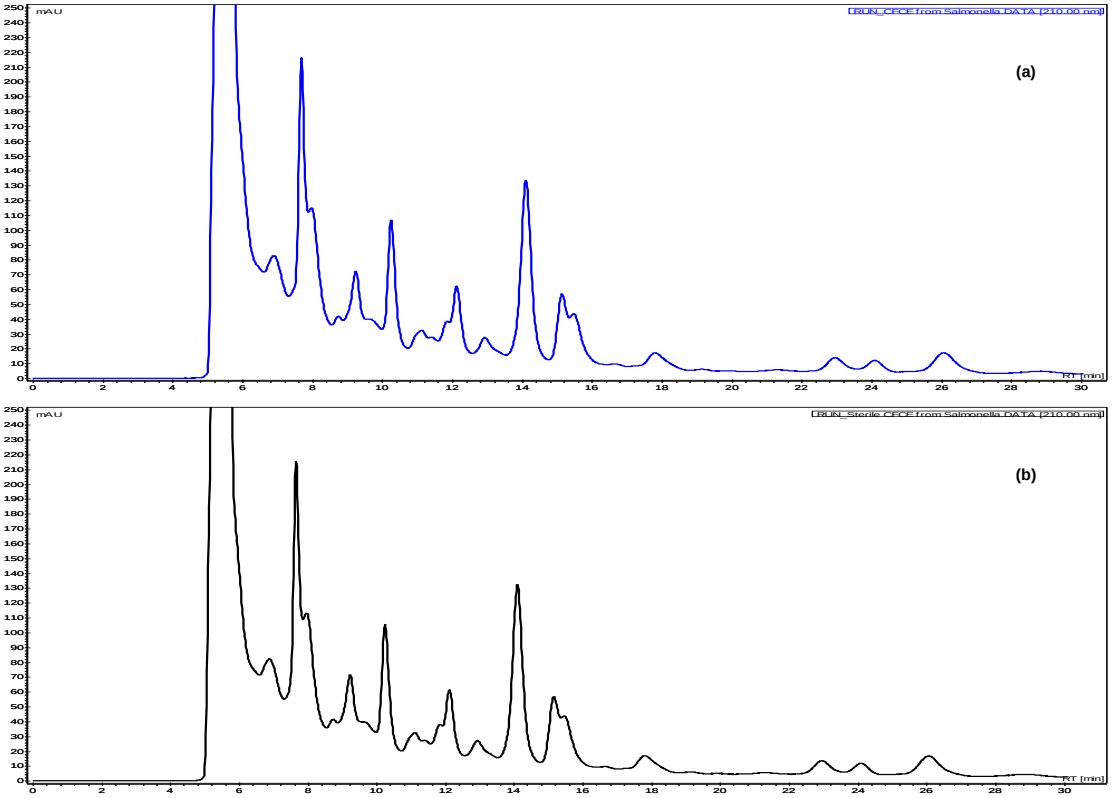

Figure 3.29. HPLC metabolic profiles of **(a)** CFCE derived from the AI-2 producer *Salmonella* Typhimurium 4/74, and **(b)** heat treated CFCE from the same *Salm.* Typhimurium strain.

Chapter 4

Discussion

4.1 Survey of minced beef

Fifty-seven minced beef samples purchased from the Athens area during a seven month period were analysed microbiologically. It was found that *Pseudomonas* spp., *Brochotrix thermosphacta*, lactic acid bacteria (LAB), H_2S-producing bacteria and *Enterobacteriaceae* contributed to the microbial succession. The prevalence of a particular microbial succession on stored meat is affected by various factors persisting during processing, transportation and storage in the market (Nychas *et al.,* 2008).

As presented in Section 3.1, the minced beef samples (32 from supermarkets and 25 from butcher shops) had a total viable count range between 4.18 - 8.17 log CFU g^{-1}. The samples obtained from supermarkets (mean value 6.58 log CFU g^{-1}) exhibited a slightly higher mean microbial population load than butcher shops (mean value 6.30 log CFU g^{-1}). These values were lower when compared with previous studies (Nychas *et al.,* 1991; Tsigarida, 2000). The numbers of *Pseudomonas* spp. were higher in samples obtained both from supermarkets and butcher shops, followed by *Br. thermosphacta* and LAB especially in the supermarkets' samples. This tendency is in accordance with previous observations (Tsigarida, 2000), and may be attributed to the fact that supermarkets manipulate big parts of meat pre-packaged under modified atmospheres, which induce the growth of *Br. thermosphacta* and LAB (Stanbridge & Davis, 1998). The main differences between supermarkets and butcher shops were observed in the numbers of *Enterobacteriaceae* and H_2S-producing bacteria, which appeared lower in butcher shops than supermarkets. *Enterobacteriaceae* can be introduced onto meat from work surfaces, whereas their numbers increase when stored aerobically at chill temperatures. The climatic conditions (warm: May – July and cold: October – January) affected the level of

microbial contamination also. Counts during the warm period were larger than those obtained during the cold period. Nychas *et al.* (1991) have suggested that season is a parameter, except for physicochemical and microbiological ones, that must be taken into account in order to specify the microbial quality of beef meat. All counts obtained in this survey are quite common when compared with initial microbial populations in other studies using minced beef from the Athens area (Nychas & Arkoudelos, 1990; Lambropoulou *et al.,* 1996; Skandamis & Nychas, 2001).

4.2 Microbiological analysis of beef meat

The purpose of the present experiment was to monitor the changes of the spoilage-related microbial flora during the storage of beef at various temperatures (0, 5, 10, 15 and 20 °C). The microbiological analysis revealed that the initial microbial flora of beef pieces consisted of pseudomonads, *Br. thermosphacta*, LAB and *Enterobacteriaceae*. The succession of these groups and their contribution to the final microbiota was greatly influenced by the storage temperature. This was exemplified by the calculated kinetic parameters, i.e., initial and final population, lag phase duration and maximum specific growth rates of the microbial association for each of the storage temperature assayed. Aerobic storage allowed total aerobic counts to reach high levels, with pseudomonads being the dominant microorganism, followed by *Br. thermosphacta* and then LAB. Similar results for meat have been reported in previous studies (Tsigarida *et al.,* 2000; Ercolini *et al.,* 2006). Pseudomonads, *Br. thermosphacta*, LAB and *Enterobacteriaceae* had the highest growth rate at 20 °C and the longest lag-phase at 0 °C. Generally the growth rate of all microorganisms increased with increasing temperature. This was also reported by Liu *et al*. (2006), in

pork stored aerobically and under modified atmosphere at three different temperatures (-2, 4 and 10 °C). Such changes have previously been found in microbial successions in meat (Skandamis & Nychas, 2002).

The shelf life of beef decreased with increasing storage temperature. The type of muscle spoilage was characterized by putrefaction which is related to proteolytic activity and off-odour production by Gram-negative bacteria that dominate. Generally, the sensory changes are related to the composition and population of the microbial association and the type and availability of energy substrates in meat (Tassou & Nychas, 1997; Nychas *et al.*, 2008).

The pH changes at the beginning of the storage were within the normal range for fresh beef (Borch *et al.*, 1996). An increase in pH values was evident for all samples throughout storage. It has been reported in previous studies that meat pH affects the growth kinetics of pseudomonads, *Br. thermosphacta* and *Enterobacteriaceae* (Koutsoumanis *et al.*, 2006). This could be supported by the fact that in meat, small differences in pH can be translated into significant differences in lactate concentration and thus affect the growth of pseudomonads which are sensitive to lactic acid (Lowe *et al.*, 2004; Nakai & Siebert, 2004). In contrast, pH did not affect the growth kinetics of LAB due to their well established higher acid tolerance compared to other spoilage bacteria (Koutsoumanis *et al.*, 2004).

4.3 Detection of Quorum Sensing signals during minced beef spoilage

It was recently shown that QS signal molecules (AHLs and AI-2) can be found in a wide range of foods i.e., fish, meat and vegetable products. These signal

molecules have been suggested to be produced by certain members of the microbial association (Gram *et al.,* 1999; Cloak *et al.,* 2002; Bruhn *et al.,* 2004; Lu *et al.,* 2004; Lu *et al.,* 2005; Liu *et al.,* 2006; Nychas *et al.,* 2009). However, the role of QS and factors affecting QS signal molecule production are not very clear, since available data are scarce. Meanwhile, several phenotypes (pectinolyitc, lipolytic, proteolytic and chitinolytic activities) potentially involved in spoilage of different foods have been linked to AHL regulation in several bacteria (Nychas *et al.,* 2008). The contribution of QS in the spoilage process of fresh meat products stored under aerobic refrigerated conditions, and in the biofilm formation appearing as slime at their surfaces has been suggested (Jay *et αl.,* 2003). Furthermore, AHL production has been detected and appears concomitantly with proteolytic activity in aerobically chill-stored ground beef, fish, chicken and milk (Liu *et αl.,* 2006).

In this study the microbiota throughout the aerobic storage of minced beef was dominated by pseudomonads at all tested temperatures as found previously (Tsigarida *et al.,* 2000; Skandamis & Nychas, 2001; Ercolini *et al.,* 2009). Under these conditions, the tested samples (CFME) induced AHL biosensor strains when pseudomonas and *Enterobacteriaceae* populations ranged between 10^8 and 10^9 CFU g^{-1}, populations significantly higher than the detection threshold of 10^5 to 10^6 CFU g^{-1} previously reported for vacuum-packaged cold-smoked salmon (Gram *et al.,* 1999). This observation is in contrast to previous work reporting that the earliest detectable AHL signals appeared when pseudomonad and *Enterobacteriaceae* concentrations were approximately 10^8 to 10^9 CFU g^{-1} and 10^3 to 10^4 CFU g^{-1} respectively (Liu *et al.,* 2006). Considering that AHL production is common among *Enterobacteriaceae* isolated from foods and that pseudomonads produce undetectable quantities of AHL molecules using biosensor strains, it is suggested that the AHL signals detected in the

minced beef stored aerobically were mainly produced by *Enterobacteriaceae* (Gram *et al.*, 1999; Ferrocino *et al.*, 2009).

On the other hand, the growth of all members of the microbial association was delayed when minced beef was packaged under modified atmospheres. Under these packaging conditions the dominance of *Br. thermosphacta* and LAB was favoured. These microorganisms belong to the Gram-positive bacteria, which do not produce AHL signals commonly found in numerous Gram-negative bacteria (Whitehead *et al.*, 2001). Gram-positive bacteria produce AI-2 and/or autoinducing peptides which do not trigger the AHL biosensor strains (Sturme *et al.*, 2002; Schaefer *et al.*, 2008). AHL signals were detected at the very final storage period of beef stored at relatively high temperatures (10 and 15 °C), when both pseudomonads and *Enterobacteriaceae* concentrations were approximately 10^7 CFU g^{-1}, concentrations considerably lower than those reported previously for the same meat samples stored aerobically. Accordingly, in chill-stored vacuum-packed meat the microbiota typically consists of *Enterobacteriaceae* and LAB at levels of 10^6 and 10^8 CFU g^{-1}, respectively, suggesting that the spoilage is a result of an interaction between *Enterobacteriaceae* and LAB (Bruhn *et al.,* 2004).

Packaging under modified atmospheres in the presence of volatile compounds of oregano essential oil resulted in lower counts of all the members of the microbial association, with exception of LAB, compared with the samples stored under modified atmospheres. None of the samples (CFME) collected from this packaging condition induced any of the AHL biosensor strains used in this study, which can be attributed to the fact that under modified atmospheres, and even more when volatile compounds of oregano essential oil were present, the inhibition of *Enterobacteriaceae*

was observed. Similar studies with fish and beef showed that modified atmosphere packaging acts synergistically with the essential oil, since only a selected proportion of microbiota, when compared to aerobic storage is allowed to develop (Skandamis & Nychas, 2001). Additionally, various natural and synthetic compounds, including plant-derived essential oils, not only inhibit bacterial growth but affect QS (Rasmussen *et al.,* 2005; Choo *et al.,* 2006; Szabó *et al.,* 2010). More significantly, among the tested oils, rose, geranium, lavender and rosemary oils inhibited QS, while oregano essential oil was unable to modify QS, at least in the laboratory environment (Szabó *et al.,* 2010). All these findings suggest that AHL signal production in minced beef is modulated by the indigenous microbial association, whereas the presence of volatile compounds of oregano essential oil has no inhibiting effect on this QS-phenomenon.

In this study, various biosensor strains responding to AHLs with different structural features were used, in order to better estimate the total AHL content of the tested CFME samples. The induction of *A. tumefaciens* A136 and *E. coli* JM109 (pSB401) was similar among the same tested CFME samples, because these biosensor strains have similar detection specificities and both can detect a broad range of medium-side-chain AHLs (Liu *et al.,* 2006). Moreover, none of the samples induced the biosensor strains *C. violaceum* CV026 and *V. harveyi* BAA-1118 at any time during storage at all tested conditions. *C. violaceum* CV026 is induced by AHLs evaluated with *N*-acyl side chains from C4 and C8 in length (McClean *et al.,* 1997), whereas *V. harveyi* BAA-1118 responds to *N*-(3-hydroxybutanoyl)-L-homoserine lactone (Bassler *et al.,* 1997). Induction of *E. coli* JM109 (pSB536) biosensor was seen on meat extracts collected at the final storage period of aerobically packed minced beef, while *E. coli* JM109 (pSB1075) was induced by samples taken at the

very final storage period of beef stored under modified atmospheres at relatively high temperatures (10 and 15 °C). The *E. coli* JM109 (pSB536) and *E. coli* JM109 (pSB1075) biosensor strains detect short-side-chain and long-side-chain AHLs respectively (Winson *et al.,* 1998). These results suggest that AHLs produced in minced beef stored aerobically and under modified atmospheres were mainly medium-side-chain AHLs.

The TLC analysis performed on ethyl acetate extracts obtained from various CFME samples collected mainly at the final storage periods of minced beef, indicated the presence of *N*-(ß-ketocaproyl)-L-homoserine lactone. These profiles were coupled with the *A. tumefaciens* A136 biosensor strain and compared to AHL standards. The presence of other types of AHLs in CFME samples cannot be excluded, considering that each AHL molecule has a different detection limit (Holden *et al.,* 1999). Liu *et al.* (2006) detected a broad range of AHLs, including *N*-(ß-ketocaproyl)-L-homoserine lactone, in aerobically chill-stored beef. Bruhn and co-workers have only detected an AHL spot similar to *N*-(ß-ketocaproyl)-L-homoserine lactone in vacuum-packed meat, suggesting that it was produced from *H. alvei* strains (dominant members of the *Enterobacteriaceae*), which elicited identical TLC profiles (Bruhn *et al.,* 2004).

The low levels of AI-2 activity observed in all CFME samples are similar to those in previous findings. Lu *et al.* (2004) reported very low levels of AI-2 activity (less than one fold induction of luminescence compared to the negative control) in meat products. Also, it has recently been reported that fatty acids (linoleic acid, oleic acid, palmitic acid and stearic acid) isolated from ground beef can inhibit AI-2-based cell signaling (Soni *et al.,* 2008). The presence of these compounds in minced beef would also explain the results reported in this study.

4.4 *N*-acyl homoserine lactone signal production of *Enterobacteriaceae* isolated from minced beef

Although members of the family *Enterobacteriaceae* do not become a numerically dominant part of the microbial association on meat and meat products, and rarely contribute to spoilage, they have been considered as indicators of food safety (Nychas *et al.,* 2007). AHL signal molecules were detected in aerobically chill-stored ground beef when pseudomonads populations were approximately 10^8 and 10^9 CFU g^{-1} and *Enterobacteriaceae* populations were 10^3 and 10^4 CFU g^{-1} (Liu *et al.,* 2006), populations considerably lower than the detection threshold of 10^5 to 10^6 CFU g^{-1} previously reported for vacuum-packaged cold-smoked salmon (Gram *et al.,* 1999). Additionally, several food-relevant *Enterobactericeae* produce AHL signal molecules, which regulate various behaviours. In *Serratia proteomaculans*, *N*-(ß-ketocaproyl)-L-homoserine lactone is involved in the production of exoenzymes (Christensen *et al.,* 2003). In *Ser. liquefaciens*, *N*-butanoyl-L-homoserine lactone and *N*-hexanoyl-L-homoserine lactone are involved in the production of exoenzymes and swarming motility (Eberl *et al.,* 1996, Riedel *et al.,* 2001). Also, antibiotic production in *Erwinia carotovora* is regulated by *N*-(ß-ketocaproyl)-homoserine lactone (Bainton *et al.,* 1992). Some of these AHL-behaviours have been suggested to affect the quality of food products (Bruhn *et al.,* 2004).

Throughout storage of minced beef, various *Enterobacteriaceae,* and *Pseudomonas* spp. isolates were recovered and assayed for QS signal production *in vitro*. Several biosensor strains, responding to AHLs with different structural features, were used in order to obtain a better estimation of the total AHL content of the bacteria and their culture supernatants as previously described. In this study it was found that 17 out of 19 *Enterobacteriaceae* fingerprints were AHL producers, those

strains were assigned to *Serratia* spp., *Ser. proteomaculans*, *Ser. liquefaciens*, *Citrobacter freundii* and *Hafnia alvei*, whereas the strains of *Proteus vulgaris* did not exhibit detectable levels of AHL production when propagated under standard growth conditions. More precisely, the strains *Citro. freundii*, *Serratia* spp., *Ser. liquefaciens* (VK23, VK40, VK74 and VK75) and *Ser. proteomaculans* (VK25, VK32 and VK113) were detected to produce long-chain AHLs using the assay based on inhibition of the induced *C. violaceum* CV026. All *H. alvei* strains and the *Ser. liquefaciens* VK17 elicited the same response to the *A. tumefaciens* A136, *C. violaceum* CV026 and *E. coli* JM109 (pSB401) biosensor strains. The biosensor strain *E. coli* JM109 (pSB401) has similar detection specificities to *A. tumefaciens* A136 detecting a relatively broad range of medium-side-chain AHLs, whereas *C. violaceum* CV026 is induced by AHLs evaluated with *N*-acyl side chains from C4 to C8 in length. *Ser. proteomaculans* (VK5 and VK6) induced all the biosensor strains used, except for *E. coli* JM109 (pSB536) and (pSB1075), which detect small acyl side-chain and long acyl side-chain AHLs respectively. The final profiles of AHLs in strains responding positively to *A. tumefaciens* A136 and *E. coli* JM109 (pSB401) biosensors strains, as determined by TLC coupled with *A. tumefaciens* A136 were similar. All these strains were found to produce *N*-(ß-ketocaproyl)-L-homoserine lactone compared to the AHL standards used. The presence of other types of AHLs in strains responding to induced *C. violaceum* CV026 cannot be excluded, taking into account that each AHL has a different detection limit (Holden *et al.,* 1999). These results are similar to those reported by other researchers (Ravn *et al.,* 2001; Gram *et al.*, 1999), demonstrating that AHL production is common among *Enterobacteriaceae* isolated from foods and that they produce a multitude of different AHLs. More specifically, *H. alvei* and *Serratia* spp. have been shown to be the dominating species

among the AHL-producing *Enterobacteriaceae* isolated from vacuum-packed meat. These strains were capable of producing AHLs, mainly *N*-(ß-ketocaproyl)-homoserine lactone (Gram *et al.*, 1999; Ravn *et al.*, 2001; Bruhn *et al.*, 2004). It is worth noting, however, that *Enterobacteriaceae* strains producing AHL signal molecules were isolated from initial, mibble and final stage of minced beef storage regardless the condition (packaging and temperature) and the indigenous microbial load. The AHL-producing strain *Ser. liquefaciens* VK17 and the *Ser. liquefaciens* VK23, which did not respond to any of the biosensor strains used in this study, were the most frequently isolated strains throughout storage.

In contrast, in a preliminary screening of *Pseudomonas* spp. isolated from the same meat samples, was found that none of these isolates produced detectable levels of AHL production using the same biosensors. This comes in accordance with previous findings, where none of the *Pseud. fragi* stains isolated from fresh and spoiled meat were able to produce AHL signal molecules (Ferrocino *et al.*, 2009). Additionally, only a small fraction of *Pseudomonas* spp. recovered from proteinaceous raw foods and bean sprouts had detectable AHL signals. Generally, most *Pseudomonas* spp. isolates from food products did not produce AHL signal molecules in sufficient quantities to be detectable in the assays used (Bruhn *et al.*, 2004; Rasch *et al.*, 2005; Liu *et al.*, 2006). According to these observations, AHL based QS was suggested to play no role in the spoilage of aerobically packed meat where pseudomonads dominate.

4.5 Autoinducer-2-like activity in lactic acid bacteria isolated from minced beef

To our knowledge, no researchers have documented AI-2 production in LAB isolated from meat and/or meat products. Few studies demonstrate the production of AI-2 signaling molecules in LAB isolated from milk, dairy products and human or animal gastrointestinal tract, such as probiotic strains of *Lactobacillus* spp. and the pathogen *Streptococcus suis* Serotype 2 (SS2) commonly associated with disease in pigs and humans (De Keersmaecker & Vanderleyden, 2003; Moslehi-Jenabian *et al.*, 2009; Han & Lu, 2009). In this study, fifteen different fingerprints (assigned to *Leuconostoc* spp., *Leuc. mesenteroides*, *Weissella viridescens*, *Leuc. citreum* and *Lactobacillus sakei*) out of 89 LAB isolated from minced beef stored under modified atmospheres at various temperatures (0, 5, 10 and 15 °C) were screened for their ability to exhibit AI-2-like activity.

The isolates were propagated under certain growth conditions, taking into account that AI-2 production has been revealed to be affected by the growth medium and external environmental factors such as temperature (Surette & Bassler, 1999; Cloak *et al.*, 2002). It has been shown that components of the culture medium may lead to false-negative or false-positive results (De Keersmaecker & Vanderleyden, 2003). At the same time intervals as the microbiological analysis and the recovery of isolates, cell-free meat extracts (CFME) were collected and tested for presence of AI-2-like molecules. All bioassays were conducted using the *V. harveyi* BAA-1117 (sensor 1^-, sensor 2^+) biosensor strain. AI-2-like activity was observed on *Leuconostoc* spp. isolates, whereas none of the *Lact. sakei* strains produced detectable AI-2-like activity. The *luxS* gene is responsible for the production of AI-2 signal molecules, and is present in the genomes of a wide variety of Gram-negative and

Gram-positive bacteria (Xavier & Bassler, 2003; Gobbetti *et al.*, 2007). Various LAB studied so far have been shown to possess a *luxS* gene, like *Leuc. mesenteroides*, *Lact. gasseri*, *Lact. plantarum*, *Lactococcus lactis* and *Leuc. oenos* (Federle & Bassler, 2003). On the other hand, many Gram-positive bacteria communicate via QS autoinducing peptides, which do not trigger the AI-2 biosensor strain (Sturme *et al.*, 2002). Among LAB, some strains of *Lact. sakei* produce this category of signal molecules, which induce bacteriocin (sakacin P) production (Eijsink *et al.*, 1996; Brurberg *et al.*, 1997; Møretrø *et al.*, 2005). The absence of AI-2 production mechanism and/or the presence of autoinducing peptides in the tested isolates would explain the results reported in this study. The AI-2-like activity was evident mainly among *Leuconostoc* spp. (B233) strain which was the dominant isolate recovered from storage at 10 and 15 °C, and at the initial and middle stage of storage at chill temperatures (0 and 5 °C). All the tested CFME samples displayed low amounts of AI-2-like compared to the control (negative) sample, regardless the indigenous microbial load. The control sample was CFME from 0 h minced beef sample, which had AI-2-like activity similar to that of CFME from a "sterile" meat sample (obtained as previously described by Nychas *et al.* (2009)) and sterile growth medium. The low values of AI-2-like activity led us to evaluate the possible inhibitory effect of CFME against the biosensor strain's activity. The inhibitory effect ranged approximately between 51.11 and 91.09% without regard to the indigenous bacterial populations. Similar results e.g., very low levels of AI-2 activity (less than a fold induction of luminescence compared to the negative control) have been reported in a recent study with beef steak, beef patties, chicken breast, and turkey patties although their indigenous population loads were high (6.4 to 8.0 log CFU mL^{-1}) (Lu *et al.*, 2004). Comparable results were also reported in a previous study where certain meat

matrices were tested for inhibiting AI-2-like activity. Beef steak and beef patties showed high levels of inhibition, 90.6 and 84.4%, when indigenous bacterial populations were 7.4 and 6.4 log CFU mL^{-1}, respectively (Lu *et al.*, 2004). Various compounds from food matrices may lead to incorrect results. Previous findings suggest that the presence of fatty acids (linoleic acid, oleic acid, palmitic acid and stearic acid) isolated from ground beef and poultry meat can inhibit AI-2 activity (Widmer *et al.*, 2007; Soni *et al.*, 2008). Additionally, food additives such as sodium propionate, sodium benzoate, sodium acetate and sodium nitrate may influence AI-2 production (Lu *et al.*, 2004).

Taking into account the above, a correlation between the LAB displaying AI-2-like activity and the storage temperatures was performed. Concisely, among the 89 isolated LAB (fifteen different fingerprints were obtained totally), 68 (76.4%) of the isolates were exhibiting AI-2-like activity, whereas the variety of different strains retrieved depended on storage time and temperature. At chill temperatures (0 and 5 °C) eleven different fingerprints were recovered, whereas at relatively high temperatures (10 and 15 °C) the strain diversity reduced, since five different fingerprints were detected. At 10 and 15 °C the AI-2-producing *Leuconostoc* spp. (B232) represented the dominant biota, while at 0 and 5 °C almost fifty percent of the tested isolates did not exhibit any light induction, mainly *Lact. sakei*. Nychas *et al.* (2009) have reported the effect of CFME containing QS signal molecules on the kinetic parameters of Gram-negative bacteria isolated from meat. Data concerning the effect of AI-2 molecules on bacterial growth and their role in food spoilage are scarce. Further studies are needed to explore the possible effect of these molecules produced by the ephemeral spoilage organisms on the domination of different bacterial strains during food storage. The probability that temperature strongly affects the expression

of genes, encoding molecules that produce AI-2 activity and thus affects the diversity of the LAB population should be taken into consideration.

4.6 Effect of microbial quorum sensing signals on the growth of spoilage bacteria

Experiments obtained so far have mainly focused on the molecular perspectives of QS phenomenon, in other words how QS affect virulence, biofilm formation, toxin production, antibiotic synthesis, sporulation or conjugation, and less attention has been paid to the ecological context of why bacteria produce signal molecules and respond to these signals (Bassler *et al.,* 1994; Taga *et al.,* 2001; Burgess *et al.,* 2002). The confirmation of presence/absence or the determination of the levels of signal molecules in foods does not answer the key question in what way they influence food spoilage and in which way food compounds are affecting QS (Nychas *et al.,* 2009). Gram-negative bacteria are associated with the production of AHL and AI-2 signal molecules, while these molecules have been found in a wide range of foods in which the dominant microbial association consists of Gram-negative bacteria (i.e., pseudomonads and *Enterobacteriaceae*) or LAB. Taking into account these observations, it has been suggested that evaluating the effect of the QS signal molecules on the behaviour of the ephemeral spoilage microorganisms useful information could be provided for the potential role of these signals in the spoilage of foods (Nychas *et al.,* 2009).

Pseud. fluorescens and *Ser. liquefaciens* are among the most currently isolated strains dominating in a wide variety of foods (e.g., meat, dairy and vegetable products) stored under different conditions. *Pseud. fluorescens* strain 395 and *Ser.*

liquefaciens were therefore used as representatives in order to study the effect of microbial QS signals on the growth of these bacteria individually. The addition of cell-free culture extracts (CFCE) with QS signal molecules (AHLs and AI-2) affected differently the estimated growth kinetic parameters (i.e., lag phase duration and maximum specific growth rate) of the spoilage bacteria, *Pseud. fluorescens* and *Ser. liquefaciens*. More precisely, the presence of $CFCE_{AHL}$ from AHL-producing strain *H. alvei* 718 resulted in both growth rate and lag phase duration increase of *Pseud. fluorescens*, whereas the *Ser. liquefaciens* was partly influenced (only lag phase), compared to the control $CFCE_{MUT}$ derived from the AHL-lacking mutant *H. alvei* 718 *halI*. On the other hand, the addition of low concentration of $CFCE_{AI2}$ from the AI-2 producer *Salmonella* Typhimurium resulted in growth rate and lag-phase reduction of both examined spoilage bacteria, while no significant growth was observed using higher concentration of $CFCE_{AI2}$, compared to the control (heat treated CFCE derived from *Salmonella* Typhimurium). Heat treatment (autoclaving, 121 °C for 15 min) has been previously shown to destroy AI-2 activity (Surette & Bassler, 1998).

In a recent study, Nychas *et al.* (2009) found that cell-free meat extract derived from spoiled minced pork meat stored aerobically contained AHL and AI-2 signals. It was also observed, that the addition of cell-free meat extract from spoiled meat (containing QS signal molecules) to an 18 h culture of *Pseud. fluorescens* and *S. marcescens* resulted in an extension of the lag phase of *Pseud. fluorescens* but not of *S. marcescens* when compared to control samples and in an increase of the metabolic activity for both strains as revealed by the maximum slope of conductance changes, which corresponds to tested bacterial growth rate. The observed increase in metabolic activity was suggested to be related to the presence of some compounds in cell-free meat extract, including QS signal molecules (Nychas *et al.*, 2009). Additionally, Soni

and co-workers (2008) reported that the presence of AI-2 molecules promoted the survival of *E. coli* O157:H7 cells, whereas the protective effect of AI-2 molecules was negated in the presence of ground beef extracts that contained significant amount of inhibitory activity (Soni *et al.*, 2008). In this study, we show that AHL signal molecules of microbial origin encouraged bacterial growth of *Pseud. fluorescens* and *Ser. liquefaciens*, while the universal AI-2 molecules when present in low amounts reduced bacterial growth and in higher amounts retained the initial viable cell numbers of both tested bacterial strains. These findings imply that signal molecules seem to play a role in modulating the bacterial ecology and consequently might play a role in spoilage as already have been reported in other studies (Gram *et al.*, 2002; Bruhn *et al.*, 2004; Smith *et al.*, 2004; Pillai & Jesudhasan, 2006; Ammor *et al.*, 2008).

Chapter 5

Conclusions and Future Work

5.1 Conclusions

The findings of this study are summarized as follows:

✓ The levels of microbial contamination were determined on minced beef purchased from retail shops (Butcher shops and Supermarkets) in the Athens area, and was found that mainly pseudomonads, *Enterobacteriaceae*, *Brochothrix thermosphacta* and LAB contribute to the microbial association. The prevalence of a particular microbial association was suggested to be affected by the weather and the type of shop.

✓ The microbiological analysis of beef stored aerobically under various temperatures (0, 5, 10, 15 and 20 °C) revealed that the initial microbial flora consisted of pseudomonads, *Enterobacteriaceae*, *Br. thermosphacta* and LAB, which is in accordance with the above observations. The succession and the contribution in the spoilage process of these groups were affected by storage temperature. Pseudomonads were the dominant microorganisms, followed by *Br. thermosphacta* and then LAB.

✓ The presence of quorum sensing signals, AHLs and AI-2, throughout storage of minced beef under air and modified atmospheres with/without the presence of volatile compounds of oregano essential oil was detected using different biosensor strains. The biosensor strains responded in a wide range of AHLs and an AI-2 signal molecule, and the findings were correlated with the indigenous microbial populations. The packaging condition affected strongly the microbial association and consequently the observed fluctuations in the detected quorum sensing signals. More precisely, the CFME induced AHL biosensor strains when pseudomonas and *Enterobacteriaceae* populations ranged between 10^7 and 10^9 CFU g^{-1}, whereas no significant AI-2-activity

was observed in the tested CFME. Chemical concentration of cell-free meat extracts determined by TLC separation indicated presence of *N*-(ß-ketocaproyl)-homoserine lactone.

✓ *Enterobacteriaceae* were detected in high loads during minced beef storage based on the microbiological analysis, and were found to produce AHLs using different biosensor strains. Seventeen out of 19 *Enterobacteriaceae*, assigned to *Serratia* spp., *Ser. proteomaculans*, *Ser. liquefaciens*, *Citrobacter freundii* and *Hafnia alvei*, were AHL producers. Those strains were found to produce *N*-(ß-ketocaproyl)-homoserine lactone, suggesting that they are the main quorum sensing signal producers in meat samples.

✓ The AI-2-like activity was evident mainly in the *Leuconostoc* spp. (B233) strain which was the dominant isolate recovered during storage at relative high temperatures (10 and 15 °C), and at the initial and middle stage of storage at chill temperatures (0 and 5 °C), whereas none of the *Lactobacillus sakei* strains produced AI-2-like activity. The tested CFME samples, collected at the same time intervals as the microbiological analysis and the isolates recovery, displayed low amounts of AI-2-like activity and inhibited AI-2 activity regardless of the indigenous bacterial population loads. These findings demonstrated that LAB isolated during meat spoilage exhibited AI-2-like activity, whereas the variety of different strains isolated depended on storage time and temperature, suggesting that the production of AI-2-like molecules may affect the domination of different bacterial strains through storage.

✓ The microbial quorum sensing signals (AHLs and AI-2) affected the growth of two main spoilage bacteria, *Pseudomonas fluorescens* and *Ser. liquefaciens*. The presence of CFCE containing AHL signal molecules encouraged bacterial growth, whereas the

presence of CFCE exhibiting AI-2 activity reduced or retained bacterial growth depending on the concentration of the signal molecules in the growth medium. More accurately, the presence of $CFCE_{AHL}$ (20 and 50% v/v) resulted in both growth rate and lag phase duration increase of *Pseud. fluorescens*, whereas the *Ser. liquefaciens* was partly influenced (only lag phase). On the other hand, the addition of low concentration (20% v/v) of $CFCE_{AI2}$ resulted in growth rate and lag-phase reduction of both examined spoilage bacteria, while no significant growth was observed using higher concentration (50% v/v) of $CFCE_{AI2}$, compared to the control sample. These observations illustrate the potential effect of signal molecules on the behavior of spoilage bacteria.

5.2 Future Work

Further work is needed in the topics below, with the perspective to answer the key question "What is the role of QS in meat spoilage?"

• Screening of indigenous microbial populations e.g., *Br. thermosphacta* isolated from meat stored under various conditions for QS signal molecules production *in vitro* and *in vivo*.

• Co-culturing of strains producing or not QS signal molecules *in vitro* and *in vivo*, with or without the addition of synthetic QS or QS signals extracted from QS-producing strains isolated from meat.

• It may be useful to explore the possible effect of QS signal molecules produced by the ephemeral spoilage organisms on the domination of different bacterial strains through meat storage.

Chapter 6

References

Ammor, M. S., Michaelidis, C. & Nychas, G.-J. E. (2008). Insights into the role of quorum sensing in food spoilage. *J Food Prot* **71**, 1510-1525.

Argyri, A., Panagou, E. Z., Tarantilis, P. A., Polysiou M. & Nychas, G.-J. E. (2010). Rapid qualitative and quantitative detection of beef fillets spoilage based on Fourier transform infrared spectroscopy data and artificial neural networks. *Sens Actuators B* **145**, 146-154.

Andersen, J. B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B. B., Molin, S. & Givskov, M. (2001). Gfp-based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Appl Environ Microbiol* **67**, 575-585.

Atkinson, S., Throup, J. P., Stewart, G. S. & Williams, P. (1999). A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol Microbiol* **33**, 1267-1277.

Bainton, N.J., Bycroft, B.W., Chhabra, S.R. & 8 other authors (1992). A general role for the lux autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. *Gene* **116**, 87-91.

Baranyi, J. & Roberts, T.A. (1994). A dynamic approach to predicting bacterial growth in food. *Int J Food Microbiol* **23**, 277-294.

Bassler, B. L. (2002). Small Talk: Cell-to-Cell Communication in Bacteria. *Cell* **109**, 421-424.

Bassler, B. L., Greenberg, E. P. & Stevens, A. M. (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol* **179**, 4043-4045.

Bassler, B. L., Wright, M. & Silverman, M. R. (1994). Multiple signaling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol Microbiol* **13**, 273-286.

Borch, E., Kant-Muermans, M.-L. & Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. *Int J Food Microbiol* **33**, 103-120.

Boddy, L. & Wimpenny, J. W. (1992). Ecological concepts in food microbiology. *Soc Appl Bacteriol Symp Ser* **21**, 23S-38S.

Bruhn, J. B., Christensen, A. B., Flodgaard, L. R., Nielsen, K. F., Larsen, T. O., Givskov, M. & Gram. L. (2004). Presence of acylated homoserine lactones (AHLs) and AHL-producing bacteria in meat and potential role of AHL in spoilage of meat. *Appl Environ Microbiol* **70**, 4293-4302.

Brurberg, M. B., Nes, I. F. & Eijsink, V. G. H. (1997). Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. *Mol Microbiol* **26**, 347-360.

Burgess, N. A., Kirke, D. F., Williams, P., Winzer, K., Hardie, K. R., Meyers, N. L., Aduse-Opoku, J., Curtis, M. A. & Camara, M. (2002). LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and haemagglutinin activities but is not essential for virulence. *Microbiol* **148**, 763-772.

Burmolle, M., Hansen, L. H., Oregaard, G. & Sorensen, S. J. (2003). Presence of *N*-acyl homoserine lactones in soil detected by a whole-cell biosensor and flow cytometry. *Microb Ecol* **45**, 226-236.

Byers, J. T., Lucas, C., Salmond, G. P. & Welch, M. (2002). Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *J Bacteriol* **184**, 1163-1171.

Cataldi, T. R., Bianco, G., Palazzo, L. & Quaranta, V. (2007). Occurrence of *N*-acyl-L-homoserine lactones in extracts of some gramnegative bacteria evaluated by gas chromatography–mass spectrometry. *Anal Biochem* **361**, 226-235.

Chen, X., Schauder, S., Potter, N., van Dorsslaer, A., Pelczer, I., Bassler, B. L. & Hughson, F. M. (2002). Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**, 545-549.

Choo, J. H., Rukayadi, Y. & Hwang, J.-K. (2006). Inhibition of bacterial quorum seingng by vanilla extract. *Lett Appl Microbiol* **42**, 637-641.

Chorianopoulos, N. G., Giaouris, E. D., Kourkoutas, Y. and Nychas, G.-J. E. (2010). Inhibition of the early stage of *Salmonella enterica* serovar *enteritidis* biofilm

development on stainless steel by cell-free supernatant of a *Hafnia alvei* culture. *Appl Environ Microbiol* **76,** 2018-2022.

Christensen, A. B., Riedel, K., Eberl, L., Flodgaard, L. R., Molin, S., Gram, L. & Givskov, M. (2003). Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. *Microbiology* **149**, 471-483.

Cloak, O. M., Solow, B. T., Briggs, C. E., Chen, C. Y. & Fratamico, P. M. (2002). Quorum sensing and production of autoinducer-2 in *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Typhimurium in foods. *Appl Environ Microbiol* **68**, 4666-4671.

De Keersmaecker, S. C., Sonck, K. & Vanderleyden, J. (2006). Let LuxS speak up in AI-2 signaling. *Trends Microbiol* **14**, 114-119.

De Keersmaecker, S. C. & Vanderleyden, J. (2003). Constraints on detection of autoinducer-2 (AI-2) signalling molecules using *Vibrio harveyi* as a reporter. *Microbiology* Comment **149**, 1953-1956.

DeLisa, M. P., Valdes, J. J. & Bentley, W. E. (2001). Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated *Escherichia coli* K-12. *J Bacteriol* **183**, 2918-2928.

Dong, Y. H., Zhang, X. F., Soo, H. M., Greenberg, E. P. & Zhang, L. H. (2005). The two-component response regulator PprB modulates quorum-sensing signal production and global gene expression in *Pseudomonas aeruginosa. Mol Microbiol* **56**, 1287-1301.

Drosinos, E. H. (1994). Microbial associations of minced lamb and their ecophysiological attributes. PhD Thesis. University of Bath, Bath, United Kingdom.

Doulgeraki, A., Paramithiotis, S., Kagkli, D.-M. & Nychas, G.-J. E. (2010). Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions. *Food Microbiol*. DOI 10.1016/j.fm.2010.07.004.

Doulgeraki, A., Paramithiotis, S. & Nychas, G.-J. E. (2010). Development of *Enterobacteriaceae* community during storage of minced beef under aerobic or modified atmosphere packaging conditions. *Int J Food Microbiol* (submitted for publication).

Eberl, L., Winson, M.K., Sternberg, C., & 7 other authors (1996). Involvement of *N*-acyl-l-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Mol Microbiol* **20**, 127-136.

Eijsink, V. G. H., Brurberg, M. B., Middelhoven, P. H. & Nes, I. F. (1996). Induction of bacteriocin production in *Lactobacillus sakei* by a secreted peptide. *J Bacteriol* **178**, 2232-2237.

Ercolini, D., Russo, F., Nasi, A., Ferranti, P. & Villani, F. (2009). Mesophilic and psychrotrophic bacteria from meat and their spoilage potential *in vitro* and in beef. *Appl Environ Microbiol* **75**, 1990-2001.

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. *Appl Environ Microbiol* **72**, 4663-4671.

Farrand, S. K., Qin, Y. & Oger, P. (2002). Quorum-sensing system of Agrobacterium plasmids: analysis and utility. *Methods Enzymol* **358**: 452-484.

Federle, M. J. & Bassler, B. L. (2003). Interspecies communication in bacteria. *J Clin Invest* **112**, 1291-1299.

Ferrocino, I., Ercolini, D., Villani, F., Moorhead, S. M. & Griffiths, M. W. (2009). *Pseudomonas fragi* strains isolated from meat do not produce *N*-acyl homoserine lactones as signal molecules. *J Food Prot* **72**, 2597-2601.

Fuqua, W. C. & Winans, S. C. (1994). A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J Bacteriol* **176**, 2796-2806.

Fuqua, W. C., Winans, S. C. & Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR-Lux-I family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269-275.

Geisenberger, O., Givskov, M., Riedel, K., Hoiby, N., TummLer, B. & Eberl, L. (2000). Production of *N*-acyl-L-homoserine lactones by *P. aeruginosa* isolates from chronic lung infections associated with cystic fibrosis. *FEMS Microbiol Lett* **184**, 273-278.

Gill, C. O. (2005). Improving the Safety of Fresh Meat. Sources of microbial contamination at slaughtering plants, pp. 231-243. Edited by N. Sofos, CRC/Woodhead Publishing Limited, Cambridge, United Kingdom.

Gill, C. O. & Jeremiah, L. E. (1991). The storage life of non-muscle offals packaged under vacuum or carbon dioxide. *Food Microbiol* **8**, 339-53.

Gill, C. O. & Newton, K. G. (1978). The ecology of bacterial spoilage of fresh meat at chill temperatures. *Meat Science* **2**, 207-217.

Gobbetti, M., De Angelis, M., Di Cagno, R., Minervini, F. & Limitone, A. (2007). Cell-cell communication in food related bacteria. *Int J Food Microbiol* **120**, 34-45.

Gonzalez, R. H., Nusblat, A. & Nudel, B. C. (2001). Detection and characterization of quorum sensing signal molecules in *Acinetobacter* strains. *Microbiol Res* **155**, 271-277.

Gram, L., Christensen, A. B., Ravn, L., Molin, S. & Givskov, M. (1999). Production of acylated homoserine lactones by psychrotrophic members of the *Enterobacteriaceae* isolated from foods. *Appl Environ Microbiol* **65**, 3458-3463.

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B. & Givskov, M. (2002). Food spoilage - interactions between food spoilage bacteria. *Int J Food Microbiol* **78**, 79-97.

Han, X. G. & Lu, C. P. (2009). Detection of autoinducer-2 and analysis of the profile of *luxS* and *pfs* transcription in *Streptococcus suis* Serotype 2. *Curr Microbiol* **58**, 146-152.

Henke, J. M. & Bassler, B. L. (2004). Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi. J Bacteriol* **186**, 6902-6914.

Hoiseth, S. K. & Stocker, B. A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **21**, 238-239.

Holden, M. T., Ram Chhabra, S., de Nys, R. & 14 other authors (1999). Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Mol Microbiol* **33**, 1254-1266.

Holloway, B. W., Krishnapillai, V. & Morgan, A. F. (1979). Chromosomal genetics of *Pseudomonas*. *Microbiol Rev* **43**, 73-102.

Jay, J. M., Vilai, J. P. & Hughes, M. E. (2003). Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5-7 °C. *Int J Food Microbiol* **81**, 105-111.

Khan, S. R., Mavrodi, D. V., Jog, G. J., Suga, H., Thomashow, L. S. & Farrand, S. K. (2005). Activation of the phz operon of *Pseudomonas fluorescens* 2-79 requires the LuxR homolog PhzR, *N*-(3-OH-hexanoyl)-L-homoserine lactone produced by the LuxI homolog PhzI, and a *cis*-acting phz box. *J Bacteriol* **187**, 6517-6527.

Koutsoumanis, K. P., Ashton, L. V., Georgnaras, I., Belk, K. E., Scanga, J. A., Kendall, P. A., Smith, G. C. & Sofos, J. N. (2004). Effect of single or sequential hot water and lactic acid decontamination treatments on the survival and growth of *Listeria monocytogenes* and spoilage microflora during aerobic storage of fresh beef at 4, 10, and 25°C. *J Food Prot* **67**, 2703-2711.

Koutsoumanis, K., Stamatiou, A., Skandamis, P. & Nychas, G.-J.E. (2006). Development of a microbial model for the combined effect of temperature and pH on spoilage of ground meat, and validation of the model under dynamic temperature conditions. *Appl Environ Microbiol* **72**, 124-134.

Lambropoulou, K. A., Drosinos, E. H. & Nychas, G.-J. E. (1996). The effect of glucose supplementation on the spoilage microflora and chemical composition of

minced beef stored aerobically or under a modified atmosphere at 4 °C. *Int J Food Microbiol* **30**, 281-291.

Lambert, A. D., Smith, J. P. & Dodds, K. L. (1991). Shelf life extension and microbiological safety of fresh meat – a review. *Food Microbiol* **8**, 267-297.

Lazdunski, A. M., Ventre, I. & Sturgis, J. (2004). Regulatory circuits and communication in Gram-negative bacteria. *Nature Reviews* **2**, 581-592.

Lindsay, A. & Ahmer, B. M. (2005). Effect of *sdi*A on biosensors of *N*-acylhomoserine lactones. *J Bacteriol* **187**, 5054-5058.

Lithgow, J. K., Danino, V. E., Jones, J. & Downie, J. A. (2001). Analysis of *N*-acyl homoserine-lactone quorum-sensing molecules made by different strains and biovars of *Rhizobium leguminosarum* containing different symbiotic plasmids. *Plant Soil* **232**, 3-12.

Liu, F., Yang, R.-Q. & Li, Y.-F. (2006). Correlations between growth parameters of spoilage micro-organisms and shelf-life of pork stored under air and modified atmosphere at -2, 4 and 10 °C. *Food Microbiol* **23**, 578-583.

Liu, M., Gray, J. M. & Griffiths, M. W. (2006). Occurrence of proteolytic activity and *N*-acyl-homoserine lactone signals in the spoilage of aerobically chill-stored proteinaceous raw foods. *J Food Prot* **69**, 2729-2737.

Liu, F., Guo, Y. & Li, Y. (2006). Interactions of microorganisms during natural spoilage of pork at 5 °C. *J Food Eng* **72**, 24-29.

Llamas, I., Keshavan, N. & Gonzalez, J. E. (2004). Use of *Sinorhizobium meliloti* as an indicator for specific detection of long-chain *N*-acyl homoserine lactones. *Appl Environ Microbiol* **70**, 3715-3723.

Lowe, T. E., Devine, C. E., Wells, R. W. & Lynch, L. L. (2004). The relationship between postmortem urinary catecholamines, meat ultimate pH, and shear force in bulls and cows. *Meat Sci* **67**, 251-260.

Lu, L., Hume, M. E. & Pillai, S. D. (2004). Autoinducer-2-like activity associated with foods and its interaction with food additives. *J Food Prot* **67**, 1457-1462.

Lu, L., Hume, M. E. & Pillai, S. D. (2005). Autoinducer-2-like activity on vegetable produce and its potential involvement in bacterial biofilm formation on tomatoes. *Foodborne Pathog Dis* **2**, 242-249.

Lyon, G. J. & Novick, R. P. (2004). Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides* **25**, 1389-1403.

McClean, K. H., Winson, M. K., Fish, L. & 9 other authors (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for detection of *N*-acyl homoserine lactones. *Microbiology* **143**, 3703-3711.

McDougald, D., Rice, S. A. & Kjelleberg, S. (2007). Bacterial quorum sensing and interference by naturally occurring biomimics. *Anal Bioanal Chem* **387**, 445-453.

McDowell, P., Affas, Z., Reynolds, C. & 9 other authors (2001). Structure, activity and evolution of the group I thiolactone peptide quorum-sensing system of *Staphylococcus aureus*. *Mol Microbiol* **41**, 503-512.

Medina-Martinez, M. S., Uyttendaele, M., Demolder, V. & Debevere, J. (2006). Influence of food system conditions on *N*-acyl-L-homoserine lactones production by *Aeromonas* spp. *Int J Food Microbiol* **112**, 244-252.

Miller, M. B. & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu Rev Microbiol* **55**, 165-199.

Møretrø, T., Naterstad, K., Wang, E., Aasen, I. M., Chaillou, S., Zagorec, M. & Axelsson, L. (2005). Sakacin P non-producing *Lactobacillus sakei* strains contain homologues of the sakacin P gene cluster. *Res Microbiol* **156**, 949-960.

Moslehi-Jenabian, S., Gori, K. & Jespersen, L. (2009). AI-2 signalling is induced by acidic shock in probiotic strains of *Lactobacillus* spp. *Int J Food Microbiol* **135**, 295-302.

Nakai, S. A. & Siebert, K. J. (2004). Organic acid inhibition models for *Listeria innocua, Listeria ivanovii, Pseudomonas aeruginosa*, and *Oenococcus oeni*. *Food Microbiol* **21,** 67-72.

Nealson, K. H. & Hastings, J. W. (1979). Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev* **43**, 496-518.

Nealson, K. H., Platt, T. & Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial luminescence system. *J Bacteriol* **104**, 313-322.

Nychas, G.-J. & Arkoudelos, J. S. (1990). Microbiological and physicochemical changes in minced meats under carbon dioxide, nitrogen or air at 3°C. *Int J Food Scien and Technol* **25**, 389-398.

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. *Biotechnol Appl Biochem* **10**, 203-231.

Nychas, G.-J. E., Douglas, L. M. & Sofos, J. N. (2007). Food Microbiology: fundamentals and frontiers. Meat, poultry, and seafood. pp. 105-140. Edited by M.P. Doyle, and L.R. Beuchat, ASM press, Washigton, D. C.

Nychas, G.-J. E., Dourou, D., Skandamis, P., Koutsoumanis, K., Baranyi, J. & Sofos, J. (2009). Effect of microbial cell-free meat extract on the growth of spoilage bacteria. *J Appl Microbiol* **107**, 1819-1829.

Nychas, G.-J. E. & Drosinos, E. H. (2000). Encyclopedia of Food Microbiology. Spoilage of meat, pp. 1253-1260. Edited by R.K. Robinson, C.A. Batt and P.D. Patel, pp. 1253-1260. Academic Press Ltd., London, UK

Nychas, G.-J. E., Drosinos, E. H. & Board, R. G. (1998). The microbiology of meat and poultry. Chemical changes in stored meat, pp. 288-326. Edited by A. Davies and R.G. Board, Blackie Academic & Professional, London.

Nychas, G.-J. E., Robinson, A. & Board, R. G. (1991). Microbiological and physico-chemical evaluation of ground beef from retail shops. *Fleisch* **71**, 1057-1059.

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C. & Koutsoumanis, K. (2008). Meat spoilage during distribution. *Meat Sci* **78**, 77-89.

Nychas, G-J. E. & Tassou, C. C. (1997). Spoilage process and proteolysis in chicken as noted by HPLC method. *J Sci Food Agric* **74**, 199-208.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* **91**, 197-201.

Pearson, J. P., Van Delden, C. & Iglewski, B. H. (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* **181**, 1203-1210.

Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **96**, 1129-1134.

Pillai, S. D. & Jesudhasan, P. R. (2006). Quorum sensing: how bacteria communicate. *Food Technol* **60**, 42-50.

Pinto, U. M., de Souza Viana, E., Martins, M. L. & Vanetti, M. C. D. (2007). Detection of acylated homoserine lactones in gram-negative proteolytic psychrotrophic bacteria isolated from cooled raw milk. *Food Control* **18**, 1322-1327.

Rajamani, S., Zhu, J., Pei, D. and Sayre, R. (2007). A LuxP-FRET-based reporter for the detection and quantification of AI-2 bacterial quorum-sensing signal compounds. *Biochemistry* **46**, 3990-3997.

Rasch, M., Andersen, J. B., Nielsen, K. F., Flodgaard, L. R., Christensen, H., Givskov, M. & Gram, L. (2005). Involvement of bacterial quorum-sensing signals in spoilage of bean sprouts. *Appl Environ Microbiol* **71**, 3321-3330.

Rasmussen, T. B., Bjarnsholt, T., Skindersoe, M. E., Hentzer, M., Kristoffersen, P., Köte, M., Nielsen, J., Eberl, L. & Givskov, M. (2005). Screening for Quorum-Sensing Inhibitirs (QSI) by Use of a Novel Genetic system, the QSI Selector. *J Bacteriol* **187**, 1799-1814.

Ravn, L., Christensen, A. B., Molin, S., Givskov, M. & Gram, L. (2001). Methods for detecting acylated homoserine lactones produced by gram-negative bacteria and

their application in studies of AHL-production kinetics. *J Microbiol Methods* **44**, 239-251.

Ravn Flodgaard, L., Christensen, A. B., Molin, S., Givskov, M. & Gram, L. (2003). Influence of food preservation parameters and associated microbiota on production rate, profile and stability of acylated homoserine lactones from food-derived *Enterobacteriaceae. Int J Food Microbiol* **84**, 145-156.

Reading, N. C. & Sperandio, V. (2006). Quorum sensing: the many languages of bacteria. *FEMS Microbiol Lett* **254**, 1-11.

Reading, N. C., Torres, A. G., Kendall, M. M., Hughes, D. T., Yamamoto, K. & Sperandio, V. (2007). A novel two-component signaling system that activates transcription of an enterohemorrhagic *Escherichia coli* effector involved in remodeling of host actin. *J Bacteriol* **189**, 2468-2476.

Riedel, K., Hentzer, M., Geisenberger, O. & 7 other authors (2001). *N*-acylhomoserine-lactone–mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology* **147**, 3249-3262.

Schauder, S. & Bassler, B. L. (2001). The languages of bacterial. *Genes Dev* **15**, 1468-1480.

Schauder, S., Shokat, K., Surette, M. G. & Bassler, B. L. (2001). The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **41**, 463-476.

Schaefer, A. L., Hanzelka, B. L., Parsek, M. R. & Greenberg, E. P. (2000). Detection, purification, and structural elucidation of the acylhomoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Methods Enzymol* **305**, 288-301.

Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Rinehart, K. L. & Farrand, S. K. (1997). Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc Natl Acad Sci USA* **94**, 6036-6041.

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. *J Appl Microbiol* **91**, 1011-1022.

Skandamis, P. N. & Nychas, G.-J. E. (2002). Preservation of fresh meat with active and modified atmosphere packaging conditions. *Int J Food Microbiol* **79**, 35-45.

Smith, D., Wang, J. H., Swatton, J. E. & 8 authors (2006). Variations on a theme: diverse *N*-acyl homoserine lactone-mediated quorum sensing mechanisms in gram-negative bacteria. *Sci Prog* **89**, 167-211.

Smith, J. L., Fratamico, P. M. & Novak, J. S. (2004). Quorum Sensing: A Primer for Food Microbiologists. *J Food Prot* **67**, 1053-1070.

Soni, K. A., Jesudhasan, P., Cepeda, M., Widmer, K., Jayaprakasha, G. K., Patil, B. S., Hume, M. E. & Pillai, S. D. (2008). Identification of ground beef derived fatty acid inhibitors of autoinducer-2 based cell signaling. *J Food Prot* **71**, 134-138.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P. & Kaper, J. B. (2003). Bacteria-host communication: the language of hormones. *Proc Natl Acad Sci USA* **100**, 8951-8956.

Stanbridge, L. H. & Davies, A. R. (1998). The microbiology of meat and poultry. The microbiology of chill-stored meat, pp. 175-177. Edited by A. Davies and R. G. Board, Blackie Academic & Professional, London.

Steindler, L. & Venturi, V. (2006). Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* **266**, 1-9.

Sturme, M. H., Kleerebezem, M., Nakayama, J., Akkermans, A. D., Vaugha, E. E. & de Vos, W. M. (2002). Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie Van Leeuwenhoek* **81**, 233-243.

Surette, M. G. & Bassler, B. L. (1998). Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proc Natl Acad Sci USA* **95**, 7046-7050.

Surette, M. G. & Bassler, B. L. (1999). Quorum sensing in *Escherichia coli*, *Salmonella typhimurium* and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci USA* **96**, 1639-1644.

Swift, S., Karlyshev, A. V., Fish, L., Durant, E. L., Winson, M. K., Chhabra, S. R., Williams, P., Macintyre, S. & Stewart, G. S. (1997). Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. *J Bacteriol* **179**, 5271-5281.

Swift, S., Winson, M. K., Chan, N. P. F. & 11 other authors (1993). A novel strategy for the isolation of luxI homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol Microbiol* **10**, 511–520.

Szabó, M. Á., Vagra, G. Z., Hohmann, J., Schelz, Z., Szegedi, E., Amaral, L. & Molnár, J. (2010). Inhibition of Quorum-sensing Signals by Essential Oils. *Phytother Res* **24**, 782-786.

Taga, M. E., Semmelhack, J. L. & Bassler, B. L. (2001). The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium. Mol Microbiol* **42**, 777-793.

Throup, J. P., Camara, M., Briggs, G. S., Winson, M. K., Chhabra, S. R., Bycroft, B. W., Williams, P. & Stewart, G. S. (1995). Characterisation of the *yen*I/*yen*R locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acylhomoserine lactone signal molecules. *Mol Microbiol* **17**, 345-356.

Turovskiy, Y. & Chikindas, M. L. (2006). Autoinducer-2 bioassay is a qualitative, not quantitative method influenced by glucose. *J Microbiol Methods* **66**, 497-503.

Tsigarida, E. (2000). Effect of the film permeability and modified atmospheres on the meat ecosystem. PhD Thesis. Agricultural University of Athens.

Tsigarida, E. & Nychas, G.-J. E. (2001). Ecophysiological attributes of a *Lactobacillus* sp. and a *Pseudomonas* sp. on sterile beef fillets in relation to storage temperature and film permeability. *J Appl Microbiol* **90**, 697-705.

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. *J Appl Microbiol* **89**, 901-909.

Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P. & Pesci, E. C. (2005). Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **187**, 4372-4380.

Waters, C. M. & Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**, 319-346.

Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J. & Salmond, G. P. (2001). Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* **25**, 365-404.

Widmer, K. W., Soni, K. A., Hume, M. E., Beier, R. C., Jesudhasan, P. & Pillai, S. D. (2007). Identification of poultry meat-derived fatty acids functioning as quorum sensing signal inhibitors to autoinducer-2 (AI-2). *J Food Science*. **72**, M363-M368.

Winson, M. K., Camara, M., Latifi, A. & 9 other authors (1995). Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92**, 9427-9431.

Winson, M. K., Swift, S., Fish, L., Throup, J. P., Jorgensen, F., Chhabra, S. R., Bycroft, B. W., Williams, P. & Stewart, G. S. (1998). Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone–mediated quorum sensing. *FEMS Microbiol Lett* **163**, 185-192.

Winzer, K., Hardie, K. R. & Williams, P. (2003). LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria. *Adv Appl Microbiol* **53**, 291-396.

Winzer, K. & Williams, P. (2001). Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. *Int J Med Microbiol* **291**, 131-143.

Xavier, K. B. & Bassler, B. L. (2003). LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol* **6**, 191-97.

Zhang, L., Murphy, P. J., Kerr, A. & Tate, M. E. (1993). *Agrobacterium* conjugation and gene regulation by *N*-acyl-l-homoserine lactones. *Nature* **362**, 446-448.

Zhu, J., Beaber, J. W., More, M. I., Fuqua, C., Eberhard, A. & Winans. S. C. (1998). Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens. J Bacteriol* **180**, 5398-5405.

Zhu, J., Chai, Y., Zhong, Z., Li, S. & Winans, S. C. (2003). *Agrobacterium* bioassay strain for ultrasensitive detection of *N*-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in *Mesorhizobium huakuii*. *Appl Environ Microbiol* **69**, 6949-6953.

APPENDIX I

The following work has been published in the *Journal of Food Protection*

Autoinducer-2-like activity in lactic acid bacteria isolated from minced beef packaged under modified atmospheres

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Abstract

Fifteen fingerprints (assigned to *Leuconostoc* spp., *Leuconostoc mesenteroides*, *Weissella viridescens*, *Leuconostoc citreum*, and *Lactobacillus sakei*) of 89 lactic acid bacteria (LAB) isolated from minced beef stored under modified atmospheres at various temperatures were screened for their ability to exhibit autoinducer-2 (AI-2)–like activity under certain growth conditions. Cell-free meat extracts (CFME) were collected at the same time as the LAB isolates and tested for the presence of AI-2–like molecules. All bioassays were conducted using the *Vibrio harveyi* BAA-1117 (sensor 1^-, sensor 2^+) biosensor strain. The possible inhibitory effect of meat extracts on the activity of the biosensor strain was also evaluated. AI-2–like activity was observed for *Leuconostoc* spp. isolates, but none of the *L. sakei* strains produced detectable AI-2–like activity. The AI-2–like activity was evident mainly associated with the *Leuconostoc* sp. B 233 strain, which was the dominant isolate recovered from storage at 10 and 15˚C and at the initial and middle stages of storage at chill temperatures (0 and 5˚C). The tested CFME samples displayed low AI-2–like activity and inhibited AI-2 activity regardless of the indigenous bacterial populations. The LAB isolated during meat spoilage exhibited AI-2–like activity, whereas the LAB strains retrieved depended on storage time and temperature. The production of AI-2–like molecules

may affect the dominance of different bacterial strains during storage. The results provide a basis for further research concerning the effect of storage temperature on the expression of genes encoding AI-2 activity and on the diversity of the ephemeral bacterial population.

APPENDIX II

The following work has been submitted in the *International Journal of Food Microbiology*

Potential of a simple HPLC-based approach to quantify spoilage of minced beef stored in different temperatures and packaging systems

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Abstract

The shelf life of minced beef stored (i) aerobically, (ii) under modified atmosphere packaging (MAP), and (iii) under MAP with oregano essential oil (MAP/OEO) at 0, 5, 10, and 15 ºC was investigated. The microbial associations of meat and the temporal biochemical changes were monitored. Total viable counts (TVC), *Pseudomonas* spp., *Brochothrix thermosphacta*, lactic acid bacteria, *Enterobacteriaceae* and yeasts/moulds were quantified, in parallel with sensory assessment, pH measurement and HPLC analysis of the organic acid profiles. Spectral data collected by HPLC were subjected to statistical analysis, including Principal Components Analysis (PCA) and Factorial Discriminant Analysis (FDA). This allowed qualitative discrimination of the samples based on their spoilage status. Partial Least Square Regression (PLS-R) was used to evaluate quantitative predictions of TVC, *Pseudomonas* spp., *Br. thermosphacta*, lactic acid bacteria, *Enterobacteriaceae* and yeasts/moulds. Overall, the metabolic profile of organic acids, determined by HPLC analysis, was found to be a reliable method to evaluate the spoilage and microbial status of a meat sample regardless of storage conditions. This could be a very useful tool for monitoring quality of meat batches during distribution and storage in the meat food chain.

PROCEEDINGS

ORAL PRESENTATIONS

- **Blana V. A.**, Michaelidis C., Panagou S. and Nychas G.-J. E. (2008). Detection of Quorum Sensing Signals (AI-1 and AI-2) Produced in Minced Beef Samples Stored Under Various Conditions. FOODMICRO 2008, 1-4 September 2008, Aberdeen, Scotland

- Doulgeraki, A. I., **Blana, V. A.,** Panagou, E. and Nychas, G.-J. E. (2008). The role of lactic acid bacteria and quorum sensing in beef spoilage. Meat and Meat products, 10-12 October 2008, Athens, Greece

- Doulgeraki, A., **Blana, V**, Panagou, E., Sofos, J., & Nychas G.-J. (2009). Quorum Sensing compounds and the role of lactic acid bacteria in beef spoilage. Ashtown Food Research Centre in Dublin, March 2009, Ireland

- **Blana, V. A.** and Nychas, G.-J. E. (2009). Quorum sensing phenomenon through minced beef spoilage. 3rd FoodBiotech, 15-17 October 2009, Rethymno, Crete, Greece

- **Blana, V. A.**, Griffiths M. W. and Nychas, G-J. E. (2010). Cell-to-cell communication during beef spoilage. 3rd National Conference of Interdisciplinary Society of Food Hygiene Assurance, 4-6 June 2010, Thessaloniki, Greece

- **Blana, V. A.,** Le Marc, Y. and Nychas, G.J. E. (2010). Quorum sensing signal molecules and microbial interactions in gel cassette system. 3rd National Conference of Interdisciplinary Society of Food Hygiene Assurance, 4-6 June 2010, Thessaloniki, Greece

- Argyri, A. A., Doulgeraki, A. I., **Blana, V. A.,** Panagou E. Z. and Nychas, G.-J. E. (2010). The potential of HPLC analysis of organic acids on predicting the shelf life of minced beef stored under conventional and active packaging conditions. 3rd National Conference of Interdisciplinary Society of Food Hygiene Assurance, 4-6 June 2010, Thessaloniki, Greece

- **Blana V. A.**, Gkika, P., Panagou, E. Z. and Nychas, G.-J. E. (2010). Effect of quorum-sensing signaling compounds on the growth of spoilage bacteria. 3rd MIKROBIOKOSMOS Conference, 16-18 December 2010, Thessaloniki, Greece

- **Blana, V. A.**, Gkika, P., Panagou, E. Z. and Nychas G.-J. E. (2011). Effect of microbial quorum sensing signals on the growth of spoilage bacteria. 2nd Pan-Hellenenic Veterinary Congress of Reproductive animals, Hygiene-Safety of Food of Animal Origin and Consumer Protection, 18-20 March, Thessaloniki, Greece

POSTER PRESENTATIONS

- **Blana, V. A.,** Stamatiou, A., Michaelidis, C., Stergiou, V. and Nychas G.-J. E. (2007). Qualitative evaluation of QS compounds produced in pork and beef samples at different storage conditions; possible effect on kinetic characteristics on spoilage bacteria. 2nd FoodBiotech, 29-31 March, Athens, Greece

- Ammor M.S., Michaelidis C., Doulgeraki A., **Blana V.** and Nychas G.-J. E. (2007). Autoinducer-2-like activity in lactic acid bacteria isolated from modified atmosphere packaged minced beef held from freshness to spoilage at 0, 5, 10 and 15°C. CBL2007, 13-15 November, France

- Doulgeraki A., **Blana V.**, Argyri A, Ammor S., Sofos J. and Nychas G.-J.E. (2008). The Role of Lactic Acid Bacteria and AI-2 Like Activity In Spoilage of Modified Atmosphere Packaged Meat Potential use. FOODMICRO 2008, 1-4 September, Aberdeen, Scotland

- Doulgeraki A., Argyri A., **Blana V.** and Nychas G.-J. E. (2008). Potential use of Essential oils for Beef preservation. Natural Products, 3–8 August, Athens, Greece

- Doulgeraki, A., **Blana, V.,** Panagou, E., Sofos, J., & Nychas G.-J. (2009). Quorum Sensing compounds and the role of lactic acid bacteria in beef spoilage. Teagasc, Ashtown Food Research Centre, Dublin

- Argyri A. A, Doulgeraki A. I., **Blana V. A.** and Nychas G.-J. E. (2009). The potential of HPLC analysis of organic acids on predicting the self life of minced beef stored under conventional and active packaging conditions. International conference on strategic and targeted controls for microbial pathogens and chemical residues in beef. Teagasc, Ashtown Food Research Centre, Dublin

- **Blana V. A.** and Nychas G.-J. E. (2009). Presence of quorum sensing phenomenon in beef products. 2nd International Conference of Food Science and Nutrition, 9 April 2009, Athens, Greece

- **Blana, V. A.,** Le Marc, Y. and Nychas, G.-J. E. (2010) Microbial interaction and quorum sensing signaling molecules in Gel Cassette system. FOODMICRO 2010, 30 August-3 September, Copenhagen, Denmark

- **Blana, V. A.** and Nychas, G.-J. E. (2010). Effect of microbial quorum sensing signal molecules (AHLs and AI-2) on the growth of spoilage bacteria. ProSafeBeef 4th Annual General Assembly, 5-7 October, Aberystwyth, Wales, UK

Journal of Food Protection, Vol. 74, No. 4, 2011, Pages 631–635
doi:10.4315/0362-028X.JFP-10-276

Research Note

Autoinducer-2–like Activity in Lactic Acid Bacteria Isolated from Minced Beef Packaged under Modified Atmospheres

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MS 10-276: Received 2 July 2010/Accepted 2 January 2011

ABSTRACT

Fifteen fingerprints (assigned to *Leuconostoc* spp., *Leuconostoc mesenteroides, Weissella viridescens, Leuconostoc citreum,* and *Lactobacillus sakei*) of 89 lactic acid bacteria (LAB) isolated from minced beef stored under modified atmospheres at various temperatures were screened for their ability to exhibit autoinducer-2 (AI-2)–like activity under certain growth conditions. Cell-free meat extracts (CFME) were collected at the same time as the LAB isolates and tested for the presence of AI-2–like molecules. All bioassays were conducted using the *Vibrio harveyi* BAA-1117 (sensor 1^-, sensor 2^+) biosensor strain. The possible inhibitory effect of meat extracts on the activity of the biosensor strain was also evaluated. AI-2–like activity was observed for *Leuconostoc* spp. isolates, but none of the *L. sakei* strains produced detectable AI-2–like activity. The AI-2–like activity was evident mainly associated with the *Leuconostoc* sp. B 233 strain, which was the dominant isolate recovered from storage at 10 and 15°C and at the initial and middle stages of storage at chill temperatures (0 and 5°C). The tested CFME samples displayed low AI-2–like activity and inhibited AI-2 activity regardless of the indigenous bacterial populations. The LAB isolated during meat spoilage exhibited AI-2–like activity, whereas the LAB strains retrieved depended on storage time and temperature. The production of AI-2–like molecules may affect the dominance of different bacterial strains during storage. The results provide a basis for further research concerning the effect of storage temperature on the expression of genes encoding AI-2 activity and on the diversity of the ephemeral bacterial population.

Quorum sensing is a cell-to-cell signaling mechanism that allows bacterial populations to sense their environment and coordinate gene expression *(33)*. Various bacterial behaviors are regulated by quorum sensing, including symbiosis, virulence, antibiotic biosynthesis, bioluminescence, sporulation, motility, plasmid transfer, and biofilm formation *(1, 6, 11)*. Among the several signaling molecules that have been identified, autoinducer (AI)-1 quorum sensing signaling molecules (*N*-acyl homoserine lactones) are produced and used by gram-negative bacteria primarily for intraspecies communication. AI-2 signaling molecules (furanosyl borate diesters) are produced by both gram-positive and gram-negative bacteria and are thought to serve as a universal signal for both intra- and interspecies communication *(1)*. Gram-positive bacteria produce and use autoinducing peptides *(18)*. Other molecules chemically similar to *N*-acyl homoserine lactones have been described, e.g., 2(5H)-furanones, which were released by *Lactobacillus helveticus* that was exposed to oxidative and heat stresses *(21)*. The 2(5H)-furanones were released during different growth phases by gram-positive bacteria such as *Lactobacillus plantarum, Lactobacillus paraplantarum, Lactobacillus sanfranciscensis,* and *Enterococcus faecalis (30)*.

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AI-1 and AI-2 signaling compounds are present and/or increase their concentrations in various food ecosystems such as meat, milk, and vegetables as the number of spoilage bacteria increases *(4, 16, 17, 22, 24)*. These compounds may be produced by the specific spoilage organisms or a smaller fraction of them, called ephemeral spoilage organisms *(1)*. However, no direct correlations have been found between the presence of signaling compounds and the presence of specific or ephemeral spoilage organisms (mainly gram-negative bacteria), which represent most of the microbial community generally associated with these food products when stored under aerobic conditions *(23)*. The bacterial strains isolated from these products have been tested for the production of these signaling compounds *(8, 12, 14, 16)*. Similar studies have not been conducted with lactic acid bacteria (LAB), which are the specific spoilage organisms on meat stored under modified atmospheres *(23)*.

The objective of the present study was to determine whether the ephemeral LAB isolated throughout spoilage of minced beef stored under modified atmospheres at various temperatures exhibit AI-2–like activity. Cell-free meat extracts (CFMEs) were collected at the same time as were samples for microbiological analysis and isolate recovery. These CFMEs were evaluated for the presence of AI-2–like

activity that could be correlated with the indigenous microbial population.

MATERIALS AND METHODS

Bacterial strains and culture conditions. From the 89 strains of LAB used in this study, 15 fingerprints were obtained. These strains were isolated from minced beef stored under modified atmospheres (40% CO_2, 30% O_2, 30% N_2) at 0, 5, 10, and 15°C *(2)*. The strains were identified using pulsed-field gel electrophoresis (PFGE) and 16S rRNA gene sequence analysis according to the methods of Doulgeraki et al. *(9)*. Throughout the storage period, relevant petri dish cultures from the highest dilution of the minced beef samples were kept. At the end of the storage period, LAB strains were isolated from three time points (initial, middle, and final stages of storage) considering the growth kinetic parameters related to LAB populations, i.e., LAB were recovered from the lag phase (initial stage), the middle of the exponential growth phase (middle stage), and the early stationary phase (final stage of storage). Isolated LAB were purified by successive subculture in de Man Rogosa Sharpe (MRS) agar (Biolife, Milano, Italy) and stored at −80°C in MRS broth (Biolife) supplemented with 20% (vol/vol) glycerol (Merck, Darmstadt, Germany). Before experimental use, each strain was grown twice in quarter-strength brain heart infusion (BHI) broth (Lab M, Bury, UK) at 30°C with agitation (160 rpm).

The *Vibrio harveyi* BAA-1117 (*luxN*::Tn*5* sensor 1^- sensor 2^+) biosensor strain, which only senses the AI-2 molecule, and the AI-2–producing *V. harveyi* BAA-1119 (*luxL*::Tn*5* AI-1^- AI-2^+) strain were used for the AI-2 activity bioassay; both strains were purchased from LGC Promochem (Teddington, Middlesex, UK) *(3)*. The *V. harveyi* strains were stored at −80°C in cryovials (Lab M). The working stock cultures were streaked onto autoinducer bioassay (AB) plates, and cells from a single colony were grown for 16 h at 30°C with agitation (160 rpm) in AB medium. The AB medium was prepared as described by Lu et al. *(17)*.

An exogenous source of AI-2–like molecules in the inhibition assays was used. The cell-free culture supernatant (CFCS) from *Salmonella enterica* serovar Typhimurium strain 4/74 ($CFCS_{ST}$) had previously produced AI-2 in our laboratory.

Preparation of CFCSs. LAB isolates were grown in quarter-strength BHI broth to avoid the effects of glucose repression on the luminosity of the *V. harveyi* BAA-1117 biosensor strain *(8)*. The isolates were incubated at 30°C with agitation (160 rpm) until early stationary phase (about 20 h). $CFCS_{LAB}$ was prepared by removing the cells from the growth medium by centrifugation at 5,000 × *g* for 15 min at 4°C in a Heraeus Fresco 21 microcentrifuge (Thermo Electron Corporation, Langenselbold, Germany). The cleared culture supernatants were filter sterilized with 0.2-μm-pore-size filters (Whatman, Clifton, NJ) and stored at −20°C until the AI-2 activity bioassays were performed.

Preparation of CFMEs. CFMEs were collected throughout minced beef storage at the same time as the LAB isolates were recovered (i.e., initial, middle, and final stages of storage). Five-gram portions of minced beef samples were homogenized with 10 ml of Ringer solution (Lab M). The CFMEs were obtained by centrifugation at 5,000 × *g* for 15 min at 4°C in a Heraeus Multifuge 1S-R centrifuge (Thermo Electron) and filtered through 0.2-μm-pore-size filters (Whatman) as described by Nychas et al. *(22)*. The supernatants were stored at −20°C until the assays were performed.

Bacterial enumeration. A detailed description of the methodology used for the enumeration of the total viable bacteria and LAB in this work was presented elsewhere *(2)*. LAB counts were determined on MRS agar (Biolife) overlaid with the same medium and incubated at 30°C for 72 h.

AI-2 activity bioassay. The AI-2 activity bioassay was performed as described by Surette and Bassler *(28)*. An overnight culture of *V. harveyi* BAA-1117 was diluted 1:5,000 with fresh AB medium. Ninety microliters of this cell suspension was mixed with 10 μl of the tested sample (i.e., $CFCS_{LAB}$ or CFME) in a 96-well polystyrene microplate (μ-Clear, Greiner Bio-One, Munich, Germany). Ten microliters of sterile growth medium (quarter-strength BHI) was used as the negative control *(15)* when screening CFCS and 10 μl of CFME of the 0-h minced beef sample was used as the negative control when screening CFME. The CFCS (10 μl) of *V. harveyi* BAA-1119 was used as the positive control to verify the bioassays.

To identify inhibition of luminescence caused by the CFME in the biosensor strain *V. harveyi* BAA-1117, an equal volume (50 μl) of meat extract and CFCS of an AI-2 producer (*Salmonella* Typhimurium) were mixed, and the AI-2 activity bioassay was performed *(17)*. The $CFCS_{ST}$ was used as a positive control (50 μl of $CFCS_{ST}$ and 50 μl of AB medium).

The microplates were incubated at 30°C, and luminescence was measured every 30 min with a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT) until the negative control exhibited an increase in luminescence *(8)*. AI-2–like activity is expressed as relative AI-2–like activity, which was calculated as the ratio of luminescence of the test sample ($CFCS_{LAB}$ or CFME) to that of the control (negative) sample. The inhibition of the AI-2–like activity was expressed as a percentage of luminescence relative to the corresponding positive control: 100 − [(relative light unit of sample/relative light unit of positive control) × 100] *(17)*. All bioassays were conducted in triplicate.

Statistical analysis. Statistical analysis was performed with a nonparametric one-way analysis of variance. Differences among replicates were considered nonsignificant ($P > 0.05$).

RESULTS AND DISCUSSION

To our knowledge, no researchers have documented AI-2 production in LAB isolated from meat and/or meat products. In a few studies, the production of AI-2 signaling molecules was found in LAB isolated from milk, dairy products, and human or animal gastrointestinal tract. These LAB were probiotic strains of *Lactobacillus* (*L. rhamnosus* GG, *L. salivarious* UCC118, *L. acidophilus* NCFM, and *L. johnsonii* NCC533) isolated from human intestine or human feces *(20)*. Several strains of *L. rhamnosus* and *Lactobacillus casei* and strains *L. plantarum* NCIMB 8826 Int-1, *L. johnsonii* VPI 11088, and *Lactococcus lactis* MG1363 originally isolated from human gastrointestinal tract and/or dairy products also produce AI-2 molecules *(8)*. AI-2 signals also were produced by the pathogen *Streptococcus suis* serotype 2, which is commonly associated with disease in pigs and humans *(15)*.

Recent reports have associated meat spoilage with quorum sensing compounds *(1)*. Because LAB are considered the ephemeral and specific spoilage organisms that contribute to spoilage of modified-atmosphere-packaged meat products, the AI-2 signals have been proposed as potential compounds that may be involved directly or

indirectly with spoilage. In this study, 89 $CFCS_{LAB}$ and 13 CFME samples were tested for the production of AI-2–like activity and the presence of the AI-2–like signaling molecules, respectively. The AI-2 activity bioassay used relies on the ability of the *V. harveyi* BAA-1117 biosensor strain to produce light in response to AI-2. The tested $CFCS_{LAB}$ were collected from equal numbers of isolates (*Leuconostoc* spp., *Leuconostoc mesenteroides, Weissella viridescens, Leuconostoc citreum,* and *Lactobacillus sakei*) recovered from initial, middle, and final stage of minced beef storage. From those isolates, 15 fingerprints were obtained. Identical isolates were tested and verified for presence or absence of relative AI-2–like activity. The isolates exhibiting AI-2–like activity are shown in Table 1. The $CFCS_{LAB}$ extracted from the *Leuconostoc* sp. type B 233 isolate expressed AI-2–like activity ranging from 12.41- to 26.84-fold compared with the negative control. No significant differences ($P > 0.05$) in AI-2–like activity were found among these identical strains regardless of the stage of storage (initial, middle, and final) and the storage temperature of the minced meat. This AI-2–like activity may explain why these bacteria can survive at the last stages of storage. The *Leuconostoc* spp. (B 232 and B 240) and *L. mesenteroides* (B 243) strains also expressed AI-2–like activity (Table 1). Quantification of AI-2 signaling molecules was not possible because there is no linear relationship between luminescence values and AI-2 signaling molecule concentrations *(31)*. Eleven fingerprints assigned to *L. sakei* (B 222, B 227, B 236, B 237, B 238, B 239), *W. viridescens* (B 234 and B 235), *Leuconostoc* sp. (B 241), *L. citreum* (B 258), and *L. mesenteroides* (B 242) did not express detectable AI-2–like activity under standard growth conditions. The isolates were propagated under certain growth conditions to promote growth and the ability of the biosensor strain to detect AI-2. AI-2 production is affected by the growth medium and external environmental factors such as temperature *(7, 29),* and components of the culture medium may promote false-negative or false-positive results *(8)*. The *luxS* genes are subject to catabolic repression by glucose; consequently, AI-2 activity cannot be detected when cells with these genes are grown in the presence of glucose *(1)*. The *luxS* gene is responsible for the production of AI-2 signaling molecules and is present in the genomes of a wide variety of gram-negative and gram-positive bacteria *(13, 33)*. Various LAB, such as *L. mesenteroides, Lactobacillus gasseri*, *L. plantarum*, *Lactococcus lactis,* and *Leuconostoc oenos,* possess a *luxS* gene *(11)*. However, many gram-positive bacteria communicate via quorum sensing autoinducing peptides, which are not detected by the AI-2 biosensor strain *(27)*. Among LAB, some strains of *L. sakei* produce this category of signaling molecules, which induce bacteriocin (sakacin P) production *(5, 10, 19)*. The absence of an AI-2 production mechanism and/or the presence of autoinducing peptides in the tested isolates would explain the results reported in this study.

All the tested CFME samples had low AI-2–like activity ranging between 0.47 and 2.24 compared with the control (negative) sample (Table 2). The control sample was CFME from the 0-h minced beef sample, which had AI-2–like activity similar to that of CFME from a ''clean'' meat sample (obtained as previously described by Nychas et al. *(22)*) and sterile growth medium (data not shown). Similar results, i.e., very low levels of AI-2 activity (less than onefold induction of luminescence compared with the negative control), have been reported in a recent study with beefsteak, beef patties, chicken breast, and turkey patties, although the indigenous population loads in that study were high (6.4 to 8.0 log CFU/ml) *(17)*. The low AI-2 activity found in CFME in comparison with those from the LAB raises questions concerning the contribution of these compounds to growth of the specific LAB during meat storage and to the spoilage process. No evidence indicates that the LAB populations were related to AI-2 activity, a possible inhibitory effect of CFME should be considered. The CFME could have inhibited the ability of the biosensor strain to react to AI-2 activity, which was determined by mixing equal volumes of the CFCS of the AI-2-producing *Salmonella* Typhimurium strain with the CFME and performing the AI-2 activity bioassay. In this study, the inhibitory effect ranged from approximately 51.11 to 91.09% (Table 2). Comparable results also were reported previously, when meat matrices were tested for inhibition of AI-2–like activity. Beefsteak and beef patties produced high levels of inhibition, 90.6 and 84.4%, when indigenous bacterial populations were 7.4 and 6.4 log CFU/ml, respectively *(17)*. Various compounds from food matrices may lead to incorrect results and false conclusions *(17, 25)*. Previous findings suggest that the presence of fatty acids (linoleic acid, oleic acid, palmitic acid, and stearic acid) isolated from ground beef and poultry meat can inhibit AI-2 activity *(25, 32)*. Food additives such as sodium propionate, sodium benzoate, sodium acetate, and sodium nitrate also may influence AI-2 production *(17)*.

In this study, the majority of the LAB produced AI-2 activity. Among the 89 isolated LAB with 15 different fingerprints, e.g., B 232, B 233, B 240, and B 243, obtained by PFGE analysis *(9),* 76.4% (68) of the isolates produced AI-2–like activity. Although the LAB isolated at the same storage times and temperatures were identical and displayed similar activity patterns, the hypothesis that these signal compounds affect the dominance of these particular strains cannot be supported with confidence, and further data are needed. At chill temperatures (0 and 5°C), isolates with 11 different fingerprints were recovered *(9),* whereas at relative high temperatures (10 and 15°C) the strain diversity was reduced to 5 different fingerprints *(9)*. Two fingerprints, B 233 assigned to *Leuconostoc* sp. and B 237 assigned to *L. sakei,* were common among those isolates obtained at both chill and relative high temperatures. At the initial stage of storage (day 0), two *Leuconostoc* spp. strains (B 232 and B 233) were recovered, and both exhibited AI-2–like activity (Table 1). At 10 and 15°C, *Leuconostoc* sp. B 233 was the dominant strain, whereas at 0 and 5°C the same strain was prevalent in the initial and middle stages of storage. Forty-four (95.7%) of the tested LAB isolated at 10 and 15°C exhibited AI-2–like activity, whereas only 18 (48.6%) of the LAB isolated at 0 and 5°C displayed AI-2–like activity. Twenty-three (95.8%) and 21 (95.5%) isolates recovered

TABLE 1. *Representative lactic acid bacteria exhibiting AI-2–like activity at each storage period*

Temp (°C)	Storage period	No. of isolates	Strains exhibiting AI-2	No. of identical isolates exhibiting AI-2	AI-2–like activity of strains[a]
	Day 0, initial flora	6	*Leuconostoc* spp. (B 233)	5	25.90 ± 11.60 A
			Leuconostoc spp. (B 232)	1	2.23 ± 0.32 B
0	Initial	5	*Leuconostoc* spp. (B 233)	2	13.28 ± 1.79 A
	Middle	6	*Leuconostoc* spp. (B 233)	5	14.81 ± 1.32 A
	Final	5		0	
5	Initial	6	*Leuconostoc* spp. (B 233)	4	22.11 ± 2.13 A
	Middle	6	*Leuconostoc* spp. (B 233)	5	18.03 ± 0.85 A
	Final	9	*Leuconostoc* spp. (B 233)	2	13.86 ± 1.89 A
10	Initial	6	*Leuconostoc* spp. (B 233)	6	13.97 ± 4.73 A
	Middle	8	*Leuconostoc* spp. (B 233)	8	13.41 ± 1.58 A
	Final	10	*Leuconostoc* spp. (B 233)	8	12.41 ± 0.53 A
			L. mesenteroides (B 243)	1	3.24 ± 0.74 B
15	Initial	6	*Leuconostoc* spp. (B 233)	6	25.73 ± 10.73 A
	Middle	8	*Leuconostoc* spp. (B 233)	8	24.71 ± 9.41 A
	Final	8	*Leuconostoc* spp. (B 233)	6	26.84 ± 13.12 A
			Leuconostoc spp. (B 240)	1	3.01 ± 1.14 B
Total		89		68	

[a] AI-2–like activity was calculated as the ratio of the luminescence of the test sample ($CFCS_{LAB}$) to that of the control (negative) sample and is presented as the mean ± standard deviation ($n = 3$). Values with the same letter are not significantly different ($P > 0.05$).

from 10 and 15°C, respectively, were positive for AI-2–like activity. The isolates that exhibited positive response in the AI-2 activity bioassay were characterized as *Leuconostoc* spp. (B 233 and B 240) and *L. mesenteroides* (B 243), and those that did not exhibit AI-2–like activity were characterized as *W. viridescens* (B 234) and *L. sakei* (B 237) *(9)*. Seven (43.8%) and 11 (52.4%) of the LAB isolates recovered at 0 and 5°C, respectively, exhibited AI-2–like activity; those isolates were all identified as *Leuconostoc* sp. (B 233). The isolates that did not exhibit any light induction at chill temperatures belonged to 10 different fingerprints: *L. sakei* (B 226, B 227, B 236, B 237, B 238, B 239, and B 241), *L. mesenteroides* (B 242), *L. citreum* (B 258), and *W. viridescens* (B 235) *(9)*. These isolates were recovered mainly from the final stages of meat storage (Table 1), where only a small fraction of isolates recovered at 5°C produced luminescence.

Nychas et al. *(22)* reported the effect of CFME containing quorum sensing molecules on the kinetic parameters of gram-negative bacteria isolated from meat, suggesting that these signals may contribute at least to the physiological behavior of bacteria during the spoilage process. Considering the potential role of these molecules for modulating microbial persistence and growth, Soni et al. *(26)* reported that the presence of AI-2 molecules promoted the survival of *Escherichia coli* O157:H7 cells, whereas the protective effect of AI-2 molecules was negated in the presence of ground beef extracts that produced significant inhibitory activity. Nevertheless, data concerning the effect of AI-2 molecules on bacterial growth and their role in food

TABLE 2. *Relative CFME AI-2–like activity, bacterial counts, and inhibition of AI-2–like activity at each storage period*

Temp (°C)	Storage period	Relative AI-2–like activity of CFME[a]	Bacterial counts (log CFU/g)	% inhibition of AI-2–like activity[b]
	Day 0, initial flora		5.26 ± 0.13	89.50 ± 0.37
0	Initial	1.07 ± 0.43	5.10 ± 0.11	84.70 ± 0.04
	Middle	1.21 ± 0.30	6.31 ± 0.24	82.92 ± 4.47
	Final	1.24 ± 0.13	7.54 ± 0.11	85.35 ± 3.30
5	Initial	1.78 ± 1.23	5.60 ± 0.39	75.76 ± 2.03
	Middle	1.49 ± 0.12	6.74 ± 0.37	81.30 ± 2.88
	Final	1.00 ± 0.53	7.24 ± 0.08	91.09 ± 0.49
10	Initial	0.59 ± 0.12	5.97 ± 0.42	83.87 ± 4.31
	Middle	0.56 ± 0.18	7.02 ± 0.17	81.62 ± 4.89
	Final	0.47 ± 0.17	8.56 ± 0.15	51.11 ± 4.89
15	Initial	2.24 ± 1.22	6.86 ± 0.08	83.55 ± 1.48
	Middle	1.01 ± 0.54	7.17 ± 0.04	85.61 ± 2.98
	Final	1.69 ± 0.91	8.44 ± 0.01	78.45 ± 1.07

[a] Relative AI-2–like activity was calculated as the ratio of the luminescence of the test sample (CFME) to the control (negative) sample and is presented as mean ± standard deviation ($n = 3$).

[b] Inhibition of AI-2–like activity was expressed as a percentage relative to the activity of the corresponding positive control.

spoilage are scarce *(1)*. Further studies are needed to explore the possible effect of these molecules produced by the ephemeral spoilage organisms on the dominance of different bacterial strains during food storage and the probability that temperature strongly affects the expression of genes encoding molecules that produce AI-2 activity and thus affects the diversity of the LAB population.

ACKNOWLEDGMENT

This work was supported by the European Union project ProSafeBeef (Food-CT-2006-36241) within the 6th Framework Programme.

REFERENCES

1. Ammor, M. S., C. Michaelidis, and G.-J. E. Nychas. 2008. Insights into the role of quorum sensing in food spoilage. *J. Food Prot.* 71: 1510–1525.
2. Argyri, A., A. I. Doulgeraki, V. A. Blana, E. Z. Panagou, and G.-J. E. Nychas. Potential of a simple HPLC based approach to quantify spoilage of minced beef stored at different temperatures and packaging systems. Submitted for publication.
3. Bassler, B. L., E. P. Greenberg, and A. M. Stevens. 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J. Bacteriol.* 179:4043–4045.
4. Bruhn, J. B., A. B. Christensen, L. R. Flodgaard, K. F. Nielsen, T. O. Larsen, M. Givskov, and L. Gram. 2004. Presence of acylated homoserine lactones (AHLs) and AHL-producing bacteria in meat and potential role of AHL in spoilage of meat. *Appl. Environ. Microbiol.* 70:4293–4302.
5. Brurberg, M. B., I. F. Nes, and V. G. H. Eijsink. 1997. Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. *Mol. Microbiol.* 26:347–360.
6. Chorianopoulos, N. G., E. D. Giaouris, Y. Kourkoutas, and G.-J. E. Nychas. 2010. Early stage of biofilm development by *Salmonella* Enteritidis on stainless steel is inhibited by the cell-free culture supernatant of *Hafnia alvei*. *Appl. Environ. Microbiol.* 76:2018–2022.
7. Cloak, O. M., B. T. Solow, C. E. Briggs, C. Y. Chen, and P. M. Fratamico. 2002. Quorum sensing and production of autoinducer-2 in *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Typhimurium in foods. *Appl. Environ. Microbiol.* 68:4666–4671.
8. De Keersmaecker, S. C. J., and J. Vanderleyden. 2003. Constraints on detection of autoinducer-2 (AI-2) signalling molecules using *Vibrio harveyi* as a reporter. *Microbiology* 149:1953–1956. (Comment.)
9. Doulgeraki, A. I., S. Paramithiotis, D.-M. Kagkli, and G.-J. E. Nychas. 2010. Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging. *Food Microbiol.* 27:1028–1034.
10. Eijsink, V. G. H., M. B. Brurberg, P. H. Middelhoven, and I. F. Nes. 1996. Induction of bacteriocin production in *Lactobacillus sakei* by a secreted peptide. *J. Bacteriol.* 178:2232–2237.
11. Federle, M. J., and B. L. Bassler. 2003. Interspecies communication in bacteria. *J. Clin. Invest.* 112:1291–1299.
12. Ferrocino, I., D. Ercolini, F. Villani, S. M. Moorhead, and M. W. Griffiths. 2009. *Pseudomonas fragi* strains isolated from meat do not produce *N*-acyl homoserine lactones as signal molecules. *J. Food Prot.* 72:2597–2601.
13. Gobbetti, M., M. De Angelis, R. Di Cagno, F. Minervini, and A. Limitone. 2007. Cell-cell communication in food related bacteria. *Int. J. Food Microbiol.* 120:34–45.
14. Gram, L., A. B. Christensen, L. Ravn, S. Molin, and M. Givskov. 1999. Production of acylated homoserine lactones by psychrotrophic members of the *Enterobacteriaceae* isolated from foods. *Appl. Environ. Microbiol.* 65:3458–3463.
15. Han, X. G., and C. P. Lu. 2009. Detection of autoinducer-2 and analysis of the profile of *luxS* and *pfs* transcription in *Streptococcus suis* serotype 2. *Curr. Microbiol.* 58:146–152.
16. Liu, M., J. M. Gray, and M. W. Griffiths. 2006. Occurrence of proteolytic activity and *N*-acyl-homoserine lactone signals in the spoilage of aerobically chill-stored proteinaceous raw foods. *J. Food Prot.* 69:2729–2737.
17. Lu, L., M. E. Hume, and S. D. Pillai. 2004. Autoinducer-2–like activity associated with foods and its interaction with food additives. *J. Food Prot.* 67:1457–1462.
18. Miller, M. B., and B. L. Bassler. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55:165–199.
19. Møretrø, T., K. Naterstad, E. Wang, I. M. Aasen, S. Chaillou, M. Zagorec, and L. Axelsson. 2005. Sakacin P non-producing *Lactobacillus sakei* strains contain homologues of the sakacin P gene cluster. *Res. Microbiol.* 156:949–960.
20. Moslehi-Jenabian, S., K. Gori, and L. Jespersen. 2009. AI-2 signalling is induced by acidic shock in probiotic strains of *Lactobacillus* spp. *Int. J. Food Microbiol.* 135:295–302.
21. Ndagijimana, M., M. Vallicelli, P. S. Cocconcelli, F. Cappa, F. Patrignani, R. Lanciotti, and M. E. Guerzoni. 2006. Two 2[5H]-furanones as possible signalling molecules in *Lactobacillus helveticus*. *Appl. Environ. Microbiol.* 72:6053–6061.
22. Nychas, G.-J. E., D. Dourou, P. Skandamis, K. Koutsoumanis, J. Baranyi, and J. Sofos. 2009. Effect of microbial cell-free meat extract on the growth of spoilage bacteria. *J. Appl. Microbiol.* 107:1819–1829.
23. Nychas, G.-J. E., P. N. Skandamis, C. C. Tassou, and K. Koutsoumanis. 2008. Meat spoilage during distribution. *Meat Sci.* 78:77–89.
24. Pinto, U. M., E. S. Viana, M. L. Martins, and M. C. D. Vanetti. 2007. Detection of acylated homoserine lactones in gram-negative proteolytic psychrotrophic bacteria isolated from cooled raw milk. *Food Control* 18:1322–1327.
25. Soni, K. A., P. Jesudhasan, M. Cepeda, K. Widmer, G. K. Jayaprakasha, B. S. Patil, M. E. Hume, and S. D. Pillai. 2008. Identification of ground beef–derived fatty acid inhibitors of autoinducer-2–based cell signaling. *J. Food Prot.* 71:134–138.
26. Soni, K. A., P. Jesudhasan, M. E. Hume, and S. D. Pillai. 2008. Influence of autoinducer-2 (AI-2) and beef sample extracts on *E. coli* O157:H7 survival and gene expression of virulence genes *yadK* and *hhA*. *J. Food Sci.* 73:136–139.
27. Sturme, M. H. J., M. Kleerebezem, J. Nakayama, A. D. L. Akkermans, E. E. Vaughan, and W. M. de Vos. 2002. Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antoine Leeuwenhoek* 81:233–243.
28. Surette, M. G., and B. L. Bassler. 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* 95:7046–7050.
29. Surette, M. G., and B. L. Bassler. 1999. Quorum sensing in *Escherichia coli, Salmonella typhimurium* and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc. Natl. Acad. Sci. USA* 96:1639–1644.
30. Vannini, L., M. Ndagijimana, P. Saracino, P. Vernocchi, A. Corsetti, M. Vallicelli, F. Cappa, P. S. Cocconcelli, and M. E. Guerzoni. 2007. New signaling molecules in some gram-positive and gram-negative bacteria. *Int. J. Food Microbiol.* 120:25–33.
31. Vilchez, R., A. Lemme, V. Thiel, S. Schulz, H. Sztajer, and I. Wagner-Döbler. 2007. Analysing traces of autoinducer-2 requires standardization of the *Vibrio harveyi* bioassay. *Anal. Bioanal. Chem.* 387:489–496.
32. Widmer, K. W., K. A. Soni, M. E. Hume, R. C. Beier, P. Jesudhasan, and S. D. Pillai. 2007. Identification of poultry meat–derived fatty acids functioning as quorum sensing signal inhibitors to autoinducer-2 (AI-2). *J. Food Sci.* 72:M363–M368.
33. Xavier, K. B., and B. L. Bassler. 2003. LuxS quorum sensing: more than just a numbers game. *Curr. Opin. Microbiol.* 6:191–197.