CRANFIELD UNIVERSITY

Cranfield Health

PhD Thesis

AGAPI I. DOULGERAKI

MONITORING THE SUCCESSION OF BACTERIAL COMMUNITIES DURING STORAGE OF RAW MEAT

Supervisors:

Dr. David Aldred

Prof. Naresh Magan

Prof. George - John Nychas

2010

Abstract

Fresh meat is exposed to various factors which cause microbiological contamination during handling, processing, packaging and storage. Furthermore, the storage conditions applied may affect the microbial association of the product and consequently the spoilage process. Therefore, the purpose and importance of this study was to identify areas that should be addressed to monitor the succession of bacterial communities during storage of raw meat. The improvement of the microbiological quality and safety of meat was also studied.

The observed differences in microbial quality of samples showed that packaging can play a significant role in extending the shelf life of fresh meat, since the growth of aerobic microorganisms was prevented in meat under modified atmosphere packaging (MAP). When the minced beef was stored aerobically all the microbial groups showed viable counts higher than those of the other packaging conditions adopted (MAP-, MAP+). More specifically, total viable counts levels were suppressed for 1.9 and 2.15 log cfu g^{-1} under MAP- and MAP+ at 0°C, respectively. Additionally, growth of *Listeria monocytogenes* occurred in minced beef stored aerobically, although limited growth was observed under MAP with or without volatile compounds of oregano essential oil. These results revealed that, volatile compounds of oregano essential oil in conjunction with MAP could be used to control the microbial loads and colour change to acceptable levels and as a more effective system for extending the shelf life and increase the safety of meat.

Culture – dependent (PFGE, species specific PCR, SDS-PAGE, sequencing analysis) and - independent (PCR-DGGE) methods were applied to provide an insight of the population dynamics of bacteria in relation to storage conditions. Nevertheless, the main findings of the present study were based on PFGE. This culture dependent approach has provided important information in relation to the strain distribution of the microbiota which would have not been acquired if strain typing had not been

performed. In the latter case, a modified PFGE protocol i.e. addition of thiourea after the proteinase treatment, was successfully developed with all *Enterobacteriaceae* isolates that were previously untypeable now producing high quality fingerprints; this is the first time that thiourea was introduced in a step during the preparation of agarose inserts.

It has been shown that storage temperature combined with packaging conditions induced the selectivity of the spoilage microbiota at a species and/or strain level, while the microbiota recovered from the initial stage of storage was markedly different from that at the final stage of storage at chill temperatures. More accurately, within the LAB population obtained, *Leuconostoc* spp. (*Ln. mesenteroides* in the case of beef fillets) and *Lactobacillus sakei* were identified as significant members of the microbiota at abuse and chill temperatures, respectively. Moreover, *Serratia liquefaciens* represented the dominant isolate of *Enterobacteriaceae* during storage of minced beef for most conditions adopted, but 10 and 15 °C under MAP + and 10 °C under MAP –; in the latter case, *Hafnia alvei* represented the dominant fingerprint. In the case of beef fillets, *S. liquefaciens*, *Serratia* spp., *Klebsiella oxytoca*, *Enterobacter ludwigii* and *E. cloacae* were common at 0, 5, 10, 15 and 20°C. Additionally, four *Enterobacteriaceae* strains isolated from beef fillets could not be assessed to genus level, leading to the possibility that new bacterial species were detected. Furthermore, different pseudomonads strains dominated the Pseudomonas Agar Base (PAB) community of beef, whereas *Ps. fragi* was recovered from fresh beef.

The overall outcome of the present study has been clearly demonstrated that certain species and/or strains are present or dominant only under certain conditions. These observations seem to be of great importance and fundamental in understanding the spoilage process in order to widen the knowledge of the spoilage related bacterial succession during storage of meat.

Acknowledgements

I would like to express my appreciation to Prof. Naresh Magan, Dr. David Aldred and Prof. George – John Nychas for their advice and time all the way through this thesis.

I am grateful to my colleagues and best friends Anthoula A. Argyri and Vasiliki A. Blana for our collaboration, good atmosphere and the meaningful time that we spent throughout.

A big thank to my closest friends for being a great soul and for their love, patience and understanding in the last years.

I wish to thank my parents Ioannis and Evaggelia and my lovely brother Markos for their continual support and for providing me with the opportunity to become this thesis real.

Table of contents

List of Tables

List of Figures

Chapter 1

Introduction and Literature Review

1.1. An overview

Meat is recognized as one of the most perishable foods. Apart from the physical damage, oxidation and colour change, the other spoilage symptoms are due to the undesirable growth of microorganisms to unacceptable levels. Many groups of organisms contain members that potentially contribute to meat spoilage under appropriate conditions (Ercolini et al. 2006). Within a certain range of environmental conditions, often only one microbial species from the total microbiota is responsible for spoilage (specific spoilage organisms - SSO or ephemeral spoilage organisms - ESOs) (Nychas et al. 2007). One of the main factors that influences microbial spoilage is the temperature, which is related to the maximum specific growth rate and lag phase duration (LPD) of microorganisms (Mataragas et al. 2006).

The populations of specific species which cause spoilage are determined by the packaging conditions. *Pseudomonas* spp. and *Brochothrix thermosphacta* have been reported as ESOs in raw meat stored under aerobic and vacuum (VP)/modified atmosphere (MA) conditions respectively, while lactic acid bacteria (LAB) have been identified as ESOs in cooked and cured meat products (Mataragas et al. 2006; Nychas et al. 2007). Moreover, it is well known that during storage of meat and meat products, depending on the environmental conditions the range of bacterial species changes. This was emphasised by Ercolini et al. (2006) who reported that different spoilage related bacterial species were found according to packaging conditions and time of storage, although similar viable counts were observed. Also it has been reported that only a small fraction of microorganisms is analyzed by conventional methods and often the isolated strains do not seem to represent the real spectrum of microorganisms (Engelen et al. 1998; Ampe et al. 1999). In contrast, culture independent methods seem to overcome problems associated with selective

cultivation and isolation of bacteria from natural samples (Fontana et al. 2006). On the other hand, the latter methods are estimated to detect only the dominant microbial community representing a small fragment of the total microbial population in an environmental sample (Nocker et al. 2007).

This chapter will focus on the microbiology of meat, trends in meat consumption, extension of shelf life of meat, future developments in modified atmosphere packaging, as well as the strategies for detection and identification of bacteria in meat.

1.2. Microbiology of meat

The microbial quality of meat depends on the physiological status of the animal at slaughter, the spread of contamination during slaughter and processing, the temperature and other conditions of storage during distribution (Nychas et al. 2008). When large numbers of undesirable microorganisms are present in raw meat, contaminated cooked or fermented food supplies, they compete for space and utilization of food nutrients; these are considered to be spoilage microorganisms. Occasionally, the effect of consuming the food is minimal. However, if the contamination of bacteria levels is too high, then there will be undesirable physical, chemical and biochemical changes such that the food becomes unappealing and unsuitable for human consumption (Fung 2010).

Spoilage occurs as a direct consequence of the development of a microbial association. Competition between species under the selective conditions found on meat surfaces tends to produce a climax population, the spoilage microbiota, e.g., ESOs (Stanbridge & Davies 1998; Nychas et al. 2008; Vasilopoulos et al. 2010).

These ecological strategies, developed by the ESO, are the consequence of environmental determinants and allow them to proliferate in all available niches (Nychas et al. 2008). In this study emphasis will be given to bacteria associated with meat spoilage, sources of microbial contamination on meat as well as the influence of environmental factors on bacterial growth and shelf life.

1.2.1. Bacteria associated with meat spoilage

Spoilage bacteria commonly found on meat include *Pseudomonas* spp., *Acinetobacter* spp., *Moraxella* spp., *Psychrobacter* spp., *Aeromonas* spp., *Shewanella putrefaciens*, *Enterobacteriaceae*, *Br. thermosphacta*, *Micrococcaceae*, *Clostridium* spp. and lactic acid bacteria (LAB) (Dainty & Mackey 1992; Borch et al. 1996; Lambert et al. 1991; Adams & Moss 1995; Drosinos & Board 1995; Nychas et al. 2007; Table 1.1). Most spoilage bacteria found in meat are saprophytic Gram-negative and include aerobic and facultative anaerobic psychrotrophic strains (*Pseudomonas* and related genera), while Gram-positive (LAB, *Micrococcus*) can also be found in high numbers (Samelis 2006). However, few species dominated spoilage. The spoilage bacteria considered in this study are the main groups of bacteria responsible for the spoilage of meat i.e. pseudomonads, *Enterobacteriaceae* and LAB as well as *Br. thermosphacta*.

A. Pseudomonads are Gram negative rods which constitute a large genus of bacteria that exists in fresh foods. The genus *Pseudomonas* consists of five phylogenetic groups based on rRNA similarity studies (Palleroni 1993) with the most important meat spoilage species being assigned to the first group including *Ps. aeruginosa*, *Ps. fluorescens*, *Ps. putida*, *Ps. chlororaphis*, *Ps.*

cichorii, *Ps. viridiflava* and *Ps. syringae* (Garcia-Lopez et al. 1998). They are by far the most important group of bacteria that bring about the spoilage of refrigerated fresh food as well as the most common saprophytic organisms found in meat (Jay 2000). Phenotypic and molecular characterization of the phsychrotrophs isolated from fresh and spoiled meat revealed the presence of three major species of *Pseudomonas* (*Ps. fragi*, *Ps. fluorescens* and *Ps. lundensis*) (Liao 2006). Their phychrotrophy, very fast rate and high affinity for oxygen have been suggested as the main reasons for the predominant growth of the above pseudomonas species in air packed fresh meats (Gill & Newton 1977). These Gram negative, strict aerobes use glucose as their primary substrate. Once the glucose present in the meat system has been totally consumed by the bacteria, amino acids are utilized and malodorous compounds such as sulfides, esters, acids, and others are formed as by-products (Miller et al. 1973). A large proportion of pseudomonads are capaple of producing extracellular proteases, lipases, thus the spoilage caused by these bacteria is indicated by slimy or mushy appearance, production of off odours and partial or complete degradation of animal tissues (Liao 2006). More accurately, the ability of *Ps. fluorescens*, *Ps. lundensis* and *Ps. fragi* strains to cause spoilage of proteinaceous foods is in part due to their ability to produce proteases and lipases for degradation of protein or lipid components in meat, milk, poultry and seafood (Odagami et al. 1994).

B. Lactic acid bacteria are a heterogenous group of Gram positive organisms. In common they are catalase negative, non-spore forming, strictly fermentative, facultative anaerobic and producing lactic acid as a major product of glucose fermentation (Stanbridge & Davies 1998). Genetic diversity and habitat

variation are considerably wide within LAB, while species reported to dominate in meat and meat products include the genera of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, and *Weissella* **(**Schillinger & Holzapfel 2006). More accurately, *Lactobacillus* spp., *Carnobacterium* spp. and *Leuconostoc* spp. can play an important role in the spoilage of refrigerated raw meat (Labadie 1999) and are also recognized as important competitors of the other spoilage related microbial groups under VP/MAP conditions (Gill 1996; Tsigarida et al. 2000; Castellano et al. 2004; Nychas & Skandamis 2005). They can also become dominant throughout storage including under reduced O_2 tension environment (Lambert et al. 1991). Sour flavour and off odour, formation of CO_2 resulting in bulging of packages, slime formation and discolorations are typical sensory changes involved in LAB meat spoilage (Schillinger & Holzapfel 2006). Spoilage LAB are not associated with formation of cadaverine and putrescine as the Gram negative meat spoilage bacteria are. In general they do not produce malodourous substances (Dainty et al. 1975), but tyramine is produced by *Carnobacterium* spp. (Edwards et al. 1987). Small concentrations of dimethylsulphide and methanethiol have been associated with sour odour typical of VP/CO_2 stored meat stored for long period (Dainty & Mackey 1992).

C. *Enterobacteriaceae* is a large group of Gram negative, rod shaped, non-spore forming, and facultative anaerobic bacteria. Currently, the family comprises at least 34 genera, 149 species and 21 subspecies (Baylis 2006). Members of the *Enterobacteriaceae* are widely distributed in nature and the environment, thus it is therefore inevitable that some members of the *Enterobacteriaceae* will enter the food chain where they can be responsible for causing food-borne

disease and food spoilage. Although more attention is generally paid to the pathogenic properties of the particular genera of *Enterobacteriaceae*, some members of the family constitute an important spoilage group when conditions favour their growth (Stanbridge & Davies 1998; Nychas et al. 2008). Members of the family *Enterobacteriaceae* do not become a numerically dominant part of the microbial association on meats, but they may contribute to spoilage (Stanbridge & Davies 1998). Several workers have detected enterobacteria of the genera *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Kluyera* (less commonly), *Proteus*, and *Serratia* on raw beef, lamb, pork, and poultry products, as well as on offal meats (Garcia-Lopez et al. 1998). With regard to their meat spoilage potential, the most important *Enterobacteriaceae* are the species *Serratia liquefaciens*, *Hafnia alvei* and *Enterobacter* (*Pantoea*) *agglomerans* (Samelis 2006). Similarly to pseudomonads, *Enterobacteriaceae* utilize glucose and glucose-6-phosphate as substrates and some strains produce sulfides and malodorous diamines (putrescine and cadaverine) as by-products (Gill 1986, Lambert et al. 1991). More accurately, *H. alvei* and *S. liquefaciens* produce malodorous diamines (putrescine and cadaverine), while a green discoloration of the meat was associated with the growth of these two organisms (Stanbridge & Davies 1998). Furthermore, synergism was found between *S. liquefaciens* and *H. alvei* and those lactic acid bacteria utilizing arginine to produce orthithine, which is converted to putrescine (Edwards et al. 1985).

D. *Brochothrix thermosphacta* is a Gram positive facultative anaerobic rod that can occur either singly or in chains. It is found in soil and water and is a common inhabitant of the intestinal track of animals, while it has been isolated

from a wide range of food types including beef, lamb, pork, fish, frozen vegetables and dairy products (Betts 2006). *Br. thermosphacta* is an important meat spoilage bacterium and often associated with the spoilage of fresh and cured meats (Samelis 2006). How and why fresh meat has become the main ecological niche of *Br. thermosphacta* is still unclear, given that this species has a very low incidence in farm samples (soil, hay, faeces) (Labadie 1999). The spoilage potential of the latter in fresh meat is fairly high, and seems to be due to production of acetic and butyric acids, acetone and alcohols and a range of fatty acids from aerobic spoilage, which significantly contributes to off odour development, i.e., giving rise to sour, musty, acidic or sweaty odours (Betts 2006; Samelis 2006). These compounds are produced by *Br. thermosphacta* only during aerobic metabolism, while anaerobic metabolism's major product is lactic acid (Gill 1986).

1.2.2. Sources of microbial contamination on meat

In muscle tissues of healthy live animals, the bacteria are absent, undetectable, or at extremely low populations (Gill 2005). The defensive mechanisms (e.g. skin, hair, mucous membrane) which present barriers to the entry of microorganisms into the muscle of live animals, are destroyed at slaughter. The resulting meat becomes exposed to increasing levels of contamination and may undergo rapid microbial decay (Nychas et al. 2007).

Table 1.1. Bacteria commonly found on meats and poultry.

Microorganisms	Gram reaction	Fresh	Processed
Achromobacter	−	X[1]	
Acinetobacter	−	XX[1]	X
Aeromonas	−	XX	X
Alcaligenes	−	X	
Alteromonas	−	X	X
Arthrobacter	±	X	X
Bacillus	+	X	X
Brochothrix	+	X	X
Campylobacter	−	X	
Carnobacterium	+	X	
Chromobacterium	−	X	
Citrobacter	−	X	
Clostridium	+	X	
Corynebactenum	+	X	X
Enterobacter	−	X	X
Enterococcus	+	XX	X
Escherichia	−	X	
Flavobacterium	−	X	
Hafnia	−	X	X
Janthinobacterium	−		X
Klebsiella	−	X	
Kluyvera	−	X	
Kocuria	+	X	X
Kurthia	+	X	
Lactobacillus	+	X	XX
Lactococcus	+	X	
Leuconostoc	+	X	X
Listeria	+	X	X
Microbacterium	+	X	X
Micrococcus	+	X	X
Moraxella	−	XX	
Paenibacillus	+	X	X
Pantoea	−	X	
Proteus	−	X	
Providencia	−	X	X
Pseudomonas	−	XX	X
Shewanella	−	X	X
Staphylococcus	+	X	X
Streptococcus	+	X	X
Vibrio	−	X	
Weissella	+	X	X
Yersinia	−	X	X

Based on Nychas et al. 2007
[1]X = known to occur, XX = most frequently isolated.

Microbial contamination of raw meat results from processing, and starts during slaughter, when the carcass becomes contaminated with microorganisms residing on external surfaces, the gastrointestinal tract and lymph nodes of the animal,

and in the plant environment (Samelis 2006). Sources of these microbial contaminants include the slaughter animals themselves (external surfaces of the animal and the gastrointestinal tract), slaughter facility equipment (grinders, belts, saws), process workers (hand contact, knives) and exposure to other environmental sources (water, faeces and the animal hides) (Ayres 1955; McMeekin 1981; Gill 1998; Narashima Rao et al. 1998; Samelis 2006; Nychas et al. 2007; Fernandes 2009). Airborne contamination of carcasses is also important (Gustavsson & Borch 1993; Rahkio & Korkeala 1997). Furthermore, certain processing steps increase contamination by spreading the existing contaminants attached to the fresh meat surface to its entire mass or by introducing additional contaminants. For example, meat chopping or grinding results in greater microbial loads because of larger areas of exposed surface, more readily available water and nutrients, additional processing time, and contact with more sources of contamination such as equipment (Hedrick et al. 1994).

A wide spectrum of Gram negative bacteria (*Pseudomonas*, *Acinetobacter*, *Serratia*, *Enterobacter*, *Proteus* and *Vibrio*) were recovered from hides and work surfaces within the abattoir, from carcasses, butchered meat as well as from environmental samples in meat processing plants (von Holy et al. 1992; Gill 2005; Nychas et al. 2008). Moreover, members of the family *Enterobacteriaceae* are successful colonizers of wet environments in the structural and work surfaces within the abattoirs (Newton & Gill 1978). In a survey of microbial levels for incoming raw beef, environmental sources, and ground beef in a red meat processing plant, microorganisms have been isolated from beef during all steps of ground beef processing including the outer surfaces of beef carcasses, from boxed beef, from retail cuts and from ground beef (Eisel et al. 1997). Commonly, isolated spoilage microorganisms include genera in the family *Enterobacteriaceae*, *Shewanella*

putrefaciens, *Br. thermosphacta*, *Pseudomonas* spp. *Acinetobacter* spp., *Moraxella* spp., *Aeromonas* spp. and lactic acid bacteria (Silliker 1980; McMeekin 1981; Gill 1983; Nychas et al. 2007). Many different types of pathogenic microorganisms have also been isolated from raw beef, most notably *Salmonella* spp. *Listeria monocytogenes*, *Escherichia coli*, and *Campylobacter jejuni* (Silliker 1980; Bracewill et al. 1985; Cottin et al. 1985).

Perhaps the most common group of bacteria employed as indicator organisms by the food industry are the coliforms, which can be regarded as a subgroup within the *Enterobacteriaceae*; besides their use as indicator organisms, some groups of bacteria or individual species are used to assess the potential risk of closely related pathogens being present in food and water (Baylis 2006). The minimization of microbial contamination is essential in meat handling systems in order to retard meat spoilage as well as to prevent health hazards that may arise from meat consumption.

1.2.3. Environmental factors influences on bacterial growth and shelf life

The survival and growth of spoilage specific bacteria on foods can be affected by a diversity of environmental factors in the physical and chemical environment. These factors including temperature, pH, water activity, meat constituents, atmospheric oxygen (packaging atmosphere) and competing microbiota are important in maintaining a quality meat product over an extended period of time (Lambert et al. 1991; Koutsoumanis et al. 2006). The main factors consider in this study were temperature and packaging atmosphere.

A. Temperature

Temperature is considered the most important factor affecting meat spoilage. Microbial growth can occur over a temperature range from – 5°C up to 90°C at atmospheric pressure (Table 1.2). The most important requirement is that water should be present in the liquid state and thus available to support growth (Adams & Moss 1995). Temperature influences the microbial spoilage by affecting the lag phase duration, the maximum specific growth rate and the final cell numbers (Labuza & Fu 1993; Mataragas et al. 2006).

In food microbiology, mesophilic and phychrotrophic organisms are generally of greatest importance. Spoilage of perishable products stored in the mesophilic growth range is more rapid than spoilage under chill conditions (Adams & Moss 1995). The growth rate of psychotropic species is temperature dependent and becomes increasingly slower as the temperature is reduced (Abd El-Rhman et al. 1998). Therefore, shelf time or the rate of quality loss and subsequent spoilage of a refrigerated food is also highly temperature dependent (Tompkin 1973). It is presumed that the temperature will also affect the multiplication rate below the detection limit, but how this is affected is unknown (Shironi & Labuza 2000).

Although most countries have established regulations with maximum temperature limits for refrigeration storage, in practice these are often violated. In South European countries 30% of refrigerated foods were kept above 10°C in retail cabinets and household refrigerators and even in North Europe 5% were above 13°C in retail and 21% above 10°C in households (Kennedy et al. 2005). Abuse temperatures during any stage of the chill chain may result in an unexpected loss of quality and a significant decrease of meat shelf life (Koutsoumanis et al. 2006).

Table 1.2. Cardinal temperatures for microbial growth (ICMSF 1980).

Group	Temperature (°C)		
	Minimum	**Optimum**	**Maximum**
Thermophiles	40-45	55-75	60-90
Mesophiles	5-15	30-40	40-47
Psychrophiles	-5-+5	12-15	15-20
Psychrotrophs	-5-+5	25-30	30-35

B. Packaging atmosphere

Growth and survival of spoilage specific microorganisms are greatly affected by the gaseous composition of the atmosphere surrounding foods (Table 1.3). For example, aerobic storage of chilled red meat unwrapped or covered with an oxygen permeable film, is suitable for the psychrotrophic aerobes with non fermentative Gram negative rods grow rapidly and dominate the spoilage microbiota (Adams & Moss 1995). The principal genera are described as *Pseudomonas* spp., *Acinetobacter* spp. and *Psychrobacter* spp., with *Ps. fragi*, *Ps. lundensis* and *Ps. fluorescens* generally predominating. Their psychrotrophy and high affinity for oxygen have been suggested as the main reasons for the predominant growth of the above pseudomonad species in air packaged or high-O_2 MAP fresh meats, since these properties presumably lead to rapid glucose uptake (Gill & Newton 1977; Gill 1982; Gill & Molin 1991).

At the start of the 19th Century it was believed that contact with air caused putrefaction and that food preservation techniques worked by excluding air (Adams & Moss 1995). It has been known also, that the shelf life of meat can be extended by an

increase in the concentration of carbon dioxide in the storage atmosphere (Stanbridge & Davies 1998). Packaging can be an effective method for meat shelf life extension that avoids the use of chemical preservatives (Brody 1996; Nattress & Jeremian 2000). Also, packaging plays a significant role in meat handling practices, imparts attractiveness to the product and protects the meat from moisture loss, contamination by microorganisms, changes in colour and physical damage.

Table 1.3. Specific spoilage microorganisms found in raw meat at 0 - 4°C stored under different packaging systems[1].

Packaging system	Meat and Poultry
Air	*Pseudomonas* spp.
>50% CO_2 with O_2	*Brochothrix thermosphacta*
50% CO_2	*Enterobacteriaceae*, lactic acid bacteria
<50% CO_2 with O_2	*Br. thermosphacta*, lactic acid bacteria
100% CO_2	lactic acid bacteria
Vacuum	*Pseudomonas* spp., *Br. Thermosphacta*

[1]Modified table based on Nychas et al. 2007

In modified atmosphere packaging, a pack is flushed through with a gas mixture usually containing some combination of carbon dioxide, oxygen and nitrogen in order to inhibit the different spoilage-related bacteria and are often associated with the use of low temperatures during storage (Faber 1991).The initial gas composition is chosen so that the changes which occur do not have a profound effect on the product stability. Carbon dioxide is included for its inhibitory effect and to retard the growth of organisms produced by aerobic spoilage, nitrogen is non-inhibitory but has low water solubility and can therefore prevent pack collapse when high concentrations of carbon dioxide are used (Adams & Moss 1995, Gill 2003). Oxygen is included in gas

mixtures for the retail display of red meats to maintain the bright red appearance of oxymyoglobin (Young et al. 1988; Jeremiah 2001).

A main objective of modified atmosphere packaging is to exert its effect principally through the inhibition of aerobic Gram negative bacteria, especially pseudomonads which are responsible for quick spoilage of meat (Insausti et al. 2001). The use of CO_2 often allows the growth of LAB such as *Lactobacillus* spp. and *Leuconostoc* spp. that develop a homolactic or heterolactic metabolism (Lee et al. 1983) and thus outcompeting *Enterobacteriaceae*, *Pseudomonas* spp., and *Br. thermosphacta* (Stanbridge & Davies 1998). Moreover, the use of high CO_2 concentrations, together with low pH and chill storage, can more readily inhibit the growth of food pathogens than vacuum packaging (Garcia de Fernando et al. 1995). Overall, it should be stressed that LAB spoilage is far less offensive than putrid types of spoilage caused by Gram negative meat borne bacteria not only because of the shift from aerobic to vacuum or MAP conditions, but also because LAB are weakly proteolytic (Law & Kolstad 1983).

In conclusion, MAP contributes to the extension of the shelf life of meat: (i) by reducing the growth rate of specific spoilage bacteria (ii) by delaying the deterioration of meat colour and retaining the fresh meaty odour and (iii) by decreasing the rate of consumption of glucose and lactate, the limitation of which also affected the metabolic products produced by the microbial association of meat (iv) by producing relatively inoffensive compounds compared to typical spoilage odours produced by pseudomonads (Nychas et al. 1998; Skandamis & Nychas 2001). Nevertheless limited information are provided for the characterization of the specific spoilage microorganisms at species and/or strain level.

1.3. Trends in meat consumption

Meat has played a significant role in the human diet. This is due to its desirable texture and flavour characteristics although meat protein also has a high biological value. Furthermore, meat consumption is often something of a status symbol and is generally far greater in wealthy societies. Growing numbers of people world-wide consume red meat, particularly beef. This has resulted in large amounts of meat being transported long distances to satisfy the market.

In the latter part of 20th century, with the development of vast warehouses, a wide range of food and other domestic household goods are sold in the same place. Stanbridge & Davies (1998) noted that the general change in the lifestyle of the people of Western Europe has tended to decrease the patronage of local butchers shops, with supermarkets assuming dominant role. Also it is important for the consumers to be able to easily select the fresh meat they want to buy, based on meat colour, the quantity, quality and the price. The consumers demand has resulted in the re-orientation of the supermarkets and the change in packaging of fresh meat, either in grease – proof paper wrapping or plastic bags, to the display of meat on a foam tray with a covering of gas permeable film or in modified atmosphere package. Those packaging systems also protect the meat surfaces from casual contamination, retain moisture and colour changes. The industries commonly use the modified atmosphere packaging to enable the promotion of an acceptable product. Nowadays, as the industries increase their supplies to the supermarkets with fresh meat packed in modified atmosphere, it is more feasible for the consumers to be aware of the meat origin.

1.4. Extending shelf life of meat - future developments in modified atmosphere packaging

Numerous methods are available to control spoilage and thus extend the shelf life of meat. Novel preservation technologies include the application of the concept of active packaging, bioprotective cultures and natural antimicrobial compounds such as essential oils and other phytopreservatives, enzymes and bacteriocins, while all preservation treatments may also be used in combination to make use of synergististic or additive effects (Schillinger & Holzapfel 2006). For example, modified atmosphere packaging (MAP) has gained considerable popularity as a modern method for food preservation. The combination of carbon dioxide, nitrogen and oxygen in MAP packs is able to suppress the aerobic spoilage biota of perishable foods, such as meat, and to sustain their visual appearance (Davies 1995). However, it is important to store the foods at 10°C or below to maximize the effect of the increased concentration of CO_2 (Liao 2006). To date, most efforts to determine spoilage by chemical/biochemical means have questionable results under practical application, probably due to the fact that such measurements are likely to be influenced by the packaging method (e.g. VP/ MAP), or the use of preservatives, including essential oils, since the latter act as additional hurdles on the microbial association (Davies 1995; Tassou et al. 1996; Tsigarida et al. 2000).

The excessive use of chemical preservatives, many of which are suspect because of their potential carcinogenic and teratogenic attributes or residual toxicity, has resulted in increasing pressure on food manufacturers either to completely remove chemical preservatives from their food products or to adopt alternatives that consumers conceive as “natural”. Consequently, there is considerable research interest in the possible use of natural products, such as essential oils and extracts of edible and

medicinal plants, herbs, and spices, for the development of alternative food additives in order to prevent the growth of food-borne pathogens or to delay the onset of food spoilage (Skandamis & Nychas 2001; Skandamis & Nychas 2002; Chorianopoulos et al. 2004). Researchers have also examined the use of LAB as biopreservatives in foods because they are suitable as antagonistic microorganisms and are capable of inhibiting other food-borne or spoilage bacteria by producing bacteriocins (Aguirre & Collins 1993; Mauriello et al. 2004).

1.4.1. Essential oils

The use of essential oils from herbs and spices in foods as preservatives is limited because of flavour considerations, since effective antimicrobial doses may exceed organoleptically acceptable levels (Nychas et al. 2003), although the majority of them are classified as Generally Recognized As Safe (GRAS) (Kabarana 1991). According to Conner et al. (1984) the antimicrobial action of essential oils may be due to impairment of a variety of enzyme systems including those involved in energy production and structural component synthesis. The antimicrobial action of essential oils in model or real food systems is well documented in the literature (Tassou et al. 1996; Koutsoumanis et al. 1998; Koutsoumanis et al. 1999; Skandamis & Nychas 2000; Tsigarida et al. 2000). The efficacy of essential oils in vitro is often much greater that *in vivo* (Davidson 1997; Nychas & Tassou 2000). The type of oil or fat present in a food and the bounds between active compounds of essential oils and food components (e.g. proteins, fats, sugars, salts) can affect the antimicrobial efficacy of essential oils (Nychas et al. 2003).

The addition of essential oils (e.g. oregano, mint, finely ground rosemary) in foods, such as liver sausages, aubergine salad, fish, pate, tarama salad, tzatziki and

sterile beef extracts has been found to inhibit *L. monocytogenes*, *Staphylococcus aureus, Esch. coli, Salmonella* spp. under aerobic conditions (Aureli et al. 1992; Pandit & Shelef 1994; Tassou et al. 1995; Tassou et al. 1996; Cutter 2000; Skandamis & Nychas 2000; Tsigarida et al. 2000). The Gram positive bacteria (*Staph. aureus*, L. *monocytogenes*, and *Bacillus cereus*) are more susceptible to essential oils than the Gram negative bacteria (*Esch. coli* and *Salm. enteritidis*) (Ouattara et al 1997; Mangena & Muyima 1999). Moreover, Chorianopoulos et al. (2004) reported that volatile compounds of essential oils from *Satureja* plants had a strong inhibitory effect against Gram positive bacteria while in the case of Gram negative bacteria, a differentiation on the rate of their metabolic activity was observed.

Essential oils derived from plants of *Origanum* and *Thymus* species (Lamiaceae family) have been found to possess significant antifungal, insecticidal, and antimicrobial activities (Cosentino et al. 1999; Aligiannis et al. 2001). Studies of the effect of oregano and rosemary essential oils on spoilage biota and pathogenic microorganisms in meat and fish, stored aerobically, revealed that bacterial counts were significantly suppressed in both (Tassou et al. 1996; Tsigarida et al. 2000). Moreover, oregano essential oil, as a potential `hurdle', was found to affect the contribution of spoilage microorganisms to the microbial association as well as to the physico-chemical changes of the minced meat (Skandamis & Nychas 2001; Burt 2004). The volatile compounds of oregano essential oil are also capable of affecting both growth and metabolic activity of microbial association of meat stored at modified atmospheres (Skandamis & Nychas, 2002), however, such inhibition is not as strong as that due to the contact of pure essential oil with microorganisms when this is added directly on the surface of meat (Skandamis & Nychas, 2001; Tsigarida et al. 2000). The volatile compounds of oregano essential oil can expand its application

to extend the shelf life of meat (Skandamis & Nychas 2002) by (1) delaying of growth of specific spoilage organisms, (2) inhibiting or restricting their metabolic activity that cause spoilage through the production of spoilage microbial metabolites and (3) by minimizing the flavour consideration.

Axelsson (1998) concluded that the addition of oregano essential oil influenced the metabolic activity of LAB. More specifically, the initial heterofermentative microbiota was substituted by a homofermentative one at the end of storage. However, despite the antimicrobial action of essential oil on biota, there is less information about the effect of such compounds on the microbial diversity of the LAB or bacteria in general isolated from meat at species and strain level. The only information available relates the essential oil effect *in vitro* on growth of meat spoilage bacteria such as *Lb. sakei*, *Lb. curvatus* and *Carnobacterium piscicola, Br. thermosphacta, Ps. fluorescens* and *S. liquefaciens* (Ouattara et al. 1997).

1.5. Strategies for detection and identification of bacteria in meat

1.5.1. Enumeration

The traditional methods to examine a food for the presence of a specific microbiota and detect the organisms responsible for the spoilage of meat, uses plating methodology on appropriate media. A number of limitations of this methodology may lead to the failure to detect the organisms responsible for the sensory defects, to miss-identify or underestimate of the number of spoilage bacteria. The results depend on several factors such as the correct choice of the media for the organisms expected to contribute to the spoilage, on the physiological state of these bacteria and the identification methods used (Schillinger & Holzapfel 2006). However, the

shortcomings associated with the use of enumeration methods cannot be ignored, which include insufficient selectivity of the media and failure to recover viable but non culturable (VBNC) or sub lethally injured bacteria (Liao 2006). In foods, many adverse conditions such a nutrient depletion, low temperature and other stresses can induce this VBNC state, while these bacteria are known to be still metabolically active (Fleet 1999).

1.5.2. Identification based on phenotypic methods

Classical characterization requires a battery of morphological, physiological and biochemical features (Vandamme et al. 1996). Therein lies the greatest disadvantage of classical tools for identification of organisms, as even the most sophisticated array of tests can often lead to uncertainties in the classification of isolates. The confidence level of the species identification will increase with the more tests that are carried out. In most cases, colonies should be picked from the plates and after sub culturing of the isolates, several procedures for the identification could be applied (Schillinger & Holzapfel 2006). Most identification keys includes features such as cell morphology, growth at certain tempetarures, pH values, salt concentrations, in the presence of various substances (i.e. antimicrobial agents), production of gas from glucose, metabolization of compounds or fermentation patterns of a number of carbohydrates (obtained from the Bergey's Manual of Systematic Bacteriology, Bergey 1986; Vandamme et al. 1996; Schillinger & Holzapfel 2006). Another method, based on comparison of the whole cell protein patterns obtained by highly standardized sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) has been proven to be extremely reliable for comparing and grouping large numbers of closely related isolates (Vauterin et al. 1993; Kersters

et al. 1994; Pot et al. 1994). The latter method helped to identify wine spoilage LAB (Patarata et al. 1994), non-starter LAB from Italian ewe cheeses (De Angelis et al. 2001), LAB from spoilage associations of cooked and brined shrimps (Dalgaard et al. 2003) and sourdough lactic acid bacteria (Corsetti et al. 2003). However, the use of SDS-PAGE for general identification purposes is hampered by the fact that it yields only discriminative information at or below the species level (Vandamme et al. 1996).

Phenotyping methods are still being used on a routine basis for the identification of bacteria, due to the fact that they are cheaper compared to genotyping methods and no special skills are required to carry out most tests (O' Sullivan 1999; Temmerman et al. 2004). In general, identification and characterization by classical methods has many shortcomings, in particular, lack of accuracy, reproducibility, ambiguity of some techniques (often caused by complex growth conditions), discriminatory power, labor-intensive and time consuming (O' Sullivan 2000; Temmerman et al. 2004; Schillinger & Holzapfel 2006; Rantsiou & Cocolin 2006). Also, the use of conventional phenotypic methods does not always allow efficient characterization of microbiota at species level (Holzapfel 1998; Stanbridge & Davies 1998) and are ineffective in comparing the relatedness between species from different individuals (O' Sullivan 2000).

1.5.3. DNA based methods for identification of bacteria

Several molecular typing techniques have been developed during the last two decades for the culture dependent or independent identification and classification of bacteria at or near the strain level, as applied molecular biology is a fast moving area (Ercolini 2004). While the culture dependent methods are commonly used to identify and molecularly characterize microbial isolates, the culture independent methods are

used to directly profile the microbial populations of the sample (Rantsiou & Cocolin 2006). Ideally, these techniques are far more consistent, universally applicable, rapid, reliable and reproducible and can discriminate even between closely related groups of species, which are otherwise indistinguishable on the basis of their phenotype (Vandamme et al. 1996).

It has been reported that only a small fraction of microorganisms is analyzed by conventional methods, and often the isolated strains often do not represent the real spectrum of microorganisms and their genes active in the habitat of choice (Ampe et al. 1999; Engelen et al. 1998; Ward et al. 1990). With the traditional cultivation methods only 0.1–3% of the total bacterial population can be cultivated (Amann et al. 1995). On the other hand, culture independent methods are believed to overcome problems associated with selective cultivation (e.g. the inability to detect some bacteria on the known media, the lack of knowledge of the real conditions under which most of bacteria are growing in their natural habitat and the difficulty to develop media for cultivation accurately resembling these conditions), isolation of bacteria from natural samples (Fleet 1999; Warriss et al. 2000; Ercolini 2004) and interactions between bacterium species (Molin 2000).

The most powerful methods deal with DNA fragment sizing which is arguably the most widely used analytical method in molecular biology, biochemistry, and microbiology and provide a profile representing the genetic diversity of a microbial community from a specific environment. Basically, these methods rely on the detection of DNA polymorphisms between species or strains and differ in their dynamic range of taxonomic discriminatory power, reproducibility, each of interpretation and standardization (Ben Amor et al. 2007). During the last few years,

bacterial identification based on molecular methods, especially those including the sequencing of genes coding for 16S rRNA, has become a very important tool in studying bacterial communities in environmental samples (Ercolini 2004; Maukonen & Saarela 2009). This is because the microbial species have the same length of 16S rRNA gene fragments although their DNA sequences differ (Ercolini 2004; Ercolini et al. 2006).

The advances in molecular techniques are expected to widen the knowledge of spoilage-related bacterial succession during storage of foods (Chenoll et al. 2003; Ercolini et al. 2006) and considered capable of providing a more realistic view of microbial diversity (Ampe et al. 1999). Further studies are needed to provide more information about the specific spoilage bacteria at species and/or strain level, while a comparative study of the culture dependent (bacteria isolation, cultivable community) and independent methods is missing. The molecular methods considered in this task are Pulsed Field Gel Electrophoresis (PFGE), Polymerase Chain Reaction (PCR) as well as Denaturing Gradient Gel Electrophoresis (PCR-DGGE).

A. Pulsed Field Gel Electrophoresis

A variation of agarose gel electrophoresis, called Pulsed Field Gel Electrophoresis was developed in 1984 by Schwartz and his co-workers. PFGE makes possible the ability to separate even extremely long DNA molecules resulting from the digestion of whole genomic DNAs with rare-cutting restriction endonucleases (Tenover et al. 1995). Ordinary gel electrophoresis fails to separate such molecules, because the steady electric field stretches them out so that they travel end first through

the gel in snakelike configurations at a rate that is independent of their length. In contrast, the direction of the electric field in PFGE is changed periodically, which forces the molecules to reorient before continuing to move snakelike through the gel. This reorientation takes much more time for the larger molecules, so that longer molecules move more slowly than the shorter ones.

This technique is considered the "gold standard" for the characterization of strains, since it is very precise, reproducible, and reliable. In the field of food borne pathogens, a database of PFGE profiles was created (PulseNet, http://www.cdc.gov/pulsenet/), thereby allowing epidemiologic analysis of food borne disease outbreaks. This is possible because the results obtained by PFGE are comparable between different laboratories thanks to the reproducibility of the method (Cocolin et al. 2008). Currently available PFGE protocols for Gram positive or negative microorganisms require embedding intact cells in agarose, cell lysis, restriction digestion of DNA and gel electrophoresis. The result obtained is a band pattern that is specific to the strain that was subjected to the PFGE analysis (Cocolin et al. 2008).

An application of PFGE has been strain level bacterial fingerprinting through the sizing of DNA fragments (Ferris et al. 2004) while, in association with PCR-based methods are commonly used for strain monitoring (Singh et al. 2009). The former method has been used to differentiate members of several genera including *Lactococcus* (Tanskanen et al. 1990), *Clostridia* (Hielm et al. 1998), *Streptomyces* (Leblond et al. 1990), probiotic lactobacilli (Yeung et al. 2004), *Staph*. *xylosus* starter cultures (Di Maria et al. 2002), *Lactobacillus* strains to be used as potential probiotic (Pennachia et al. 2006) and to compare the genomic restriction patterns of five

Bifidobacterium breve strains (Bourget et al. 1993). It is considered to be a discriminating and reproducible method to differentiate strains of intestinal bacteria (O' Sullivan 1999) and for chromosome size estimation in *Lb. acidophilus* (Roussel et al. 1993; Sanders et al. 1996), *Lb. plantarum* (Daniel 1995), and other LAB (Tanskanen et al. 1990). Considering the above studies, PFGE could be a useful tool to monitor the bacterial strains succession during storage of foods.

B. Polymerase Chain Reaction

PCR is a fast, accurate, sensitive and easy operating technique. PCR allows the DNA from a selected region of a genome to be amplified a billion fold, effectively "purifying" this DNA away from the remainder of the genome. Every cycle doubles the amount of DNA synthesized in the previous cycle. The specificity of this technique is directly associated with the primers selection and the primers annealing temperature. The introduction of the PCR methodology into the microbiology laboratory has opened a vast array of applications, because of their universal applicability, simplicity, and rapidity (Vandamme et al. 1996). The PCR based method was reported to allow differentiation at the species (Welsh & McClelland 1992) and intra-species level (Seal et al. 1992) depending on the stringency of the PCR condition.

The differences between strains can be detected by exploiting primers that are annealing in various regions of the genome to identify it thereby producing a band pattern mainly represented by RAPD-PCR (Randomly Amplified Polymorphic DNA) and Rep-PCR (repetitive extragenic palindromic PCR) (Welsh & McClelland 1990; Versalovic et al. 1991; Cocolin et al. 2008). RAPD – PCR has been intensively used

in a study of genomic diversity among bacterial species (Byun et al. 2001; Yost & Nattress 2002; Ertas & Seker 2005) and to examine the diversity of *Lb. sakei* in naturally fermented Italian sausages (Urso et al. 2006). Similarly REP-PCR used to differentiate between closely related bacterial strains in several studies (Gevers et al. 2001; Ventura et al. 2003; Kostinek et al. 2005) and in order to characterize *Staph. xylosus* strains isolated from fermentation processes in Northern Italy (Iacumin et al. 2006). The main drawback of the RAPD – PCR is its low reproducibility while the profiles obtained from Rep-PCR analysis are highly specific for a species and they are highly reproducible as well (Cocolin et al. 2008).

The techniques that allow strain grouping based on differences in a DNA sequence are RFLP (restriction fragment length polymorphism) analysis of the 16S rRNA gene (Lee et al. 2004) and DGGE (denaturing gradient gel electrophoresis; this method will be described extendedly below) (Cocolin et al. 2001; Ercolini et al. 2001). With the RFLP approach, a specific pattern for a species is given by using a restriction endonuclease that is cutting the DNA in specific restriction sites. Different patterns represent different organisms. However, identical patterns do not necessarily indicate the same strain (Ludwig 2007). This method has been used to differentiate *Leuconostoc* strains (Lyhs et al. 2004) as well as *Pseudomonas* species (Widmer et al. 1998).

Alternatively, the PCR-sequencing methodology is coupled with techniques that are able to differentiate strains based on fingerprinting profiles, allowing grouping of the isolates and reducing the number of strains requiring sequencing (Cocolin et al. 2008).

C. Denaturing Gradient Gel Electrophoresis

PCR-DGGE of ribosomal DNA was proposed into microbial ecology by Muyzer et al. (1993). The technique is based on the electrophoretic separation of PCR amplicons of the same size but different sequences in an acrylamide gel containing a gradient of a denaturant. As the DNA encounters an appropriate denaturant concentration, a sequence-dependent partial separation of the double strands occurs. This is because these fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profile (Muyzer & Smalla 1998, Ercolini 2004). This conformational change in the DNA tertiary structure causes a reduced migration rate and results in a DNA band pattern representative of the sampled microbial community (Silger et al. 2004). Every single band that is visible in DGGE gels represents a component of the microbiota (Cocolin et al. 2008). The resulting banding patterns can be digitally captured and normalised using reference patterns, allowing the identification of band positions though comparison of those present in a database of well-characterised type and reference strains (Temmerman et al. 2004).

PCR - DGGE is usually employed to assess the structure and dynamics of microbial communities in food samples without cultivation in response to environmental variations (Ercolini 2004; Ercolini et al. 2004; Fontana et al. 2005; Rantsiou et al. 2005) and in a wide range of environmental samples (Muyser et al. 1995; Ferris et al. 1996; Teske et al. 1996; Gomes et al. 2001). In recent years, this technique has been applied in many fields such as sausage (Cocolin et al. 2001; Fontana et al. 2005; Villani et al. 2007), cheese (Cocolin et al. 2004), beef (Ercolini et al. 2006; 2009; 2010; Fontana et al. 2006), food waste (Shin & Youn 2005), kimchi (Lee et al. 2005), soil (Avrahami et al. 2003), and pig faeces (Konstantinov et al.

2003). The advantages of the method are its affordability for ordinary laboratories and the relative ease in interpreting the results (Nocker et al. 2007). Despite the success of DGGE to provide a rapid survey of the bacterial community is not always suitable for the identification of all species, but can be used for screening and grouping the isolates and reducing the number of cultures to identify by molecular or biochemical methods (Ercolini 2004).

1.6. Aim and objectives

The aim of the present thesis was to determine the microbial quality of raw meat, provide strategies that should be addressed to improve the latter and identify the microorganisms that are present in meat under several conditions. To realize the aim and to test the stated hypothesis the study considered the following objectives:

- To compare the differences in microbiological load on meat at the retail level
- To investigate the efficacy of volatile compounds of oregano essential oil in combination with the use of modified atmosphere packaging conditions on microbial quality of meat
- To determine the influence of storage temperature and packaging conditions on the succession of spoilage related bacteria
- To compare the dynamics of the different molecular tools available for the study of microbial communities

The thesis is presented in the traditional format with Materials and methods in Chapter 2, the Results described in Chapter 3, and the Discussion in Chapter 4. The summary Conclusions and future work will be presented in Chapter 5.

Chapter 2

Materials and Methods

2.1. Survey of microbial levels for minced beef sold in supermarkets

A total of 37 minced beef samples were collected randomly and periodically from Greek supermarkets in Athens. Twenty four samples were sold in modified atmosphere packaging and thirteen in traditional packaging (styrofoam tray wrapped with permeable film).

2.1.1. Microbiological analysis

Samples (25 g) of minced beef were weighed aseptically, added to sterile quarter strength Ringer's solution (225 mL) (LAB100Z, LAB M, UK) and homogenized in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at room temperature. Decimal dilutions in quarter strength Ringer's solution were prepared and duplicate 1 or 0.1 mL samples of appropriate dilutions poured or spread on the following media: (i) Plate Count Agar (PCA, 402145, Biolife, Italiana S.r.l., Milano, Italy) for total viable count (TVC), incubated at 30°C for 48 h, (ii) MRS Agar (pH 5.8 and pH 5.2) (401728, Biolife) for enumeration of LAB, overlaid with the same medium and incubated at 30°C for 72 h, (iii) Pseudomonas Agar Base (PAB, 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 (402079 supplemented with selective supplement 4240052, Biolife) for the enumeration of *Brochothrix thermosphacta*, incubated at 25°C for 48 h, (v) Violet Red Bile Glucose agar (VRBG, 402188, Biolife) for the enumeration of *Enterobacteriaceae*, overlaid with the same medium and incubated at 37 ˚C for 24 h and (vi) Iron agar (made from basic ingredients, Oxoid), for the enumeration of hydrogen sulfide-producing bacteria, overlaid with the same medium and incubated at 25 ˚C for 72 hours.

2.1.2. pH measurement

The pH value was recorded by a pH meter (Metrohm 691 pH meter), the glass electrode being immersed in the homogenate of minced meat at the end of microbiological analysis.

2.1.3. Sensory analysis

The sensory evaluation of the meat samples was performed according to Gill & Jeremiah (1991) with a 4 person panel from the Laboratory of Microbiology & Biotechnology of Foods, Agricultural University of Athens.

2.1.4. Statistical analysis

The data (mean log cfu g^{-1}, Standard Deviation) of minced meat were analysed using the XLSTAT (2006) computer software.

2.2. The effect of oregano essential oil in microbial association of minced beef

Minced beef (approximately 40 kg) was obtained from the central market in Athens and transported to the laboratory within 30 min.

2.2.1. Essential oil

The oregano essential oil was kindly provided by Ecopharm Hellas S.A. The essential oil was distributed on Whatman paper (Figure 2.1.a) at a final concentration of 2 % v/w (Chorianopoulos, 2007).

2.2.2. Preparation of inoculum and inoculation procedure

The stock culture of *Listeria monocytogenes* Scott A was sub-cultured twice in Brain Heart Infusion Broth (LAB49, LAB M), incubated aerobically at 30˚C for 24 and 18 h respectively. Cells were harvested and washed by centrifugation (5min, 10000 rpm, Heraeus Multifuge 15-R) with sterile quarter-strength Ringer's solution.

Portions of 1kg minced beef was further mixed with the inoculum (final concentration 3 log cfu g^{-1}) and then divided into 75 g portions.

2.2.3. Packaging

Two portions of 75 g were placed onto upturned styrofoam trays (Figure 2.1 b) (either in the presence or absence of *Listeria monocytogenes* Scott A). These samples were packaged under three packaging conditions, i.e. air, MAP (40% CO_2/30% O_2/30% N_2) and MAP with the presence of volatile compounds of oregano essential oil, and stored at 0, 5, 10 and 15˚C. For aerobic storage, the samples were placed into polyethylene bags for domestic use (280 mm x 460 mm, Fino, Sarantis S.A., Greece). For the MAP the samples were packed into plastic pouches (Flexo-Pack S.A., Greece) 90μm thick, gas permeability at 20˚C and 50% relative humidity. ca. 25,90, and 6cm^3 /m^2 per day/ 10^5 Pa for CO_2,O_2 and N_2, respectively, using a HenkoVac 1900 Machine (Howden Food Equipment B.V., The Netherlands).

The particular type of MAP (40% CO_2/30% O_2/30% N_2) was chosen as the next best effective packaging condition (100% CO_2 is considered to be the best) with the synergistic action of the essential oil to determine the shelf life of beef because of its acceptability in terms of colour to consumers (Skandamis & Nychas, 2002, Chorianopoulos 2007).

2.2.4. Microbiological analysis

For microbiological analysis 25 g sample was added to 225 mL of sterile quarter strength Ringer's solution and homogenized in a stomacher for 60 s 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. Total viable counts were determined on Plate Count Agar (PCA) , incubated at 30˚C for 48 h, lactic acid bacteria in MRS agar (pH = 5.7) overlaid with the same medium and incubated at 30 ˚C for 72 h, *Brochothrix thermosphacta* on STA agar (from basic ingredients (CM881, Oxoid) made in the laboratory and supplemented with streptomycin sulfate, thallous acetate and cycloheximide), incubated at 25 ˚C for 48 h, *Enterobacteriaceae* on VRBG agar overlaid with the same medium and incubated at 37 ˚C for 24 h, yeasts on Rose Bengal Chloramphenicol Agar Base (LAB 36 supplemented with selective supplement X009, LAB M), incubated at 25 ˚C for 72 h, *Pseudomonas* spp. on PAB (supplemented with selective supplement), incubated at 25˚C for 48 h and *Listeria* spp. on Listeria PALCAM Agar Base (4016042, supplemented with selective supplement 4240042, Biolife) incubate at 37˚C for 48 h. For listeria detection, enrichment was done by suspending 25 g of sample in 225 mL Listeria Fraser Broth Base Half Concentration (401594, supplemented with selective supplement 4240056, Biolife) followed by incubation at 30 ˚C for 24 h, then, 0.1 mL of the culture enrichment were streaked on Listeria PALCAM Agar Base and incubated at 37 ˚C for 48 h. Also, 1 mL of the culture enrichment suspending in 10 mL of Fraser broth (LAB164, supplemented with selective supplement X165, LAB M) and incubated at

30 ˚C for 48h, then 0.1 mL of the culture enrichment were streaked on PALCAM and incubated at 37 ˚C for 48h.

2.2.5. pH measurement

The pH value was measured as it was described in Section 2.1.2.

2.2.6. Sensory analysis

The sensory evaluation was performed as it was described in Section 2.1.3.

2.2.7. Data analysis

The growth data (log cfu g^{-1}) of the different spoilage bacteria of minced meat were modelled as a function of time using the model of Baranyi and Roberts (1994), and the kinetic parameters (μ_{max} and lag) were estimated. For curve fitting the in-house Institute of Food Research program DMFit, kindly provided by J. Baranyi (Institute of Food Research, Norwich, United Kingdom), was used.

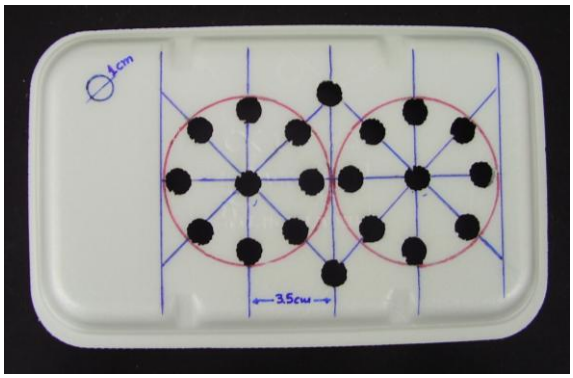
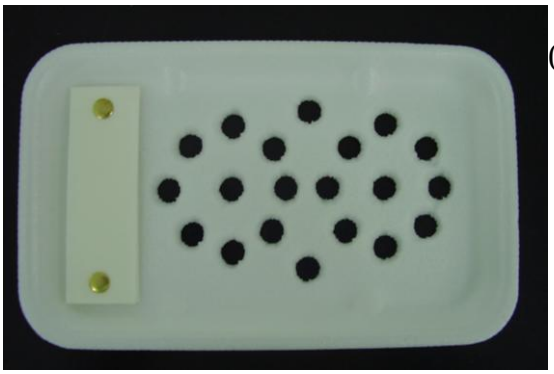
(a)   (b)

Figure 2.2. Styrofoam tray (a) upturned*, (b) with whatman paper*, where essential oil was distributed. ***Designed by the author, Anthoula Argyri and Vasiliki Blana**

2.3. Study of the microbiota during storage of meat

2.3.1. Study of the development of microbiota in minced beef stored under different conditions

A. Identification of lactic acid bacteria

LAB isolated from minced beef (Task 2.2) from three different time points (initial, middle and final stage of storage). Briefly, colonies (10%) were randomly selected (Harrigan 1998) from the highest dilution and purified by successive subculture on MRS agar at 30°C. Gram positive, catalase and oxidase negative isolates were stored at -80˚C in MRS broth (401729, Biolife) supplemented with 20% (w/v) glycerol (Merck) until further use. Before experimental use each strain was grown twice in MRS broth at 30°C for 24 and 16h respectively. Purity of the culture was always checked on MRS agar plates before use.

PFGE: PFGE was performed according to Kagkli et al. (2007). Briefly, cells were harvested by centrifugation at 14000 rpm for 5 min and washed with 10 mM Tris-HCl (pH 7.6) containing 1 M NaCl; resuspended in 100 μL of the same solution, heated at 37°C for 10 min and mixed with an equal volume of 2% (w/v) certified low melting-point agarose (161-3111, Bio-Rad, Hercules, CA, USA) in 0.125 M EDTA pH 7.6 before letting them to solidify in CHEF plug moulds (170-3713, Bio-Rad). The cells were lysed *in situ* in a solution containing 10 mg mL^{-1} of lysozyme (A4972, Applichem GmbH, Darmstadt, Germany) in EC buffer (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 1% (w/v) Sarkosyl, pH 7.6) for 16 h at 37°C. The lytic treatment was repeated with the same solution containing 2U mL^{-1} mutanolysin (M9901, Sigma, Chemical Co., St. Louis, Mo. USA). After treatment with proteinase K (P2308,

Sigma) (0.5M EDTA containing 1% sarkosyl, pH 8) for 24h at 55°C, the agarose blocks were washed twice for 1 h with 1 mM phenylmethylsulfonyl fluoride (PMSF, P7626 Sigma) in 10 mM Tris-HCl containing 1 mM EDTA, (pH 8.0) at 37°C and then stored at 4°C in 10 mM Tris-HCl containing 100 mM EDTA (pH 8.0) until further use. The agarose blocks were cut with sterile coverslips and slices (1 - 2 mm thick) of the blocks were washed three times at room temperature in 10 mM Tris-HCl containing 0.1 mM EDTA (pH 8.0) for 30 min with gentle agitation. The restriction enzymes *Apa*I and *Sma*I (10U) (New England Biolabs, Ipswich, MA, USA) were initially selected to digest the slices of a limited number of strains. The enzyme that resulted in the production of clearer and sharper PFGE digestion profile was used for the digestion of all isolates. Digestions were performed according to the recommendations of the manufacturer. Following digestion, slices were loaded into wells of a 1% PFGE grade agarose gel (162-0137, Bio-Rad) and the gel was run in 0.5 mM Tris-Borate buffer (45 mM Tris–HCl, 45 mM Boric acid, 1 mM EDTA) using a CHEF-DRII PFGE apparatus and cooling module (Bio-Rad) at 6 Volt cm^{-1} for 16h, with a pulse time ramped from 1 to 10s. Gels were then stained with ethidium bromide (0.5 μg mL^{-1}, 160539, Sigma) in water for 1 h and destained for 2 h before being photographed using a GelDoc system (Bio-Rad). Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Gel compare software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

DNA extraction: DNA was extracted according to the recommendations of the manufacturer of GenElute Bacterial Genomic DNA Kit (NA2120, Sigma).

Detection of the heme - dependent catalase (*kat*A) gene: All isolates were screened by PCR for the presence of the *kat*A gene, encoding heme-dependent catalase (Knauf et al. 1992, Hertel et al. 1998) (Table 2.1). PCR amplifications were conducted in a final volume of 25 μL containing 2U of thermostable (*Taq*) DNA polymerase (M0273, NEB), 2.5 μL *Taq* buffer, 0.8 mM dNTP's (NEB), 0.8 μM of each primer and 20 ng μL^{-1} of DNA template. PCR reaction consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles (denaturation at 94°C, 1 min, primer annealing at 56°C, 1 min, primer extension at 72 °C, 1 min), a final extension step at 72°C, and cooling to 4°C for 7 min. A positive control (*Lb. sakei* 20087) and a negative control (without DNA template) were included in parallel. Five microliters of the PCR products were submitted to electrophoresis at 100 V cm^{-1} for 1 h on a 1.5% agarose gel (15510-027, Invitrogen, Ca., USA) in 0.5 mM TAE buffer. The gel visualized under U.V. after staining the gel with ethidium bromide. A 100-bp ladder was used as a size marker.

Species identification: Representative number of isolates per distinct PFGE cluster were selected and subjected to species identification by sequencing the V1-V3 variable region of the 16S rRNA gene (Table 2.1) as described previously (Paramithiotis et al. 2008). Briefly, PCR amplifications were conducted in a final volume of 50 μL containing 2.5U of thermostable (*Taq*) DNA polymerase, 5 μL *Taq* buffer, 0.8 mM dNTP's, 0.2 μM of each primer and 20 ng μL^{-1} of DNA template. PCR reaction consisted of an initial denaturation step at 94°C for 10 min, followed by 35 cycles (denaturation at 94°C, 1 min, primer annealing at 42°C, 1 min, primer extension at 72 °C, 2 min), a final extension step at 72°C for 10min.

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (http://www.macrogen.com). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are GU998850 to GU998881.

B. Identification of *Enterobacteriaceae*

Enterobacteriaceae isolated from minced beef (Task 2.2). In brief, approximately 10 colonies were selected randomly (Harrigan 1998) from the highest dilution of VRBG (Biolife, Italiana S.r.l., Milano, Italy) from different time points (fresh meat, middle and final stage of storage). Pure cultures included in this study were stored at -80^{o}C in BHI (Merck, Darmstadt, Germany) supplemented with 20% glycerol (Serva, Heidelberg, Germany). Before experimental use they were sub-cultured twice at 37^{o}C for 16h and 6h respectively.

Whole cell protein profiling: The whole cell proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE) in 12% polyacrylamide gel according to Paramithiotis et al. (2000) on Protean II xi (Biorad). Briefly, cells were collected and washed with sodium phosphate buffer (NaBPs pH 7.3). Cell extracts were prepared by sonicating (3 min, 50W) 5 mL of bacterial culture in 800 μL sample buffer (62.5mM Tris – HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulphate, 5 % β – mercaptoethanol, 0.025% bromophenol blue). The lysate was heated at 95° C for 10 minutes and centrifuged for 10 min at 14000 rpm. The supernatant obtained (protein extract) was stored at - 20° C until further use, while 25 μL used for SDS page analysis. Protein bands were visualized by using brilliant blue

R -250 (B8647, Sigma) staining before being photographed using a Model GS-800 Calibrated Imaging Densitometer (Biorad).

PFGE: Genomic DNA was prepared from all isolates according to previously described method (Herschleb et al., 2007). Briefly, cells were harvested by centrifugation at 14000 rpm for 5 min and washed with 10 mM Tris-HCl (pH 7.6) containing 1 M NaCl; resuspended in 100 µL of the same solution, heated at 37°C for 10 min and mixed with an equal volume of 2% (w/v) low melting-point agarose in 0.125 M EDTA pH 7.6 before letting them to solidify in moulds. The cells were lysed *in situ* in a solution containing 10 mg mL^{-1} of lysozyme in EC buffer (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 1% (w/v) Sarkosyl, pH 7.6) for 16 h at 37°C. After treatment with proteinase K (0.5M EDTA containing 1% sarkosyl, pH 8) for 24h at 55°C, the agarose blocks were washed twice for 1 h with 1 mM phenylmethylsulfonyl fluoride (PMSF) in 10 mM Tris-HCl containing 1 mM EDTA, (pH 8.0) at 37°C and then stored at 4°C in 10 mM Tris-HCl containing 100 mM EDTA (pH 8.0) until further use.

The following modifications were also applied (M) (Figure 2.2):

M1: the culturing time was decreased to 6h

M2: sodium dodecyl sulfate (1%) was added in the solution used to make the agarose plugs (Hunter et al. 2005)

M3: the incubation time for proteinase K treatment was increased from 24 to 48 h, changing the buffer solution daily (Herschleb et al. 2007).

M4: after the proteinase K treatment the plugs were incubated for 1 h at room temperature in TE solution, containing 50 µM thiourea, with gentle agitation.

Subsequently washed with 500 μL of TE solution with gentle agitation for 30 minutes; this step was repeated 3 times.

M5: addition of 50 μM (M5a) (Romling and Tummler 2000, Silbert et al. 2003) or 100μM (M5b) (Lee et al. 2006, Liesegang and Tschape 2002) thiourea into the running buffer

M6: use of HEPES as running buffer (M6) (Ray et al. 1992, Koort et al. 2002)

M7: M1 + M5b

M8: M2 + M5b

M9: M3 + M5b

M10: M3 + M5b

M11: M1 + M4 + M5b

In all cases, the restriction enzyme *Xba*I (10U) was applied according to the manufacturer's recommendation for 16h at 37°C. Restriction fragments were separated in 1% PFGE grade agarose gel on CHEF-DRII equipment with the following running parameters: 6 Volt cm^{-1}, 2.2 s initial switching time, 54.2 s final switching time and a 20hrs of total run at 14º C (Ferris et al. 2004). Gels were then stained with ethidium bromide (0.5 μg mL^{-1}) in water for 1 h and destained for 2 hrs before being photographed using a GelDoc system.

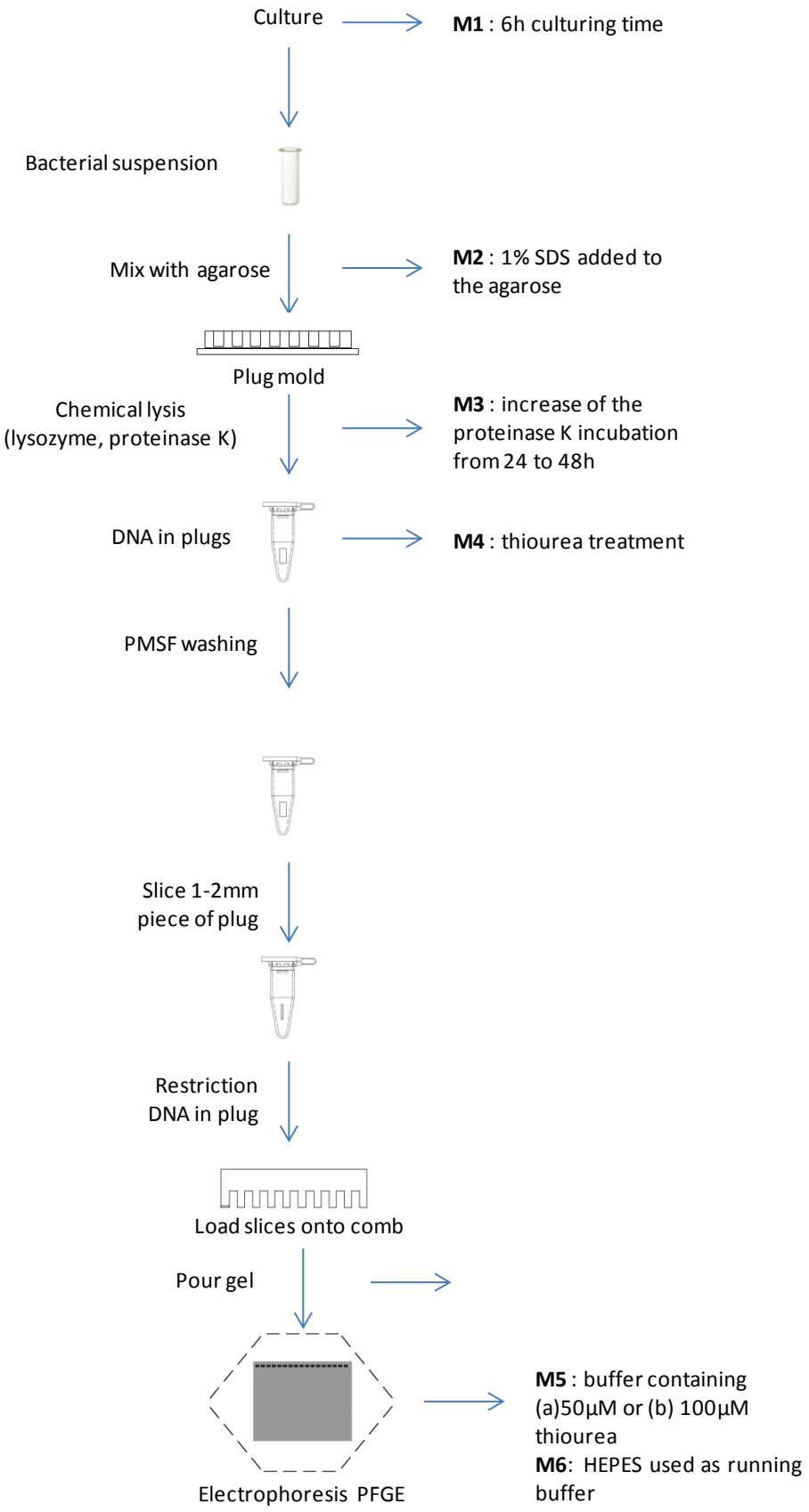


Figure 2.2. Flow diagram of the sample preparation of PFGE analysis of *Enterobacteriaceae.*

DNA extraction: DNA was extracted with a slight modification of the enzymatic method according to Ercolini et al. (2001). One milliliter of overnight culture was centrifuged at 14000 rpm for 5 min at 4°C. The pellet was resuspended in 0.5 mL buffer solution (1M sorbitol, 0.1M EDTA, pH 7.5) containing 25 mg mL^{-1} lysozyme, incubated for 2h at 37°C and centrifuged at 14000 rpm for 10 min at 4°C. After centrifugation, the pellet was resuspended in 0.5 mL of buffer (50mM Tris – HCl, 20mM EDTA, pH 7.4) and incubated for 30 min at 65°C after the addition of 50 μL 10% SDS solution. Then, the sample was mixed with 0.2 mL potassium acetate (5M), placed on ice for 30 min and centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was precipitated with 1 mL ice cold isopropanol and centrifuged 14000 rpm for 10 min at 4°C. After that, the pellet was resuspended in 0.5 mL ice cold ethanol (70%) and centrifuged at 14000 rpm for 5 min at 4°C. Finally the pellet was dried and resuspended in 50 μL sterile ddH_20.

Species identification: Representative number of isolates per distinct PFGE cluster were selected and subjected to species identification by sequencing the V1-V3 variable region of the 16S rRNA gene (as it was described at Section 2.3.2) (Table 2.1). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (**http://www.macrogen.com**). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are HM242268 to HM242286.

C. Identification of isolates from Pseudomonas Agar Base growth medium

Bacteria were isolated from PAB from minced beef (Task 2.2). Briefly, colonies (10%) were selected randomly (Harrigan 1998) from the highest dilution of PAB from different time points. Pure cultures included in this study were stored at -80°C in Brain Heart Infusion Broth (BHI, Merck, Darmstadt, Germany) supplemented with 20% (v/v) glycerol (Serva, Heidelberg, Germany). Before experimental use each isolate was subcultured twice in BHI at 37^{o}C for 16h twice, while the purity of the culture was always checked on PAB plates.

PFGE: Genomic DNA was prepared from all isolates as it was described at Task 2.3.3. Briefly, after the proteinase K treatment, the plugs were incubated for 1h at room temperature in TE solution, containing 50 μM thiourea, with gentle agitation. Subsequently washed with 500 μL of TE solution with gentle agitation for 30 minutes; this step was repeated 3 times. The restriction enzyme *Spe*I (10U) (New England Biolabs) was applied according to the manufacturer's recommendation for 16h. Restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris–Borate buffer containing 100μM thiourea on CHEF-DRII equipment with the following running parameters: 6 Volt cm^{-1}, 5.3 s initial switching time, 34.9 s final switching time and a 20 h of total run at 14° C (Khan et al. 2007). Gels were then stained with ethidium bromide (0.5 μg mL^{-1}) in water for 1 h and destained for 2 hrs before being photographed using a GelDoc system.

DNA extraction: The protocol described by the Wizard DNA purification kit (Promega, Madison, Wiscon.) was applied. One millilitre of cell culture was

centrifuged at 14000 rpm for 5 min at 4 °C, and the resulting pellet was resuspended in 100 µL of Tris – EDTA buffer (100mM Tris, 10mM EDTA). The sample was mixed with 160 µL of 0.5 M EDTA/Nuclei Lysis Solution in 1/4.16 ratio and 15 µL of proteinase K (20mg mL^{-1}, Sigma, Chemical Co., St. Louis, Mo. USA) and incubated for 90 min at 55°C. After incubation, 1 volume of potassium acetate 5 M was added to the sample which was then centrifuged at 14000 rpm for 10 min at 4 °C. The supernatant was precipitated with 0.7 volume of ice cold isopropanol and centrifuged at 14000 rpm for 10 min at 4 °C. After that, the pellet was resuspended in 0.5 mL ice cold ethanol (70%) and centrifuged at 14000 rpm for 10 min at 4°C. The pellet was dried and resuspended in 45 µL of DNA Rehydration Solution by incubation at 55 °C for 45 min. After incubation, 5 µL of RNase (10mg mL^{-1}, Promega) was added and the sample incubated for 30 min at 37 °C.

PCR amplification: PCR amplification was performed according to Ercolini et al. 2006. Briefly, primers U968 and L1401 were used to amplify the variable V6-V8 region of the 16S rRNA gene (Table 2.1), giving PCR products of about 450 bp. A GC clamp was added to the forward primer according to a method described previously by Muyzer et al. (1993). PCR amplifications were conducted in a final volume of 25 µL containing 2.5U of thermostable (*Taq*) DNA polymerase (New England Biolabs), 5 µL *Taq* buffer, 0.8 mM dNTP's, 0.2 µM of each primer, 1.0 mM $MgCl_2$ and 20 ng μL^{-1} of DNA template. PCR reaction consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles (denaturation at 94°C, 1 min, primer annealing at 56°C, 45 sec, primer extension at 72 °C, 3 min), a final extension step at 72°C for 10min. Aliquots (5 µL) of PCR products were routinely checked on 1.5% agarose gels. Reference strains included in this study consisted of

Ps. putida KT2440, *Ps. fragi* DSM 3456 and *Ps. fluorescens* GTE 015; these strains were used for the construction of a DGGE based identification database.

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

Species identification: Representative number of isolates per distinct PFGE cluster were selected and subjected to species identification by sequencing the V6-V8 variable region of the 16S rRNA gene (Table 2.1) with the primer L1401. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (**http://www.macrogen.com**). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are to HM536985 to HM536997**.**

D. Fingerprinting of bulk cells from Pseudomonas Agar Base growth medium

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

DNA extraction: DNA extraction was performed as it was described in Section 2.3.1. C.

PCR amplification: PCR amplification was performed as it was described in Section 2.3.1 C.

DGGE analysis: DGGE was performed as it was described at Section 2.3.1 C.

2.3.2. Fingerprinting of microbiota in beef fillets stored aerobically

Samples were selected from beef fillets stored aerobically at 0, 5, 10, 15 and 20˚C (work was supported by an EU project, Anthoula Argyri phD thesis) at the initial, middle and at the final stage of storage. Furthermore, bacteria were isolated at the beginning, middle and at the end of storage period.

A. Identification of bacteria

Colonies (10%) were randomly selected (Harrigan 1998) from the highest dilution of four different growth media (MRS pH 5.2, MRS pH 5.7, VRBG and PAB). The isolates were performed as it was decribed at Section 2.3.1.

PFGE: PFGE analysis was performed as it was described at Task 2.3.1 A, Task 2.3.1 B and Task 2.3.1 C for lactic acid bacteria (MRS pH 5.2, MRS pH 5.7), *Enterobacteriaceae* (VRBG) and isolates from PAB growth medium respectively.

DNA extraction: DNA extraction was performed as it was described at Section 2.3.1 B.

Species identification: Representative number of isolates per distinct PFGE cluster were selected and subjected to species identification by sequencing the V1-V3 variable region of the 16S rRNA gene (as it was described at task 2.3.2) (Table 2.1). In the case of *Enterobacteriaceae*, a number of isolates it was not possible to be assessed at genus level by sequencing the V1-V3 variable region, thus identification has supported by sequencing the *rpo*B gene (Table 2.1)**,** encoding the bacterial RNA polymerase β-subunit. Briefly, PCR amplifications were conducted in a final volume of 50 μL containing 2.5U of thermostable (*Taq*) DNA polymerase, 5 μL *Taq* buffer, 0.8 mM dNTP's, 0.2 μM of each primer and 20 ng μL^{-1} of DNA template. PCR reaction consisted of an initial denaturation step at 94°C for 10 min, followed by 35 cycles (denaturation at 94°C, 1 min, primer annealing at 56°C, 1 min, primer extension at 72 °C, 2 min), a final extension step at 72°C for 10min. In both cases, the PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (**http://www.macrogen.com**).

B. Assessment of microbiota by analysis of DNA extracted directly from beef fillets

Ten gram of beef homogenized in a stomacher bag with 20 mL of one-quarter strength Ringer's solution for 1 min; the large deposit was allowed to set for 1 min, and the supernatant was stored by freezing at -80°C. When necessary, 1 mL of the supernatant was used for DNA extraction.

DNA extraction: DNA extraction was performed as it was described in Section 2.3.1 C.

PCR amplification: PCR amplification was performed as it was described in Section 2.3.1 C.

DGGE analysis: DGGE was performed as it was described in Section 2.3.1 C.

Sequencing of DGGE fragment: DGGE band to be sequenced was purified in water according to a method described previously (Ampe et al. 1999). Briefly, DGGE band was cut out with a sterile tip. The DNA of the fragment was eluted in 20µL of sterile distilled water overnight at 4°C. Two microliters of the eluted DNA from DGGE band was reamplified as described in Section 2.3.1 C. The success of this procedure was checked in DGGE gel. PCR product which yielded single band (comigrated with an original band) was then purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (**http://www.macrogen.com**).

Table 2.1. List of PCR primers used in this study.

Primer	Sequence (5' – 3')	Position	Region/Gene	Reference
P1	GCGGCGTGCCTAATACAT GC	41 – 60	16S V1	Klijn et al. 1991
P4	ATCTACGCATTTCACCGCTAC	385– 405	16S V3	Klijn et al. 1991
U968*	AACGCGAAGAACCTTAC	968– 985	16S V6-V8	Ercolini et al. 2006; Zoetendal et al. 1998
L1401	GCGTGTGTACAAGACCC	1401– 1418	16S V6-V8	Ercolini et al. 2006; Zoetendal et al. 1998
CM_7	AACCAGTTCCGCGTTGGCCTGG	1384 – 1405	*rpo*B	Mollet et al. 1997
CM_{31b}	CCTGAACAACACGCTCGGA	2455 – 2473	*rpo*B	Mollet et al. 1997
702-F	AATTGCCTTCTTCCGTGTA	551–536	*kat*A	Knauf et al. 1992
310-R	AGTTGCGCACAATTATTTTC	127–139	*kat*A	Knauf et al. 1992

* A GC clamp was added according to Muyzer et al. 1993

2.3.3 Data analysis

The sequences were aligned with those in GenBank using the BLASTN program in order to determine their closest known relatives of the partial 16S rRNA gene sequence (Altschul et al. 1997).

Chapter 3

Results

3.1. Survey of microbial levels for minced beef sold in supermarkets

In a survey of the microbial populations in 37 pre-packed minced beef, high counts were noted (Table 3.1). The total counts ranged from 4.4 to 8.9 log cfu g^{-1} and pseudomonads from 3.8 to 7.4 log cfu g^{-1}. The sensory analysis gave a mean score 1.8 and ranged from 1 to 3, which concurred with the microbial counts. Also the mean pH value was 5.64 and ranged between 5.35 and 5.93. It is also interest to mention that the microbial load of meat differs from one region to another (data not shown).

Table 3.1. Microbial counts of minced beef sold in Athens.

	log cfu g^{-1}			
Microorganism	**Minimum**	**Maximum**	**Mean**	**Std. deviation**
Total viable counts	4.415	8.869	6.433	1.068
Pseudomonas spp.	3.748	7.418	5.687	0.861
Brochothrix thermosphacta	3.477	7.004	5.548	0.908
Hydrogen sulfide-producing bacteria	1.845	6.064	3.618	1.282
Lactic acid bacteria (pH 5.2)	1.301	6.519	4.159	1.278
Lactic acid bacteria (pH 5.7)	1.699	6.320	4.250	1.237
Enterobacteriaceae	2.204	6.090	3.850	1.058

Season did not affect the total microbial and sensory quality of the minced beef, although higher counts of *Enterobacteriaceae* and hydrogen sulfide-producing bacteria were observed in the warm season (Table 3.2). Distinct differences were observed in the microbial quality of the samples depended on the packaging system. In minced beef sold in styrofoam trays wrapped with permeable film, higher microbial counts were noted (Table 3.3). The sensory analysis also showed that the minced beef sold in MA package (mean score 1.6) was better than that in the plastic foam tray (mean score 2.1). Moreover, the pH value was strongly dependent on sample type, although no significant differences were observed between season or packaging system.

Table 3.2. Microbial counts of minced beef collected at different seasons.

		log cfu g^{-1}			
Microorganism	**season**	**Minimum**	**Maximum**	**Mean**	**Std. deviation**
Total viable counts	**cold**	4.415	8.869	6.474	1.073
	warm	4.740	7.924	6.336	1.100
Pseudomonas spp.	**cold**	4.204	7.013	5.690	0.785
	warm	3.748	7.418	5.680	1.060
Brochothrix thermosphacta	**cold**	3.477	6.663	5.454	0.940
	warm	4.362	7.004	5.768	0.826
Hydrogen sulfide-producing bacteria	**cold**	1.845	5.623	3.174	1.094
	warm	3.255	6.064	4.669	1.094
Lactic acid bacteria (pH 5.2)	**cold**	1.301	6.140	4.021	1.164
	warm	2.204	6.519	4.483	1.525
Lactic acid bacteria (pH 5.7)	**cold**	1.699	6.167	4.105	1.216
	warm	2.863	6.320	4.593	1.276
Enterobacteriaceae	**cold**	2.204	5.519	3.578	0.920
	warm	2.771	6.090	4.492	1.127

Table 3.3. Microbial counts of minced beef sold in different package.

		log cfu g^{-1}			
Microorganism	**season**	**Minimum**	**Maximum**	**Mean**	**Std. deviation**
Total viable counts	**MAP**	4.415	7.771	5.898	0.837
	Tray	5.964	8.869	7.420	0.669
Pseudomonas spp.	**MAP**	3.748	6.959	5.345	0.708
	Tray	4.513	7.418	6.318	0.772
Brochothrix thermosphacta	**MAP**	3.477	6.531	5.215	0.872
	Tray	5.041	7.004	6.162	0.623
Hydrogen sulfide-producing bacteria	**MAP**	1.845	5.881	3.158	1.140
	Tray	2.613	6.064	4.468	1.112
Lactic acid bacteria (pH 5.2)	**MAP**	1.301	5.140	3.480	0.938
	Tray	3.934	6.519	5.411	0.769
Lactic acid bacteria (pH 5.7)	**MAP**	1.699	5.813	3.671	1.039
	Tray	3.875	6.320	5.320	0.781
Enterobacteriaceae	**MAP**	2.204	6.090	3.351	0.842
	Tray	3.340	5.663	4.772	0.762

3.2. The effect of oregano essential oil on microbial associations of minced beef

3.2.1. Development of autochthonous biota

The initial biota of minced beef consisted of pseudomonads, *Br. thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria and yeasts. The contribution of these groups to the final biota depended on the packaging system used and the storage temperature. Table 3.4 summarized the estimations using the Baranyi model for the initial and final populations (log cfu g^{-1}), lag phase (lag in h) and maximum specific growth rate (μ_{max} in h^{-1}) for the total viable counts (TVC), pseudomonads, *Br. thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria and yeasts for each of the storage conditions tested.

During aerobic storage of minced beef, the TVC reached the highest levels within 338 and 78h at 0 and 15°C respectively, with the *Pseudomonas* spp. being the dominant microorganisms. Packaging under modified atmosphere delayed the growth of the pseudomonads, *Br. thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria and yeasts. Furthermore, MAP - suppressed the maximum level of the aerobic counts compared with the aerobic storage, although the lactic acid bacteria were the predominant organisms (Figure 3.1a, Figure 3.2a, Table 3.4). More accurately, lactic acid bacteria and *Br. thermosphacta* or *Enterobacteriaceae* had the higher specific growth rate at low (0, 5°C) and abuse temperatures (10, 15°C) respectively (Figure 3.1a, Figure 3.2a).

The presence of volatile compounds of oregano essential oil also influenced the microbial association of minced beef (Table 3.4). *Br. thermosphacta* was the most sensitive group in all cases, whereas inhibition occurred selectively towards pseudomonads, *Enterobacteriaceae*, lactic acid bacteria and yeasts depended on the

storage temperature (Figure 3.1b, Figure 3.2b). Indeed, despite the low yield in counts, lactic acid bacteria predominated under MAP + (Figure 3.1b, Figure 3.2b).

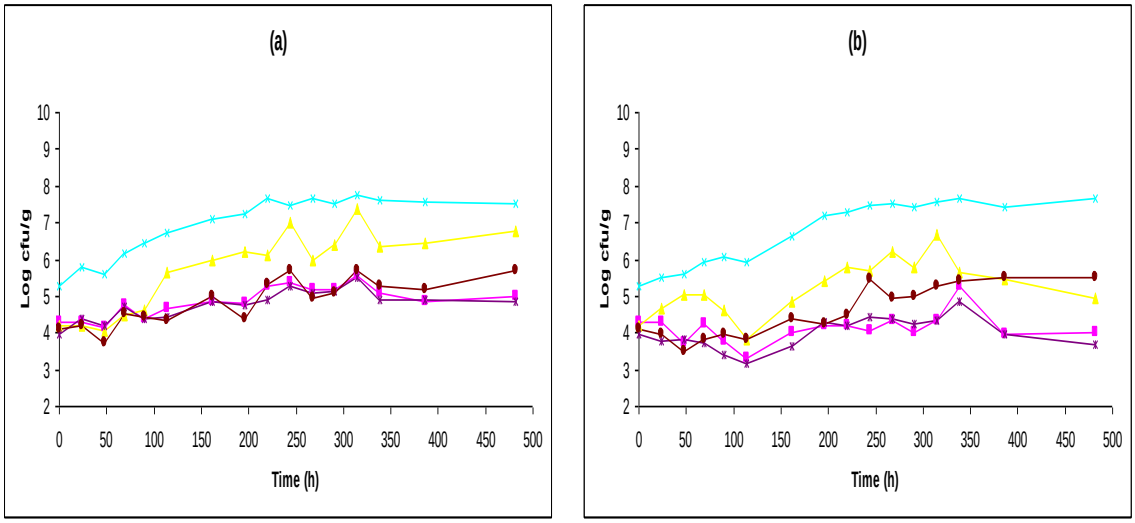


Figure 3.1. Growth of *Pseudomonas* spp. (▪), *Br. thermosphacta* (▲), *Enterobacteriaceae* (*), lactic acid bacteria (x) and yeasts (♦) on minced beef stored under MAP without (a) and with (b) volatile compounds of oregano essential oil 5 °C.

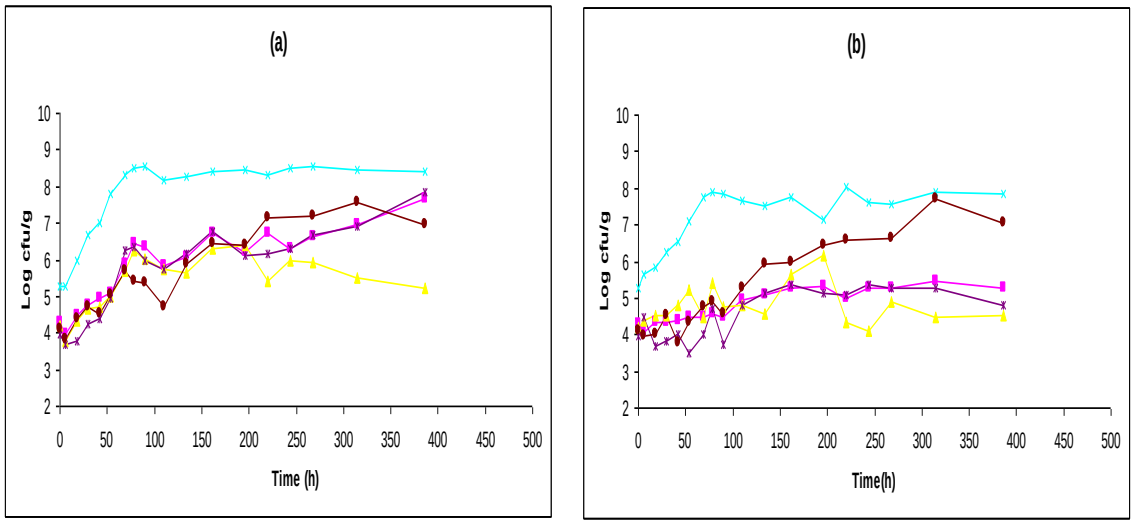


Figure 3.2 Growth of *Pseudomonas* spp. (▪), *Br. thermosphacta* (▲), *Enterobacteriaceae* (*), lactic acid bacteria (x) and yeasts (♦) on minced beef stored under MAP without (a) and with (b) volatile compounds of oregano essential oil 10 °C.

Table 3.7. The effect of packaging and volatile compounds of oregano essential oil on the final population, lag phase and maximum specific growth rate of spoilage microorganisms of minced beef stored at 0, 5, 10 and 15°C estimated by the Baranyi model.

			Air				MAP -				MAP +			
Temperature (°C)	Microorganism	Initial population (log cfu g^{-1})	Final population (log cfu g^{-1})	Time (N_{max}) (h)[3]	lag (h)	μ_{max} (h^{-1})	Final population (log cfu g^{-1})	Time (N_{max}) (h)	lag (h)	μ_{max} (h^{-1})	Final population (log cfu g^{-1})	Time (N_{max}) (h)	lag (h)	μ_{max} (h^{-1})
0	Total viable count	5.48	10.00[1] (9.50[2])	332.4	101.50	0.0542	8.16 (∞[4])	∞	209.30	0.0183	7.34 (∞)	∞	223.00	0.0136
	Pseudomonads	4.30	10.11 (9.68)	415.5	30.68	0.0500	4.73 (∞)	∞	74.75	0.0042	3.72 (3.63)	∞	0	-0.0228
	Br. thermosphacta	4.26	8.77 (8.31)	332.4	58.36	0.0562	6.57 (∞)	∞	186.70	0.0151	3.73 (∞)	∞	341.70	0.0033
	Enterobacteriaceae	3.99	7.83 (7.90)	554.0	35.61	0.0176	4.67 (∞)	∞	0	0.0012	3.71 (∞)	∞	0	-0.0005
	Lactic acid bacteria	5.26	7.37 (7.03)	387.8	31.03	0.0182	7.89 (∞)	∞	216.90	0.0219	7.29 (∞)	∞	238.90	0.0162
	Yeasts and moulds	4.18	8.02 (7.87)	332.4	101.70	0.0584	5.31 (8.19)	554.0	524.80	0.0919	3.54 (∞)	∞	0	-0.0025
5	Total viable count	5.48	9.90 (9.87)	313.3	18.29	0.0516	8.00 (7.99)	289.2	11.18	0.0285	7.85 (7.72)	337.4	52.80	0.0257
	Pseudomonads	4.30	9.99 (9.83)	241.0	16.87	0.0852	4.97 (5.17)	289.2	49.61	0.0115	4.00 (∞)	∞	0	0.0018
	Br. thermosphacta	4.26	7.86 (8.11)	168.7	50.06	0.1059	6.77 (6.60)	265.1	52.35	0.0361	4.93 (5.98)	361.5	0	0.0169
	Enterobacteriaceae	3.99	9.13 (8.91)	385.6	9.82	0.0442	4.88 (5.09)	361.5	0	0.0100	3.66 (4.16)	192.8	0	0.0057
	Lactic acid bacteria	5.26	7.70 (7.61)	192.8	0	0.0406	7.54 (7.61)	313.3	0	0.0250	7.64 (7.61)	361.5	0	0.0221
	Yeasts and moulds	4.18	8.17 (8.88)	241.0	80.97	0.0901	5.70 (∞)	∞	0	0.0077	5.53 (5.57)	482.0	88.10	0.0137
10	Total viable count	5.48	9.73 (9.60)	173.7	0	0.0914	8.66 (8.47)	115.8	10.17	0.1132	8.02 (7.85)	96.5	16.44	0.0998
	Pseudomonads	4.30	9.45 (9.53)	193.0	0	0.1063	7.66 (6.70)	173.7	0	0.0598	5.26 (5.38)	193.0	52.74	0.0227
	Br.thermosphacta	4.26	7.42 (7.01)	77.2	28.77	0.2051	5.24 (6.01)	115.8	13.26	0.0633	4.49 (∞)	∞	0	0.0010
	Enterobacteriaceae	3.99	9.32 (9.16)	154.4	19.25	0.1389	7.85 (6.59)	135.1	25.10	0.1040	4.81 (5.20)	154.4	73.39	0.0531
	Lactic acid bacteria	5.26	8.16 (8.44)	77.2	15.63	0.1538	8.42 (8.45)	96.5	4.76	0.1186	7.86 (7.77)	96.5	0	0.0836
	Yeasts and moulds	4.18	7.85 (8.43)	154.4	13.47	0.1113	6.94 (7.28)	289.5	0	0.0350	7.07 (7.52)	386.0	22.63	0.0338
15	Total viable count	5.48	9.55 (9.49)	99.0	0	0.1584	9.54 (8.38)	66	0	0.1594	8.32 (7.81)	66.0	0	0.1077
	Pseudomonads	4.30	9.35 (9.47)	110.0	0	0.1811	7.34 (∞)	∞	236.40	0.0145	7.09 (∞)	∞	61.15	0.0400
	Br. thermosphacta	4.26	7.14 (6.76)	55.0	22.18	0.2998	5.85 (∞)	∞	0	0.0093	2.59 (∞)	∞	0	-0.0260
	Enterobacteriaceae	3.99	8.78 (9.14)	99.0	0	0.1857	8.47 (8.30)	198.0	0	0.0644	6.98 (6.58)	209.0	0	0.0390
	Lactic acid bacteria	5.26	8.60 (8.42)	44.0	15.07	0.3350	8.44 (8.30)	77.0	0	0.1443	8.21 (7.85)	66.0	0	0.1470
	Yeasts and moulds	4.18	7.95 (8.21)	110.0	5.23	0.1716	7.99 (7.99)	220.0	0	0.0535	7.89 (7.89)	220.0	20.40	0.0603

[1] Determined experimentally (values recorded at the end of storage period for each condition)
[2] Estimated by the Baranyi model
[3] time needed to reach the upper asymptote
4 Fitted curve did not present upper asymptote (semisigmoidal)

3.2.2. Growth of *Listeria monocytogenes* Scott A

L. monocytogenes was inoculated on minced beef in order to evaluate the effect of volatile compounds of oregano essential oil on its growth. Prior to inoculation, the presence of *L. monocytogenes* was tested and this pathogen was not present. This pathogen grew only in samples stored aerobically (Table 3.5).

The modified atmosphere controlled the growth of *L. monocytogenes* (Table 3.5), although the presence of volatile compounds of oregano essential oil did not affect further the growth/survival of this pathogen. Indeed, in all samples stored under MAP -/ MAP + there was a reduction of about 1 log cfu g^{-1} in samples stored aerobically. The storage temperature also affected the growth of this pathogen (Table 3.5).

Table 3.8. The effect of packaging and volatile compounds of oregano essential oil on the final population, lag period and maximum specific growth rate of *Listeria monocytogenes* Scott A of minced beef stored at 0, 5, 10 and 15°C estimated by the Baranyi model.

		AIR			MAP -			MAP +		
Temperature (°C)	**initial population log cfu g^{-1}**	**final population log cfu g^{-1}**	**lag (h)**	**μ_{max} (h^{-1})**	**final population log cfu g^{-1}**	**lag (h)**	**μ_{max} (h^{-1})**	**final population log cfu g^{-1}**	**lag (h)**	**μ_{max} (h^{-1})**
0	3.20	(3.51)[1]		0.0011	(3.29)		0.0001	(3.13)		-0.0010
5	3.20	(4.22)		0.0047	(2.83)		-0.0022	(2.93)		-0.0013
10	3.20	(5.00)	133.10	0.0297	(2.48)		-0.0058	(2.49)		-0.0090
15	3.20	(5.24)	70.54	0.0339	(2.78)		-0.0024	(2.66)		-0.0080

[1] Determined experimentally

3.2.3. Sensory analysis

The microbial shelf life (TVC 7 log cfu g^{-1}) and the sensory shelf life (score 2) of minced beef stored at 0, 5, 10 and 15°C, are shown in Table 3.6. It was evident that in all cases the volatile compounds of oregano essential oil increased shelf life of minced beef compared to the samples stored in air or MAP -. The presence of oil affected the odour and colour (photographs not shown) of minced beef. It needs to be noted that sometimes, oregano

flavour was strong and might have hampered the sensory evaluation of samples by the panellists.

Table 3.9. Microbial shelf life and sensory shelf life of minced beef in air or packaged under MAP without or with volatile compounds of oregano essential oil at 0, 5, 10 and 15°C.

	AIR		MAP -		MAP +	
Temperature (°C)	**Microbial shelf life (h)**	**Sensory shelf life (h)**	**Microbial shelf life (h)**	**Sensory shelf life (h)**	**Microbial shelf life (h)**	**Sensory shelf life (h)**
0	155	291	386	422	458	458
5	90	162	114	244	176	280
10	36	69	42	78	48	100
15	24	48	15	62	33	69

3.2.4. pH changes

The effect of modified atmosphere and volatile compounds of oregano essential oil on pH values of minced beef in comparison with samples stored aerobically are shown in Figure 3.3. In both cases, the pH values were decreased, although a significant rise in pH values was obtained aerobically. The presence of oil did not affect the pH values in all cases but one. At 10°C, the highest pH values were obtained under MAP (Figure 3.3c).

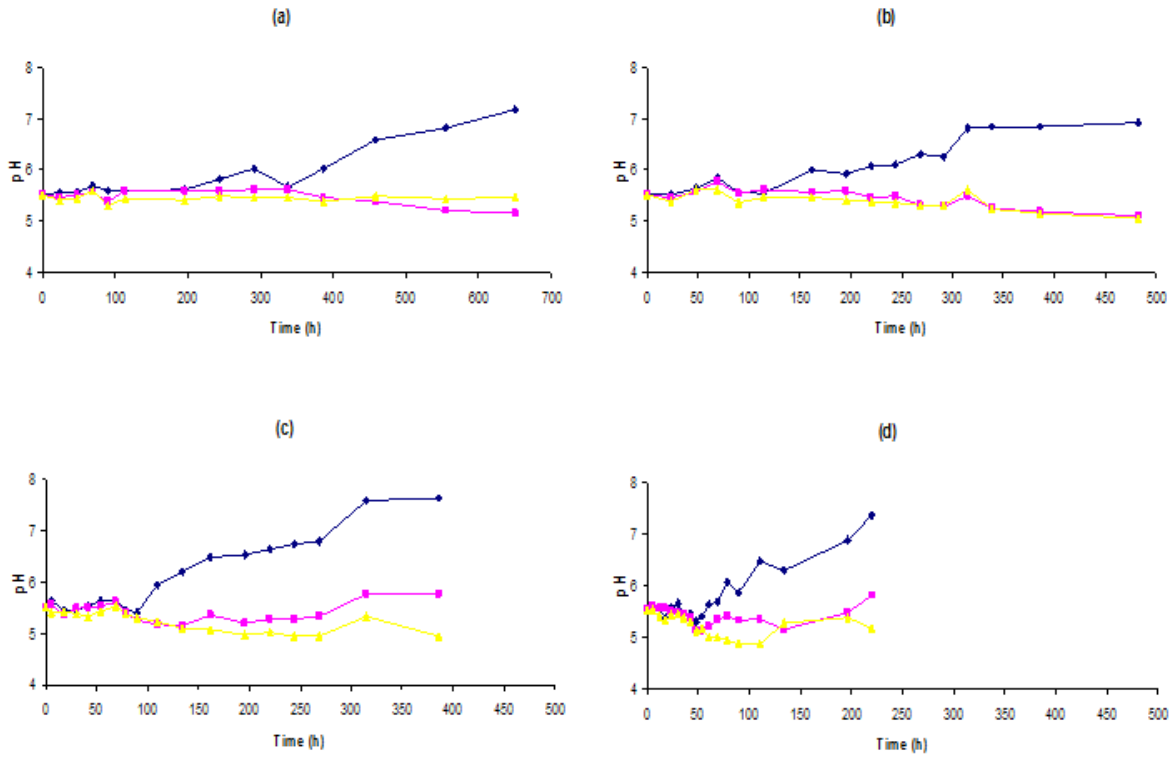


Figure 3.3. Changes in pH of minced beef stored aerobically (♦), under MAP without (▪) or with (▲) volatile compounds of oregano essential oil at 0 (a), 5 (b), 10 (c) and 15°C (d).

3.3. Study of the microbiota during storage of meat

3.3.1. Study of the development of microbiota in minced beef stored under different conditions

The development of the microbiota throughout the storage of minced beef stored under different conditions was studied. More accurately, minced beef stored under aerobic, MAP - and MAP + conditions at 0, 5, 10 and 15˚C; the experimental plan and the results are shown in Section 2.2 and Section 3.2, respectively. Briefly, bacteria were isolated from MRS growth medium (lactic acid bacteria), VRBG growth medium (*Enterobacteriaceae*) as well as from Pseudomonas agar base medium.

A. Identification of lactic acid bacteria

A total of 266 LAB isolates were recovered throughout the storage period at 0, 5, 10 and 15 °C; 99 isolates from aerobic storage, 89 isolates from MAP - and 78 isolates from MAP +. The 99 isolates from aerobic storage were subjected to PFGE to determine the strain diversity during storage. For the aforementioned isolates, high molecular weight genomic DNA was digested with two different restriction enzymes (*Apa*I and *Sma*I). *Apa*I restriction generated better distributed bands than *Sma*I allowing a more reliable analysis of the generated profiles (Figure 3.4). Therefore, *Apa*I was chosen to digest the 89 and 78 isolates from minced beef stored under MAP - and MAP +, respectively.

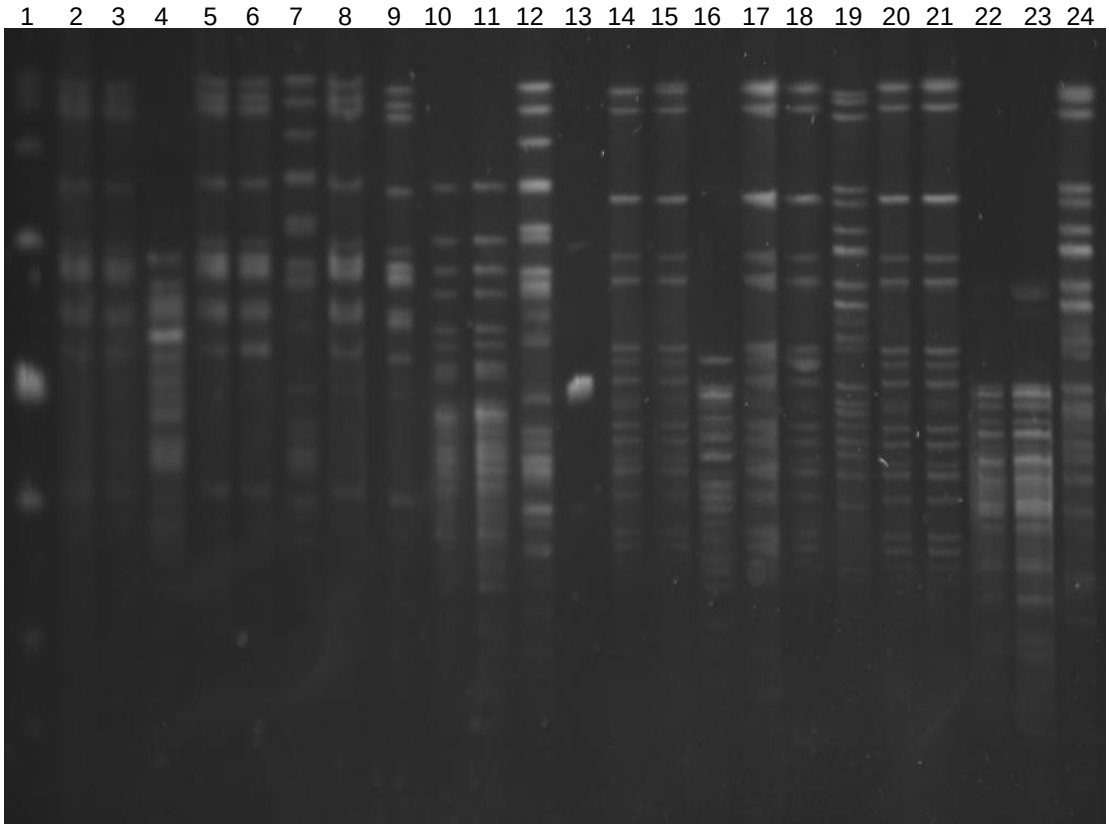


Figure 3.4. PFGE patterns of genomic DNA from the same lactic acid bacteria strains isolated from minced beef stored in air digested with *Sma*I (lanes 2-12) and *Apa*I (lanes 14-24). Low range PFG Marker at the 1st and 13th lane.

A large diversity regarding strain occurrence in the different packaging and temperature conditions was revealed (Figure 3.5, Table 3.8). The dendrogram obtained after image analysis of the different PFGE patterns, resulted into 32 different profiles, nine of which were obtained from aerobic storage, while 15 and 17 from MAP - and MAP +, respectively (Table 3.8). Each strain present in Figure 3.5 was subjected to 16S rRNA gene sequencing. Strains B 225, B 251, B 226, B 236, B 248, B 253, B 228, B 237, B 229, B 255, B 227, B 239, B 230, B 238, B 250, B 252, B 254 and B 249 were assigned to *Lb. sakei*; strains B 245 and B 246 to *Lb. curvatus*; strain B 247 to *Lb. casei*-group; strains B 234 and B 235 to *Ws. viridescens*; strains B 242 and B 243 to *Ln. mesenteroides*-group and strains B 232, B 241, B 258, B 244, B 233, B 240 and B 231 to *Leuconostoc* spp. All isolates were also screened for the presence of the *kat*A gene, specific for *Lb. sakei*. The *kat*A amplification results were in accordance with the ones already described. Two fingerprints (B 232 and B 233 assigned to *Leuconostoc* spp.) were common for all packaging conditions, two (B 226 and B 227 assigned to *Lb. sakei*) were shared between air and MAP -, one fingerprint (B 230 assigned to *Lb. sakei*) was shared between air and MAP +, and two fingerprints (B 242 and B 243 assigned to *Ln. mesenteroides*) were shared between MAP - and MAP +.

From the initial stage of storage, two different strains (B 232 and B 233) were recovered, which were assigned to *Leuconostoc* spp. Strain B 233 was the most common isolate, since it was recovered at a percentage of 83.33% of the isolates recovered from the initial stage of storage.

From the 99 isolates from the aerobic storage of minced beef, the largest group was attributed to *Leuconostoc* spp. The corresponding fingerprints were B 231, B 232 and B 233 , with the latter being the most common isolate, representing the dominant biota during storage at 5, 10 and 15°C. The rest of the fingerprints (B 225, B 226, B 227, B 228, B 229 and B 230)

were attributed to *Lb. sakei*, which were recovered from 0 and 5°C, but one (B 228) from 10°C. At 0°C, *Lb. sakei* (B 226) was the prevalent one at the final stage of storage. The frequency of isolation and prevalence of the aforementioned isolates obtained from minced beef regarding aerobic storage at 0, 5, 10 and 15°C is shown in Table 3.8.

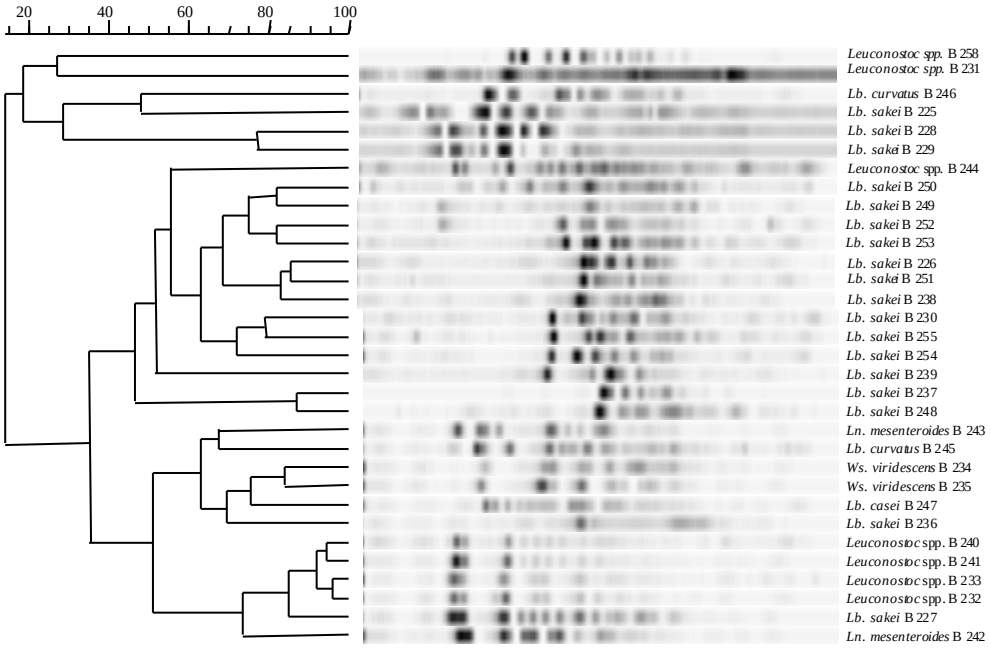


Figure 3.5. Cluster analysis of PFGE *Apa*I digestion fragments of the lactic acid bacteria isolates recovered from minced beef calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%). Strain identity is indicated by the lower and upper case letters.

Fifteen different fingerprints were detected during storage of minced beef under MAP -; they were assigned to *Lb. sakei* (B 226, B 227, B 236, B 237, B 238 and B 239), *Leuconostoc* spp. (B 232, B233, B 258, B 240 and B 241), *Ws. viridescens* (B 234 and B 235), and *Ln. mesenteroides* (B 242 and B 243). Table 3.8 shows the frequency of isolation and prevalence of the isolates regarding the storage temperature. At 10 and 15°C, *Leuconostoc* spp. (B 233) represented the dominant biota, whilst *Ws. viridescens* (B 234), *Lb. sakei* (B 237), *Ln. mesenteroides* (B 243) and *Leuconostoc* spp. (B 240) were also recovered.

At chill temperatures (0 and 5°C), the strain diversity was increased, since 11 different fingerprints were recovered. In both temperatures, *Lb. sakei* (B 237) was the prevalent strain at the final stage of storage. Moreover, *Lb. sakei* (B 236, B 238 and B 239), and *Leuconostoc* spp. (B 233 and B 258) were also recovered from storage at 0°C, whilst *Ws. viridescens* (B 235), *Lb. sakei* (B 226, B 238 and B 227), *Leuconostoc* spp. (B 233 and B 241) and *Ln. mesenteroides* (B 242) were recovered from storage at 5°C.

Out of 78 isolates, seventeen different fingerprints were obtained during storage of minced beef under MAP +, indicating the increased diversity of the isolates. The frequency of isolation and prevalence of these isolates regarding storage at 0, 5, 10 and 15°C is shown in Table 3.8. At 10 and 15°C, *Leuconostoc* spp. (B 233) was the most common isolate, representing the dominant strain, whilst *Lb. sakei* (B 252 and B 255) and *Ln. mesenteroides* (B 243) were also recovered. *Lb. sakei* (B 254) was the dominant strain at the final stage of storage at 5°C, while *Lb. sakei* (B 248 and B 255), and *Leuconostoc* spp. (B 233 and B 244) were also recovered during the storage at 5°C. At the final stage of storage at 0°C, *Leuconostoc* spp. (B 233), *Ln. mesenteroides* (B 242), *Lb. curvatus* (B 246) and *Lb. sakei* (B 249 and B 251) were equally contributed. *Lb. curvatus* (B 245), *Lb. casei* and *Lb. sakei* (B 248, B 250 and B 253) were also recovered during storage at 0°C.

Table 3.7. Identity of lactic acid bacteria isolates obtained from minced beef.

Code[1]	Closest relative	Accession Number
B 225	*Lactobacillus sakei*	GU998856
B 226	*Lb. sakei*	GU998877
B 227	*Lb. sakei*	GU998857
B 228	*Lb. sakei*	GU998850
B 229	*Lb. sakei*	GU998851
B 230	*Lb. sakei*	GU998852
B 231	*Leuconostoc* spp.	GU998853
B 232	*Leuconostoc* spp.	GU998854
B 233	*Leuconostoc* spp.	GU998855
B 234	*Weissella viridescens*	GU998858
B 235	*Ws. viridescens*	GU998859
B 236	*Lb. sakei*	GU998860
B 237	*Lb. sakei*	GU998861
B 238	*Lb. sakei*	GU998862
B 239	*Lb. sakei*	GU998863
B 258	*Leuconostoc spp*	GU998864
B240	*Leuconostoc* spp.	GU998865
B 241	*Leuconostoc* spp.	GU998866
B 242	*Ln. mesenteroides*	GU998867
B 243	*Ln. mesenteroides*	GU998868
B 244	*Leuconostoc spp.*	GU998869
B 245	*Lb. curvatus*	GU998870
B 246	*Lb. curvatus*	GU998871
B 247	*Lb. casei*	GU998872
B 248	*Lb. sakei*	GU998873
B 249	*Lb. sakei*	GU998874
B 250	*Lb. sakei*	GU998875
B 251	*Lb. sakei*	GU998876
B 252	*Lb. sakei*	GU998878
B 253	*Lb. sakei*	GU998879
B 254	*Lb. sakei*	GU998880
B 255	*Lb. sakei*	GU998881

[1] Code of different PFGE patterns of Figure 3.5

Table 3.8. Frequency (%) of isolation and distribution of lactic acid bacteria isolates recovered from minced beef stored under aerobic, MAP - and MAP + conditions.

			AIR												MAP -												MAP +											
			0 °C			5 °C			10 °C			15 °C			0 °C			5 °C			10 °C			15 °C			0 °C			5 °C			10 °C			15 °C		
Closest species	Code	Initial	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end
Lb. sakei	B 225							6.3												6.3																		
Lb. sakei	B 226			9.1	40.0														50.0																		
Lb. sakei	B 227			9.1																12.5																		
Lb. sakei	B 228								9.1																														
Lb. sakei	B 229				10.0																																	
Lb. sakei	B 230							6.3																													11.1		
Leuconostoc spp.	B 231					20.0																																
Leuconostoc spp.	B 232	16.7		9.1	20.0			31.3																														
Leuconostoc spp.	B 233	83.3	100.0	72.7	30.0	80.0	100.0	56.3	90.9	100.0	100.0	100.0	100.0	100.0	40.0	62.5		80.0	50.0	25.0	100.0	100.0	80.0	100.0	100.0	75.0	75.0	42.9	20.0	80.0	44.4	33.3	100.0	100.0	77.8	100.0	100.0	88.9
Ws.viridescens	B 234																						10.0															
Ws.viridescens	B 235																	10.0		12.5																		
Lb. sakei	B 236														20.0																							
Lb. sakei	B 237																100.0			25.0						12.5												
Lb. sakei	B 238															25.0				12.5																		
Lb. sakei	B 239														20.0	12.5																						
Leuconostoc spp.	B 240																									12.5												
Leuconostoc spp.	B 241																	10.0																				
Ln. mesenteroides	B 242																			12.5								20.0										
Ln. mesenteroides	B 243																						10.0															11.1
Leuconostoc spp.	B 244																														11.1							
Lb. curvatus	B 245																											14.3										
Lb. curvatus	B 246																												20.0									
Lb. casei	B 247																										25.0											
Lb. sakei	B 248																											14.3		20.0	33.3							
Lb. sakei	B 249																												20.0									
Lb. sakei	B 250																											14.3										
Lb. sakei	B 251																												20.0									
Lb. sakei	B 252																																		11.1			
Lb. sakei	B 253																											14.3										
Lb. sakei	B 254																															66.7						
Lb. sakei	B 255																														11.1							
Leuconostoc spp.	B 258														20.0																							

B. Identification of *Enterobacteriaceae*

A total of 232 *Enterobacteriaceae* isolates were recovered throughout the storage period (Table 3.9) and subjected to SDS – PAGE of whole cell proteins and PFGE in order to determine the species and strain diversity, respectively.

Whole cell protein profiling

Enterobacteriaceae isolates were clustered into seven groups on the basis of their SDS – PAGE profile obtained from whole cell proteins. The protein profile of each group is shown in Figure 3.6 whereas the number of the isolates as well as the storage condition and time points of isolation is presented in Table 3.9. In fresh meat, presence of two profiles, namely A and B, was detected. Profile A consisted of 139 isolates and was common for all packaging and temperature conditions, but 10°C and 15°C under MAP +. Similarly, profiles C and D, consisting of 4 and 18 isolates, respectively, were recovered from both aerobic and MAP storage; the former only at 15°C during both aerobic and MAP- storage, while omnipresence of the latter met the exceptions of 10°C aerobically, 0°C, 5°C and 15°C under MAP – and 10°C and 15°C under MAP +. Regarding the rest of the profiles, one was recovered only during aerobic storage and only 3 during MAP storage. The former, namely profile E, consisted of 2 isolates that were only isolated at 10°C, whereas the latter can be further subdivided according to the presence of oregano essential oil. Thus, profile B consisted of 10 isolates, recovered only during MAP+ storage at 0°C and 15°C, while profiles F and G consisted of 44 and 10 isolates that were isolated regardless of the presence of oregano essential oil; the former at 0°C under MAP- and 10°C and 15°C under both MAP- and MAP+, while the latter at 5°C under MAP- and at 0°C under both MAP- and MAP+.

Table 3.9. SDS groups of *Enterobacteriaceae* isolates recovered from minced beef.

Source	Tempe-rature (°C)	SDS Group							Total isolates
		A	B	C	D	E	F	G	
Fresh meat		6[3]	6						12
Meat stored aerobically	0	6			4				70
	5	16 (8,8)[4]			4 (2,2)				
	10	18 (8,10)				2 (2,-)			
	15	14 (4,10)		2 (2,-)	4 (4,-)				
Meat stored under MAP –[1]	0	14 (10,4)					2 (-,2)	4 (-,4)	74
	5	12 (2,10)						4 (4,-)	
	10	10 (10,-)			2 (-,2)		8 (-,8)		
	15	14 (6,8)		2 (2,-)			2 (-,2)		
Meat stored under MAP +[2]	0	16 (8,8)	2 (-,2)		2 (2,-)			2 (2,-)	76
	5	18 (10,8)			2 (-,2)				
	10						20 (10,10)		
	15		2 (2,-)				12 (2,10)		

[1] modified atmosphere packaging (40% CO_2/30% O_2/30% N_2)
[2] volatile compounds of 2% v/w oregano essential oil
[3] number of isolates
[4] number of isolates from different time points (middle, final)

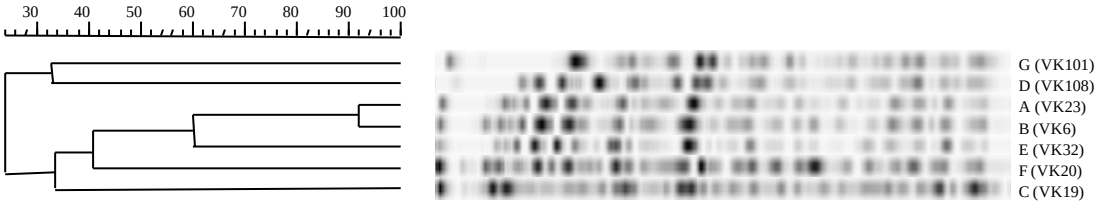


Figure 3.6. Cluster analysis of SDS – PAGE profiles from whole cell proteins of SDS groups of the *Enterobacteriaceae* isolates recovered from minced beef calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Genotypic analysis

In the present study, macrorestriction analysis by PFGE was used for strain differentiation of *Enterobacteriaceae* isolates. However, most of the *Enterobacteriaceae* isolates (group A, B, D, E, G based on SDS – PAGE analysis) could not be analysed by PFGE, when the isolation of the intact chromosomal DNA was performed according to Herschleb et al. (2007), as a continuous smear of DNA rather than well separated fragments was produced. Similar results were observed when sodium dodecyl sulfate was added in the solution used to make the agarose plugs (Hunter et al., 2005) and when the incubation time for proteinase K treatment was increased from 24 to 48 h (Herschleb et al., 2007; data not shown). Neither thiourea addition into running buffer (Romling and Tummler, 2000; Silbert et al., 2003; Lee et al., 2006; Liesegang and Tschape, 2002) nor the use of HEPES buffer as a running buffer (Koort et al. 2002; Ray et al., 1992) were able to prevent DNA degradation (data not shown). On the other hand, all isolates yielded well-separated DNA fragments with the modification of the protocol, i.e., the addition of 50 μM thiourea after the proteinase K treatment described in this study. Even better results were observed when the addition of thiourea (50 μM) after the proteinase treatment was combined with addition of thiourea (100 μM) to running buffer. Then, macrorestriction profiles with no background smearing were produced and at the same time did not alter the pattern of the strain included as a control (group C based on SDS – PAGE analysis) (i.e. typeable without thiourea) (Figure 3.7). Therefore, the modified protocol was chosen to analyse the 232 isolates.

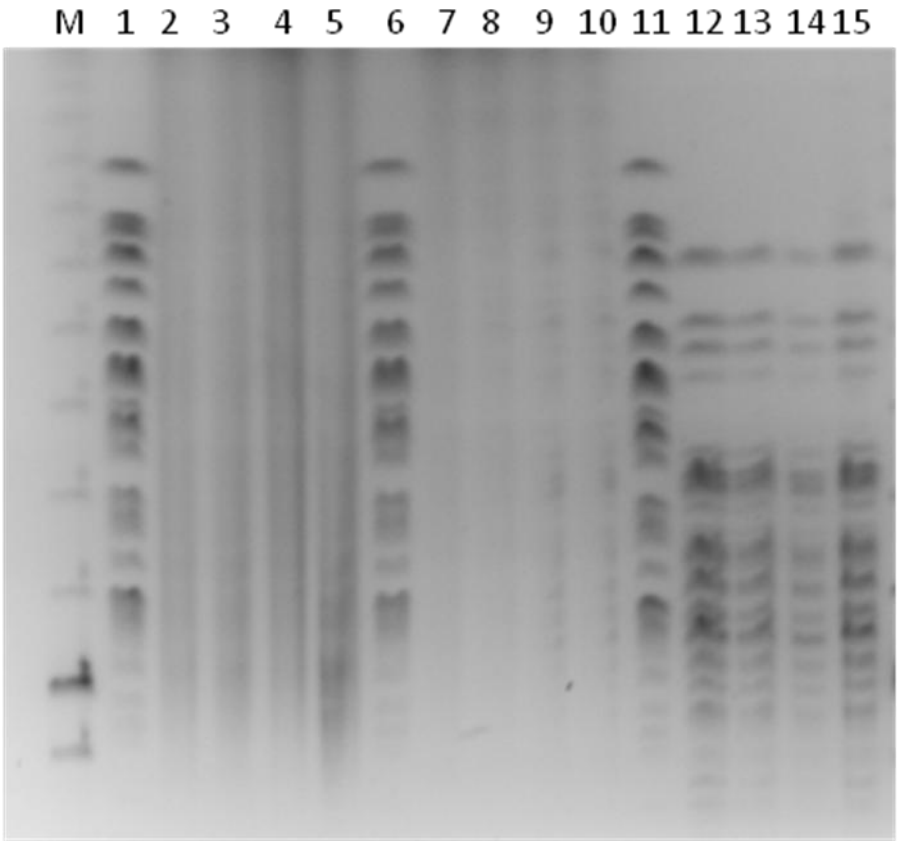


Figure 3.7. PFGE pattern of *Enterobacteriaceae* isolates recovered from minced beef after *Xba*I digestion of their genomic DNA performed a) with the standard protocol (Lanes 1-5), b) after minimize the culturing time (Lanes 6-10) and c) after thiourea treatment (Lanes 11-15). (Lanes M: Low range PFG marker, Lanes 1, 6 and 11 positive control).

The dendrogram obtained after image analysis of the different PFGE patterns from *Xba*I restriction, resulted in 19 different profiles (Figure 3.8). Each fingerprint present in Figure 3.8 was subjected to 16S rRNA gene sequencing. Five fingerprints (VK17, VK23, VK40, VK74 and VK75), which belonged to SDS – PAGE profile A, were assigned to *Serratia liquefaciens* (Table 3.10). SDS-PAGE profile B was represented by 4 different PFGE fingerprints, namely VK6, VK25, VK113 and VK5; all of them being assigned to *Serratia proteamaculans*. Similarly, fingerprints VK90 and VK108, members of SDS-PAGE profiles group D, were assigned to *Serratia* spp.. Fingerprints VK19 and VK32, belonged to SDS-PAGE profiles C and E, were attributed to *C. freundii* and *S. proteamaculans*, respectively. Four fingerprints, namely VK20, VK27, VK53 and VK60 (SDS-PAGE profile

F) were identified as *H. alvei*, while two fingerprints, namely VK101 and VK103 (SDS-PAGE profile G) were assigned to *P. vulgaris*.

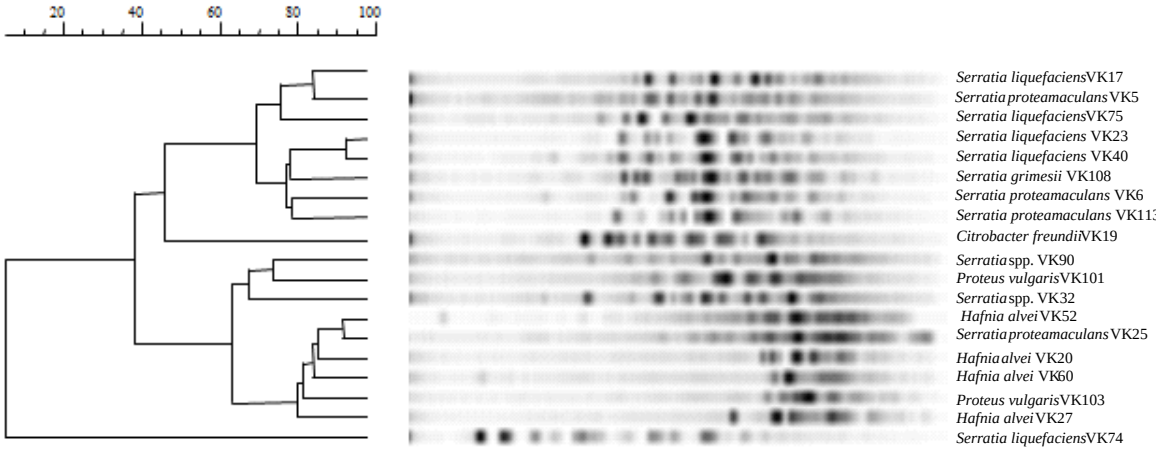


Figure 3.8. Cluster analysis of PFGE *Xba*I digestion fragments of the *Enterobacteriaceae* isolates recovered from minced beef calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

In Table 3.11 the prevalence of the different *Enterobacteriaceae* PFGE fingerprints regarding the respective storage conditions is summarized. *S. proteamaculans* (VK5 and VK6), *S. liquefaciens* (VK17 and VK23) constituted the *Enterobacteriaceae* community of fresh meat. Regarding the five different PFGE fingerprints assigned to *S. liquefaciens*, VK17 and VK23 were the most common, whereas the rest (VK40, VK74 and VK75) were only sporadically recovered. More accurately, *S. liquefaciens* VK17 was the dominant isolate in the middle stage of storage at 5 and 10°C under aerobic conditions, 15°C under MAP – and the final stage of storage at 0°C under aerobic conditions. On the other hand, *S. liquefaciens* VK23 was the dominant isolate for the rest of the storage conditions, but 10°C and 15°C under MAP +. *S. proteamaculans* VK113 and VK25 were recovered during storage under MAP + at 0°C and 15°C respectively, while VK32 was recovered in the middle stage of storage under aerobic conditions at 10°C. PFGE fingerprint VK20 was the most common

among the 42 *H. alvei* isolates and represented the dominant fingerprint under MAP – at 10°C (final stage of storage) and under MAP + at 10°C (middle stage of storage) and at 15°C (middle, final stage of storage). Additionally, *P. vulgaris* VK101 and VK103 were both isolated during storage under MAP – at 0 °C and 5 °C, while the latter was also isolated during storage under MAP + at 0°C.

Table 3.10. Species identification of *Enterobacteriaceae* isolates recovered from minced beef after sequencing of the variable V1-V3 region of the 16S rRNA genes.

Code	**Closest relative**	**GenBank accession number of closest relative**	**Identity (%)**	**GenBank accession number of sequence**
VK5	*Serratia proteamaculans*	AJ508694	99	HM242268
VK6	*S. proteamaculans*	AJ508694	100	HM242269
VK17	*S. liquefaciens*	FJ811866	99	HM242270
VK19	*Citrobacter freundii*	AB548826	100	HM242271
VK20	*Hafnia alvei*	AJ508360	100	HM242272
VK23	*S. liquefaciens*	EU880537	100	HM242273
VK25	*S. proteamaculans*	EU627690	100	HM242274
VK27	*H. alvei*	AJ508360	99	HM242275
VK32	*S. proteamaculans*	EU627690	99	HM242276
VK40	*S. liquefaciens*	EU880537	99	HM242277
VK53	*H. alvei*	AJ508360	100	HM242278
VK60	*H. alvei*	AB244473	99	HM242279
VK74	*S. liquefaciens*	AJ306725	100	HM242280
VK75	*S. liquefaciens*	AJ306725	99	HM242281
VK90	*Serratia* spp.	AJ545753	99	HM242282
VK101	*Proteus vulgaris*	AY877032	99	HM242283
VK103	*P. vulgaris*	GQ292550	99	HM242284
VK108	*Serratia* spp.	EF491959	99	HM242285
VK113	*S. proteamaculans*	AJ508694	100	HM242286

Table 3.11. Frequency (%) of isolation and distribution of *Enterobacteriaceae* isolates recovered from minced beef stored under aerobic, MAP - and MAP + conditions.

	Source	Fresh meat	Meat stored aerobically				Meat stored under MAP –[a]				Meat stored under MAP +[b]			
Closest species	**PFGE fingerprint (SDS-PAGE profile)**		**0[c]**	**5**	**10**	**15**	**0**	**5**	**10**	**15**	**0**	**5**	**10**	**15**
Serratia liquefaciens	**VK17 (A)**	33	60/60[d]	80/40	80/20		40/		20/	75/	/20	40/		
	VK23 (A)	17		/40	/60	40/100	60/40	33/60	80/	/23	67/60	60/80		
	VK40 (A)				/20									
	VK74 (A)							/20						
	VK75 (A)							/20						
S. proteamaculans	**VK5 (B)**	17												
	VK6 (B)	33												
	VK25 (B)													50/
	VK113 (B)										/20			
Citrobacter freundii	**VK19 (C)**					20/				25/				
***Serratia* spp.**	**VK90 (D)**		20/20											
	VK108 (D)		20/20	20/20		40/			/20		17/	/20		
S. proteamaculans	**VK32 (E)**				20/									
Hafnia alvei	**VK20 (F)**								/60	/20			40/20	50/80
	VK27 (F)												20/40	50/20
	VK53 (F)												20/	
	VK60 (F)						/20		/20				20/40	
Proteus vulgaris	**VK101 (G)**						/20	33/						
	VK103 (G)						/20	33/			17/			

[1] modified atmosphere packaging (40% CO_2/30% O_2/30% N_2)
[2] volatile compounds of 2% v/w oregano essential oil
[3] storage temperature
[4] percentage (%) of isolates from different time points (middle/final stage of storage)

C. Identification of isolates from Pseudomonas Agar Base growth medium

A total of 267 isolates from PAB medium were recovered throughout the storage period. The aforementioned isolates were subjected to PFGE to determine the strain diversity during storage. In Table 3.12, the different time points for each of the storage conditions that isolates were recovered are shown. The image analysis of the different PFGE patterns, resulted in eighty two different fingerprints, forty six of which were obtained from aerobic storage, while twenty seven and twenty eight from MAP - and MAP +, respectively. A large diversity regarding strain occurrence at the different packaging and temperature conditions was revealed. The most common isolates recovered during storage of minced beef under the different conditions adopted are shown in Table 3.12. Additionally, the remaining isolates recovered in these conditions are shown in Table 3.13. Because of this large observed diversity, the different fingerprints were subjected to PCR-DGGE to decrease the number of samples to be sequenced. Moreover, the results in terms of the succession of these fingerprints and their distribution in beef samples were analysed after the application of the latter method.

Sixteen different DGGE fingerprints were obtained after PCR – DGGE analysis of the 82 different fingerprints resulted from PFGE analysis. Three of them were assigned to *Ps. putida* , *Ps. fragi* and *Ps. fluorescens*; this identification was confirmed by band position analysis using *Ps. putida* KT2440, *Ps. fragi* DSM 3456 and *Ps. fluorescens* GTE 015 as reference strains**.** The rest of the fingerprints were subjected to 16S rRNA gene sequencing (Table 3.14). The DGGE fingerprint CK2 was assigned to *S. liquefaciens*; fingerprints CK19 and CK33 to C. *freundii*; fingerprint CK30 to *S. grimesii*; fingerprints CK36, CK39 and CK49 to *H. alvei*; fingerprints CK73, CK119, CK148 and CK262 to *Pseudomonas* spp.; fingerprint CK153 to *Rahnella* spp.; fingerprint CK265 to *Morganella morganii*.

Table 3.12. Common isolates recovered from Pseudomonas agar base during storage of minced beef under different packaging (air, MAP - and MAP +) and temperature conditions (0, 5, 10 and 15°C).

Temperature (°C)	Storage period (h)	Fresh Meat	Packaging condition		
			Meat stored aerobically	Meat stored under MAP –[1]	Meat stored under MAP +[2]
	0	CK2/CK4			
0	69		CK67	CK2	CK248
	196		CK185	CK2	CK2
	291		CK185	CK44	CK44
	485		CK4	CK2	CK44/CK88/ CK264/CK265[3]
5	48		CK79	CK65	CK2/CK8[3]
	69		CK120	CK87/CK124/ CK148/CK247[3]	CK2
	114		CK2/CK4/CK125/ CK126/CK127/ CK130/CK131[3]	CK14	ND[4]
	196		CK79	CK2	CK2
	244		CK4	CK14	CK8/CK14/ CK185/CK186 /CK187
10	18		CK2	CK87/CK88/ CK89[3]	ND
	54		CK4	CK14/CK88	CK2/CK88/ CK103[3]
	90		CK8	CK2	CK37
	162		CK79	CK8/CK100[3]	CK2/CK88/ CK98[3]
15	12		CK2/CK7/ CK8[3]	CK2	CK2
	36		CK2	ND	ND
	69		CK2/CK16	CK2	CK2/CK39/ CK49/CK52[3]
	110		CK19/CK20/ CK21/CK22/ CK25/CK26[3]	CK2/CK39	CK54

[1] modified atmosphere packaging (40% CO_2/30% O_2/30% N_2)
[2] volatile compounds of 2% v/w oregano essential oil
[3] equally recovered isolates
[4] no available data

Table 3.13. Isolates recovered from Pseudomonas agar base medium during storage of minced beef under different packaging (air, MAP - and MAP +) and temperature conditions (0, 5, 10 and 15°C).

Temperature (°C)	Storage period (h)	Fresh Meat	Packaging condition		
			Meat stored aerobically	Meat stored under MAP –[1]	Meat stored under MAP +[2]
	0	CK1			
0	69		CK2/CK14	CK8	CK88/CK247
	196		CK2/CK61/CK77/ CK193/CK195/ CK197/CK199/ CK203/CK204/ CK206/CK207	CK44/CK88	CK14/CK98/ CK252/CK253
	291		CK4/ CK19/CK127/ CK208	CK2	CK8/CK88/ CK261/CK262
	485		CK224	CK8/CK88/ CK197/CK244	ER[3]
5	48		CK2/CK119	CK2/CK144	ER[3]
	69		CK2/CK124	ER[3]	CK14/CK87
	114			CK4/CK153	ND[4]
	196		CK133/CK134	CK14/CK153/ CK158	CK4
	244		CK140/CK141/ CK143	CK2/CK37/ CK49/CK164	ER[3]
10	18		CK61	ER[3]	ND
	54		CK2/CK52/ CK65/CK66/ CK67/CK71/ CK73	CK87	ER[3]
	90		CK4/CK75/ CK77	CK88/CK98/ CK99	CK8/CK49/ CK109
	162		CK4/CK8/CK14/ CK85	ER[3]	ER[3]
15	12		ER[3]	CK14/CK30	CK44/CK48A
	36		CK10	ND	ND
	69		CK4/CK14/ CK19/CK20	CK4/CK33/ CK36/CK37	ER[3]
	110		ER[3]	CK8	CK2/CK49/ CK53

[1] modified atmosphere packaging (40% CO_2/30% O_2/30% N_2)
[2] volatile compounds of 2% v/w oregano essential oil
[3] equally recovered isolates (Table 3.12)
[4] no available data

Table 3.14. Species identification of isolates from Pseudomonas agar base medium recovered from minced beef after sequencing of the variable V6-V8 region of the 16S rRNA genes.

Code	Closest relative	GenBank accession number of closest relative	Identity (%)	GenBank accession number of sequence
CK2	*Serratia liquefaciens*	AY243097	99	HM536985
CK19	*Citrobacter freundii*	AB548828	100	HM536986
CK30	*S.grimesii*	AY789460	100	HM536987
CK33	*C. freundii*	AB548828	100	HM536988
CK36	*Hafnia alvei*	FM179943	100	HM536989
CK39	*H. alvei*	DQ412565	100	HM536990
CK49	*H. alvei*	AY572428	99	HM536991
CK73	*Pseudomonas* spp.	AY365075	99	HM536992
CK119	*Pseudomonas* spp.	AY365080	98	HM536993
CK148	*Pseudomonas* spp.	AY599720	99	HM536994
CK153	*Rahnella* spp.	EU275360	99	HM536995
CK262	*Pseudomonas* spp.	AY365080	98	HM536996
CK265	*Morganella morganii*	EF550572	100	HM536997

Those results revealed that members of *Enterobacteriaceae* were able to grow on PAB. Thus, the different fingerprints obtained after image analysis of PFGE were separated into two dendrograms, one for isolates that were assigned to pseudomonads (Figure 3.9) and a second for *Enterobacteriaceae* isolates (Figure 3.10).

From the initial stage of storage, two different DGGE fingerprints were recovered, which were assigned to *Ps. fragi* and *S. liquefaciens* (CK2). It needs to be noted that, *S. liquefaciens* CK2 was the most common DGGE fingerprint displayed as well as the most common isolate based on PFGE analysis in all conditions adopted.

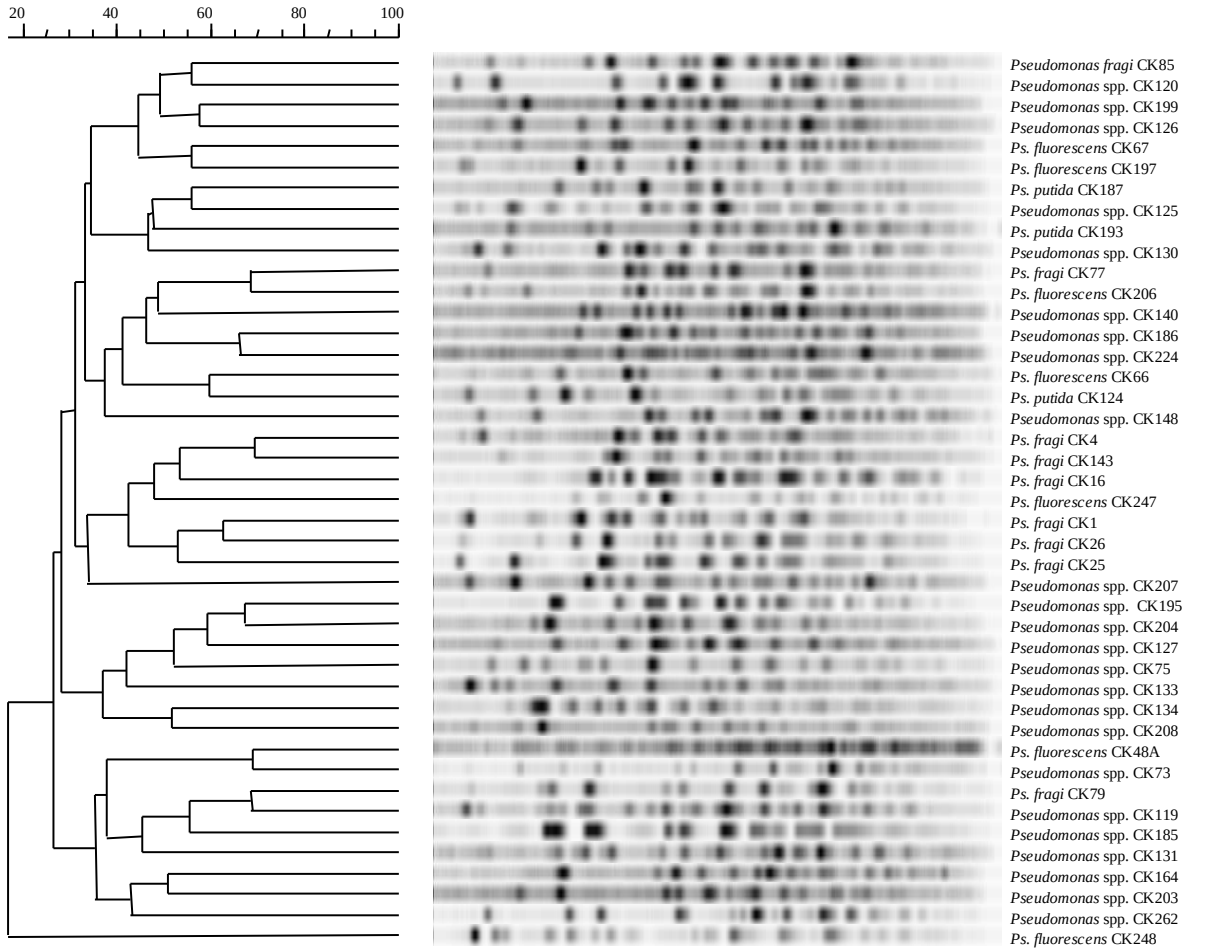


Figure 3.9. Cluster analysis of PFGE *Spe*I digestion fragments of the pseudomonads isolates recovered from Pseudomonas agar base medium during storage of minced beef under different conditions calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Ten different DGGE fingerprints were detected during storage of minced beef under aerobic conditions. *S. liquefaciens* CK2 and *Ps. fluorescens* were equally recovered after a storage period 69h at 0°C, while *Pseudomonas* spp. CK73 was common after 196 hrs. In the latter case, *S. liquefaciens* CK2, *H. alvei* CK39, *Ps. fluorescens*, *Ps. fragi* and *Ps. putida* was also detected. Additionally after 291 hrs storage, *Pseudomonas* spp. CK73 was the most common fingerprint recovered, followed by *Ps. fragi*; *C. freundii* CK19 and *Pseudomonas* spp. CK119 were also detected. *Ps. fragi* was common fingerprint recovered after a storage period of 485 hrs, while *Pseudomonas* spp. CK73 was also obtained. At 5°C, the most

common fingerprint obtained was assigned to *Ps. fragi* after 48, 196 and 244 hrs while *Pseudomonas* spp. CK119 was common after 69 and 114 hrs. Moreover *S. liquefaciens* CK2 and *Pseudomonas* spp. CK119 were also detected after 48 hrs; *S. liquefaciens* CK2 and *Ps. putida* after 69 hrs; *S. liquefaciens* CK2 and *Ps. fragi* after 114 hrs; *Pseudomonas* spp. CK119 after 196 hrs; *H. alvei* CK39 and *Pseudomonas* spp. CK119 after 244 hrs. *S. liquefaciens* CK2 was the most common fingerprint recovered from minced beef at 10°C for 18 hrs, while *H. alvei* CK39 was also obtained. *S. grimesii* CK30 was the most common fingerprint recovered after 54 hrs, followed by *Ps. fluorescens* and *Ps. fragi*. In the latter case, *S. liquefaciens* CK2 and *Pseudomonas* spp. CK73 were also recovered. Moreover, after storage for 90 hrs *S. liquefaciens* CK2 and *Ps. fragi* were equally recovered, while *Pseudomonas* spp. CK73 was also detected. *Ps. fragi* was the common fingerprint obtained after storage for 162 hrs while *S. liquefaciens* CK2 was also recovered. At 15°C, *S. liquefaciens* CK2 was the only fingerprint detected after storage for 12 and 36 hrs. The aforementioned fingerprint was common after 69 and 110 hrs, followed by *Ps. fragi*. In the latter case *C. freundii* CK19 was also recovered.

Out of thirteen different DGGE fingerprints, four, ten, four and six were detected during storage of minced beef under MAP – at 0, 5, 10 and 15°C. More accurately, *S. liquefaciens* CK2 was the only fingerprint detected after storage for 69 hrs at 0°C. The same fingerprint was the most common one obtained after 196 hrs, while *S. grimesii* CK30 and *Morganella morganii* CK265 was also recovered. After 291 hrs, *M. morganii* CK265 was the most common fingerprint recovered, followed by *S. liquefaciens* CK2. *S. liquefaciens* CK2 was common isolate recovered after storage for 485 hrs, while *S. grimesii* CK30 and *Pseudomonas* spp. CK73 was also detected. At 5°C, *S. grimesii* CK30 was the common fingerprint obtained after storage for 48 hrs, while *S. liquefaciens* CK2 and *M. morganii* CK265 was also detected. On the other hand, *S. liquefaciens* CK2, *Pseudomonas* spp.

CK148, *Ps. fluorescens* and *Ps. putida* was equally recovered after 69 hrs. *S. liquefaciens* CK2 was the common fingerprint detected after 114 hrs, followed by *Rahnella* spp. CK153; *Ps. fragi* was also recovered. Moreover, after 196 hrs, *S. liquefaciens* CK2 was the most common fingerprint, while *Rahnella* spp. CK153 was also recovered. The same fingerprint was common also after storage for 244 hrs, while *S. grimesii* CK30, *H. alvei* CK49 and *Pseudomonas* spp. were also detected. *S. liquefaciens* CK2, *S. grimesii* CK30 and *C. freundii* were equally detected at 10°C after storage for 18 hrs. After 54 and 90 hrs, *S. liquefaciens* CK2 was the most common fingerprint obtained while *S. grimesii* CK30 was also detected. Moreover, *S. liquefaciens* CK2 and *H. alvei* CK39 were equally obtained after 162 hrs. At 15°C, *S. liquefaciens* CK2 was the only fingerprint detected after storage for 12 hour. The same fingerprint was common after 69 hrs, while *S. grimesii* CK30, *C. freundii* CK33, *H. alvei* CK36 and *Ps. fragi* were also detected. Similarly, *S. liquefaciens* CK2 was the most common fingerprint recovered after 110 hrs while *H. alvei* CK39 was also detected.

Twelve different DGGE fingerprints were detected during storage of minced beef under MAP+, while six, four, five and six were obtained at 0, 5, 10 and 15°C. More specifically, *Ps. fluorescens* was the most common fingerprint obtained after storage for 69 hrs at 0°C, while *S. grimesii* CK30 was also detected. After 196 hrs, *S. liquefaciens* CK2 was the most common fingerprint recovered, while *Rahnella* spp. CK153 was also detected. *S. liquefaciens* CK2 and *M. morganii* CK265 were equally recovered after 291 hrs, while *S. grimesii* CK30 and *Pseudomonas* spp. CK148 was also recovered. After 485 hrs, *M. morganii* CK265 was the common fingerprint obtained while *S. liquefaciens* CK2 and *S. grimesii* CK30 was also detected. At 5°C, *S. liquefaciens* CK2 was the only fingerprint detected after storage for 48 and 69 hrs. The aforementioned fingerprint was common after 196 hrs, while *Ps. fragi* was also isolated. Additionally, *S. liquefaciens* CK2 and *Pseudomonas* spp. CK73 were equally obtained after 244 hrs, while *Ps. putida* was also

recovered. Furthermore, *S. liquefaciens* CK2, *S. grimesii* CK30 and *H. alvei* CK30 were equally detected after storage for 54 hrs at 10°C. After 90 hrs, *S. grimesii* CK30 were the common fingerprint obtained; *S. liquefaciens* CK2, *H. alvei* CK36 and CK49 were also detected. *S. liquefaciens* CK2 was common after 162 hrs, while *S. grimesii* CK30 was also detected. At 15°C, *S. liquefaciens* CK2 was the most common isolate recovered after storage for 12 hrs, while *M. morganii* CK265 and *Ps. fluorescens* were also detected. After 69 hrs, *S. liquefaciens* CK2, *S. grimesii* CK30, *H. alvei* CK39 and CK49 were equally detected. Moreover, *S. grimesii* CK30 was the most common fingerprint obtained after storage for 110 hrs, followed by *H. alvei* CK49; *S. liquefaciens* CK2 was also isolated.

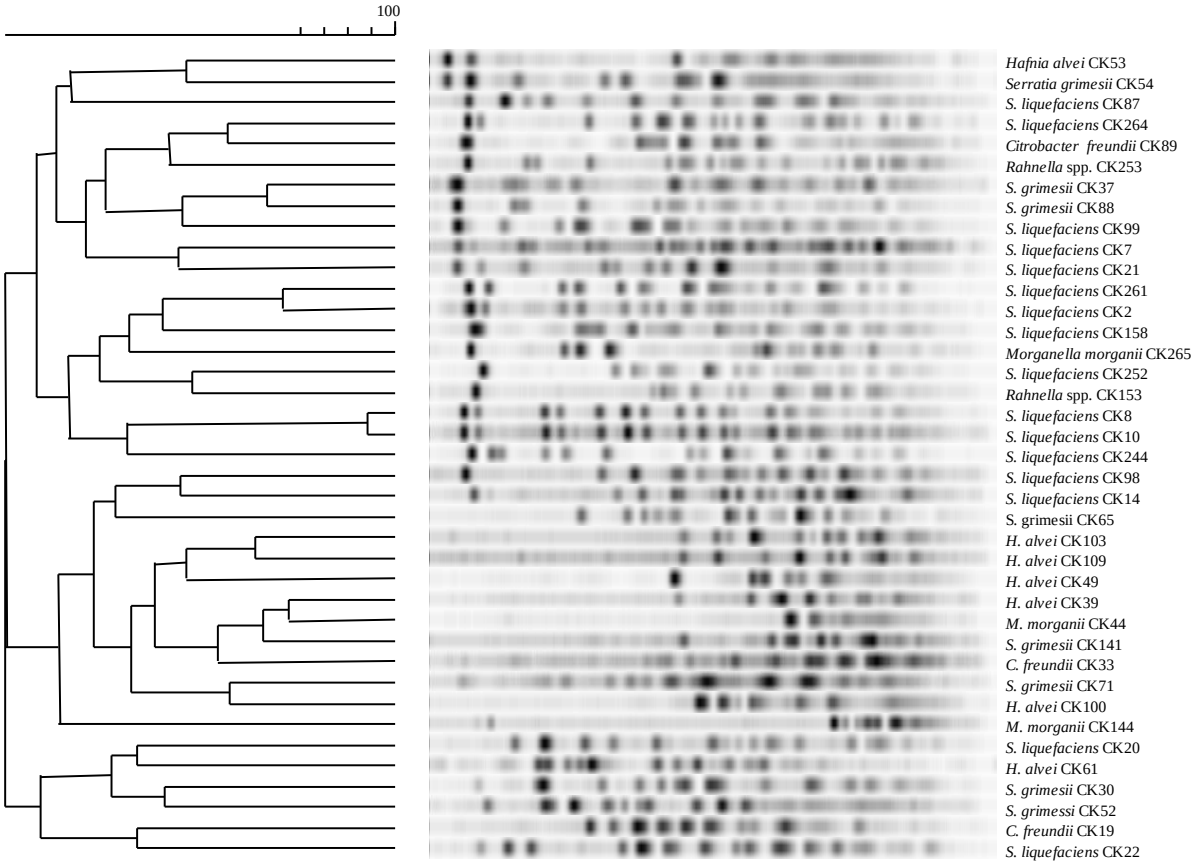


Figure 3.10. Cluster analysis of PFGE *Spe*I digestion fragments of the *Enterobacteriaceae* isolates recovered from Pseudomonas agar base medium during storage of minced beef under different conditions calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

D. Fingerprinting of bulk cells from Pseudomonas Agar Base growth medium

The cultivable fractions harvested from PAB medium were identified by PCR-DGGE of the variable V6-V8 region of the 16S rRNA gene. Twelve different bands occurred throughout the storage period of minced beef under the different conditions adopted. These bands were identified after inclusion in the same DGGE gels as the reference strains (*Ps. putida* KT2440, *Ps. fragi* DSM 3456 and *Ps. fluorescens* GTE 015) as well as the different isolates which were recovered from the same medium and exhibited different DGGE fingerprints (Figure 3.11).

The analysis of the bulk cells showed that one band occurred at the initial stage of storage; this entity was shown to migrate the same distance in DGGE gel with *S. liquefaciens* CK2. This band occurred in all conditions tested but at 0°C under aerobic (291 and 485 hrs) MAP - (291 hrs) and MAP + (69 hrs) conditions and at 10°C under MAP – (162 hrs). More accurately, two bands which identified as *S. liquefaciens* CK2 and *Ps. fluorescens* occurred in minced beef at 0°C under aerobic conditions after a storage period of 69 hrs, while two additional bands (*Ps. fragi* and *Ps. putida*) were detected after 196 hrs. However, *Ps. fragi* was the only DGGE fingerprint obtained after 291 and 485 hrs. Additionally, the minced beef stored under MAP - at 0°C for 69 hrs displayed a fingerprint containing three bands identified as *S. liquefaciens* CK2, *Pseudomonas* spp. CK73 and *Ps. fragi*. *S. liquefaciens* CK2 and *M. morganii* CK265 were identified after a storage period of 196 hrs. Similarly, bands belonging to *Pseudomonas* spp. CK73 and *M. morganii* CK265 were identified after 291 hrs, while *S. liquefaciens* CK2, *Pseudomonas* spp. CK73 and *Ps. fragi* occurred after 485 hrs. Under MAP +, the displayed DGGE fingerprint of bulk cells after 69 hrs contained the *C. freundii* CK33 and *Ps. fragi*. After 196 hrs, the only band occurred identified as *S. liquefaciens* CK2. Furthermore, *S. liquefaciens* CK2 and *M. morganii* CK265 were detected after 485 hrs, while the aforementioned entities and *Pseudomonas* spp. CK73 occurred after 291 hrs.

The bulk cells of PAB from samples stored aerobically at 5°C showed that at 48 hrs, the population was ascribable to *S. liqueafaciens* CK2 and *Ps. fragi*, while *Pseudomonas* spp. CK73 was also detected at 69, 114 and 244 hrs. The latter species along with *H. alvei* CK39 were also found after 196 hrs. In the case of MAP -, bands belonging to *S. liquefaciens* CK2 and *S. grimesii* CK30 were identified at 48 hrs. The former was only found in the DGGE profile at 69 hrs, and after 244 hrs. Similarly, *S. liquefaciens* CK2 and *Ps. fragi* were found after 114 and 196 hrs. In samples stored under MAP +, *S. liquefaciens* CK2, *C. freundii* CK33 and *Ps. fragi* were detected at 48 hrs. Furthermore, *S. liquefaciens* CK2 and *Ps. fragi* occurred at 69 hrs, while the former was the only species found at 96 hrs. In a later stage of storage (244 hrs) *S. liquefaciens* CK2 and *Pseudomonas* spp. CK73 were detected.

At 10°C, *S. liqufaciens* CK2 was present after 18 hrs in minced beef stored aerobically. At 54 and 162 hrs, the DGGE fingerprint containing three bands identified as *S. liquefaciens* CK2, *Pseudomonas* spp. CK119 and *Ps. fragi*, while *S. liquefaciens* CK2 and *Ps. fragi* were detected after 90 hrs. Under MAP -, the population was ascribable to *S. liquefaciens* CK2 after 18 hrs. The aforementioned species together with *S. grimesii* CK30 was detected after 54 and 90 hrs. In contrast, after 162 hrs ours two different bands occurred; two of these entities were identified as *H. alvei* CK39 and *Pseudomonas* spp. CK73. During storage of minced beef under MAP +, two bands were detected; *S. liquefaciens* CK2 and *S. grimesii* CK30 after 54 and 90 hrs. The latter was not found after 162 hrs whereas the former was the only species detected.

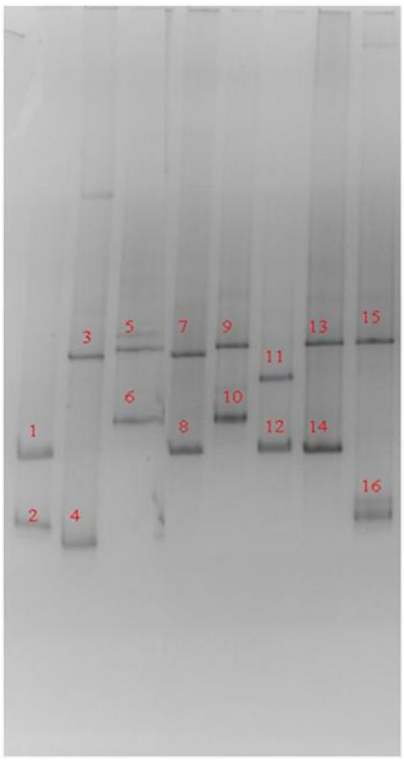


Figure 3.11. PCR – DGGE profiles of reference stains and isolates recovered from PAB medium. (1) *Ps. fragi* DSM 3456, (2) *Ps. fluorescens* GTE 015, (3) *C. freundii* CK33, (4) *Ps. putida* KT2440, (5) *Rahnella* spp. CK153, (6) *S. liquefaciens* CK2, (7) *C. freundii* CK19, (8) *Pseudomonas* spp. CK119, (9) *H. alvei* CK36, (10) *S. grimesii* CK30, (11) *M. morganii* CK265, (12-14) *Pseudomonas* spp. CK73, (13) *H. alvei* CK49, (15) *H. alvei* CK39, (16) *Pseudomonas* spp. CK148.

Based on analysis of bulk cells from PAB medium, the minced beef stored at 15°C did not show a significant degree of diversity depended on the different packaging conditions. More specifically, *S. liquefaciens* CK2 was the only band detected at 12 and 36 hrs when the samples stored aerobically. The latter along with *Ps. fragi* occurred after 69 and 110 hrs. Furthermore, *S. liquefaciens* CK2 and *H. alvei* CK36 were detected in minced beef samples stored under MAP- at 12 hrs, while the former was the only species detected at 69 and 110 hrs. The same species (*S. liquefaciens* CK2) was the only one occurred at 12 and 110 hrs of storage under MAP +. Moreover, *S. liquefaciens* CK2 and *H. alvei* CK49 were detected at 69 hrs.

3.3.2. Fingerprinting of microbiota in beef fillets stored aerobically

Samples were selected from beef fillets stored aerobically at 0, 5, 10, 15 and 20˚C (this work was supported by an EU project, Anthoula Argyri PhDthesis) at the initial, middle and final stages of storage. A total of 195 isolates were recovered throughout the storage period; 48 isolates from MRS (pH 5.7), 48 isolates from MRS (5.2), 50 isolates from VRBG and 49 from PAB. These isolates were subjected to PFGE in order to determine the strain diversity. Moreover, DNA directly extracted from beef fillets in all the conditions adopted was subjected to PCR – DGGE.

A. Identification of bacteria

A large diversity regarding the strain occurrence at the different temperature storage conditions was revealed. The dendrogram obtained after image analysis of the different PFGE patterns resulted in 6, 7, 17 and 16 different fingerprints which were obtained from MRS (pH 5.7), MRS (5.2), VRBG and PAB, respectively (Figures 3.12, 3.13 and 3.14). Each of the these fingerprints was subjected to 16S rRNA gene sequencing. Three fingerprints were obtained from MRS (MF2, MF3 and MF5) and assigned to *Ln. mesenteroides*, while five fingerprints were assigned to *Lb. sakei* (AF20, AF26, MF19, MF41, MF44) (Table 3.15). Moreover, the seventeen different profiles obtained from VRBG were assigned to *Escherichia coli* (VF2), *Klebsiella oxytoca* (VF5), *Pantoea agglomerans* (VF6), *Enterobacter* spp. (VF7 and VF33), *S. grimesii* (VF8), *S. liquefaciens* (VF15 and VF18) and *Serratia* spp. (VF17 and VF37), while eight (VF4, VF11, VF14, VF20, VF24, VF30, VF36 and VF45) could not be assessed at genus level (Table 3.16). In the latter case, identification has supported by sequencing the *rpo*B gene (Table 3.17). Thus, fingerprint VF11 was assigned to *E. cloaceae*; fingerprint VF20 to *E. ludwigii*; fingerprints VF36 and VF45 to *E. nimipresularis*. A further four fingerprints (VF4, VF14, VF24 and VF30) could not be

assessed to genus level (Table 3.17); these isolates are mentioned as Bacterium. Additionally, fingerprints which were obtained from PAB, were subjected to 16S rRNA gene sequencing and assigned to *Pseudomonas* spp. (CF1, CF3, CF5, CF6, CF8, CF9, CF10, CF12, CF25, CF26, CF36, CF45 and CF46), *Ps. fragi* (CF2 and CF43) and *Ps. putida* (CF7) (Table 3.18).

Table 3.15. Species identification of lactic acid bacteria recovered from beef fillets after sequencing of the variable V1-V3 region of the 16S rRNA genes.

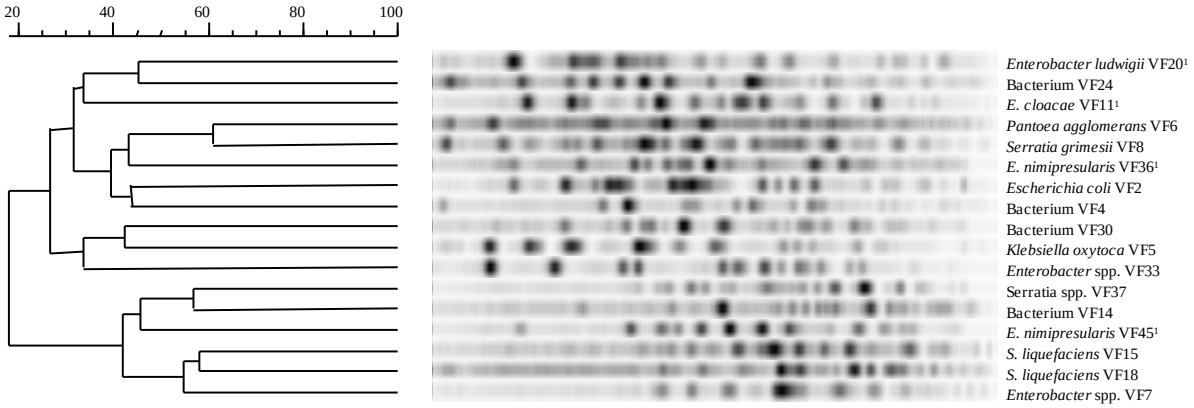
Code	**Closest relative**	**GenBank accession number of closest relative**	**Identity (%)**
AF20	*Lactobacillus sakei*	EU794737	100
AF26	*Lb. sakei*	FJ656787	100
MF2	*Leuconostoc mesenteroides*	GU344720	100
MF3	*Ln. mesenteroides*	AB023242	100
MF5	*Ln. mesenteroides*	GU344720	100
MF19	*Lb. sakei*	EU794737	99
MF41	*Lb. sakei*	AB494726	100
MF44	*Lb. sakei*	AB494726	100

In fresh meat, the presence of nine different fingerprints (MF3, MF2, MF4, MF5, VF2, VF8, CF1, CF2 and CF3) was detected. *Ln. mesenteroides* MF3 was the most common isolate from MRS (pH5.7) and MRS (pH5.2) (Table 3.19), while *Ln. mesenteroides* MF2, *Lb. sakei* MF19 and *Ln. mesenteroides* MF5 were also recovered from MRS (pH5.7) and MRS (pH5.2) respectively. *Esch. coli* VF2 and *Pseudomonas* spp. CF3 were the most common isolates recovered from each growth medium (Table 3.19), while *S. grimesii* VF8 and *Pseudomonas* spp. CF1 and *Ps. fragi* CF2 were also recovered.

Figure 3.12. Cluster analysis of PFGE *Apa*I digestion fragments of the lactic acid bacteria isolates recovered from beef fillets calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Fifteen different fingerprints were detected during storage of beef fillets at 0°C; eleven and nine fingerprints from the middle and final stage of storage respectively. *Lb. sakei* MF41 was commonly isolated from MRS (pH5.7) and MRS (pH5.2) during the middle and final stage of storage respectively (Table 3.19). *Lb. sakei* MF19 and *Ln. mesenteroides* MF5 were also recovered in these conditions while *Lb. sakei* MF9 and AF26 were recovered from MRS (pH5.7) and MRS (pH5.2) at the middle and final stage of storage respectively. Additionally, *Lb. sakei* MF41 and MF44 were common isolates recovered from MRS (pH5.7) at final stage of storage (Table 3.19), while *Lb. sakei* MF9 was also recovered. *Lb. sakei* MF19 was common isolate recovered from MRS (pH5.2) at middle stage of storage (Table 3.19), while *Ln. mesenteroides* MF5 and *Lb. sakei* MF41 were also recovered. Moreover, *S. liquefaciens* was the common isolate from VRBG at middle and final stage of storage (Table 3.19), while *S. grimesii* VF8 and *E. nimipresularis* VF45 were also recovered at middle stage of storage. At the middle stage of storage, *Ps. putida* CF7 was the most prevalent one (Table 3.19), while *Pseudomonas* spp. CF31 and CF45 and *Ps. fragi* CF43 were also recovered. *Pseudomonas* spp. CF6 was the most common isolate recovered from PAB at final stage of storage (Table 3.19), while *Pseudomonas* spp. CF46 was also recovered.

From the sixteen different fingerprints which were detected during storage of beef fillets at 5°C, nine and twelve of them were recovered during the middle and final stage of storage respectively. *Ln. mesenteroides* MF5, *Lb. sakei* MF19, MF41 and MF44 were equally recovered from MRS (pH5.7) at the middle stage of storage (Table 3.19). Similarly *Lb. sakei* MF19 and MF44 were equally recovered from the same medium at the end of storage (Table 3.19). *Ln. mesenteroides* MF5 was the only fingerprint recovered from MRS (pH5.2) at the middle stage of storage, while *Lb. sakei* AF26 was common isolate from the same medium at the final stage of storage (Table 3.19). In the later case, *Ln. mesenteroides* MF5 as well as *Lb. sakei* AF20 were also recovered. Moreover, *Kl. oxytoca* VF5 was the most common isolate recovered from VRBG at the middle stage of storage (Table 3.19) while *Enterobacter* spp. VF33 and E. *nimipresularis* VF36 were also detected. *Serratia* spp. VF37 was the most common isolate recovered from this medium at the final stage of storage (Table 3.19), while *Kl. oxytoca* VF9 was also recovered. At the middle stage of storage, *Pseudomonas* spp. CF3 was the most common fingerprint detected (Table 3.19), while *Pseudomonas* CF45 was also recovered. On the other hand, at the final stage of storage *Pseudomonas* spp. CF8, CF12, CF26, C36 and CF45 were equally recovered (Table 3.19).



[1] Identification has supported by sequencing the *rpo*B gene

Figure 3.13. Cluster analysis of PFGE *Xba*I digestion fragments of the *Enterobacteriaceae* isolates recovered from beef fillets calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Table 3.16. Species identification of *Enterobacteriaceae* isolates recovered from beef fillets after sequencing of the variable V1-V3 region of the 16S rRNA genes.

Code	Closest relative	GenBank accession number of closest relative	Identity (%)
VF2	*Escherichia coli*	AB548582	99
VF4	Bacterium[1]		
VF5	*Klebsiella oxytoca*	FJ971867	99
VF6	*Pantoea agglomerans*	AF130928	99
VF7	*Enterobacter* spp.	GQ284539	100
VF8	*Serratia grimesii*	EF491959	100
VF11	Bacterium[1]		
VF14	Bacterium[1]		
VF15	*S. liquefaciens*	AJ306725	99
VF17	*Serratia* spp.	AJ243601	100
VF18	*S. liquefaciens*	AJ306725	99
VF20	Bacterium[1]		
VF24	Bacterium[1]		
VF30	Bacterium[1]		
VF33	*Enterobacter* spp.	AF500319	99
VF36	Bacterium[1]		
VF37	*Serratia* spp.	EU734627	99
VF45	Bacterium[1]		

[1] Identification was based on *rpo*B gene sequencing (Table 3.17)

Fifteen different fingerprints were detected during storage of beef fillets at 10°C; seven and eleven fingerprints from middle and final stage of storage respectively. More accurately, *Ln. mesenteroides* MF3 was the only fingerprint detected from MRS (pH5.7) at the middle stage of storage, while *Ln. mesenteroides* MF5 was common isolate from the same medium at the final stage of storage (Table 3.19). In the latter case, *Lb. sakei* MF9 was also recovered. Moreover, *Ln. mesenteroides* MF3 and MF5 and *Lb. sakei* AF31 were equally recovered from MRS (pH5.2) at the middle stage of storage (Table 3.19). From the

aforementioned medium, *Ln. mesenteroides* MF5 was the most common isolate recovered at the final stage of storage (Table 3.19), while *Lb. sakei* AF26 was also detected. The unidentified bacterium VF24 (Tables 3.17 and 3.19) was the only isolate recovered from VRBG at the middle stage of storage. *Kl. oxytoca* VF5 was common isolate recovered from the aforementioned medium at final stage of storage (Table 3.19), while *S. liquefaciens* VF15 and the unidentified bacteria VF24 and VF30 (Table 3.17) were also recovered. *Pseudomonas* spp. CF1 and CF6 were common isolates recovered from PAB at the middle and the final stage of storage, respectively (Table 3.19). Moreover, *Pseudomonas* spp. CF25 and CF26 were also detected at the middle stage of storage, while *Pseudomonas* spp. CF1, CF12 and CF31 were also recovered at the final stage of storage.

Table 3.17. Species identification of *Enterobacteriaceae* isolates recovered from beef fillets after sequencing of the *rpo*B genes.

Code	Closest relative	GenBank accession number of closest relative	Identity (%)
VF4	Bacterium[1]		
VF11	*Enterobacter cloacae*	AJ543702	100
VF14	Bacterium[2]		
VF20	*E. ludwigii*	GU199600	99
VF24	Bacterium[3]		
VF30	Bacterium[4]		
VF36	*E. nimipresularis*	AJ566941	99
VF45	*E. nimipresularis*	AJ566948	98

[1]*Enterobacter cloacae* (96%)
[2]*Enterobacter cloacae*/*ludwigii*, *Klebsiella oxytoca* (95%)
[3] *Enterobacter cloacae*/*ludwigii*/*cancerogenus*, *Klebsiella oxytoca* (94%)
[4]*Leclercia* sp. (92%)

Out of twelve different fingerprints obtained from beef fillets stored at 15°C, eight and seven were recovered from the middle and final stage of storage, respectively. *Ln. mesenteroides* MF5 was the most common isolate recovered from MRS (pH5.7) at the middle

and final stage of storage (Table 3.19), while *Lb*. *sakei* MF19 was also detected. Similarly, *Ln*. *mesenteroides* MF5 was the only fingerprint obtained from MRS (pH5.2) at the middle stage of storage (Table 3.19). The aforementioned fingerprint was common isolate recovered from the same medium at final stage of storage (Table 3.19) while *Lb*. *sakei* MF19 and AF20 were also recovered. The unidentified bacterium VF14 (Tables 3.17 and 3.19) was common isolate recovered from VRBG at the middle stage of storage, while *S. liquefaciens* VF15 and VF18 as well as *Serratia* spp. were also detected. Moreover, *E. ludwigii* VF20 was common isolate recovered from the same medium at the end of the experiment (Table 3.19); *Enterobacter* spp. VF7 was also recovered. *Pseudomonas* spp. CF8 and CF12 was common isolates obtained from PAB at the middle and final stage of storage respectively (Table 3.19). In both cases, *Pseudomonas* spp. CF6 was also recovered.

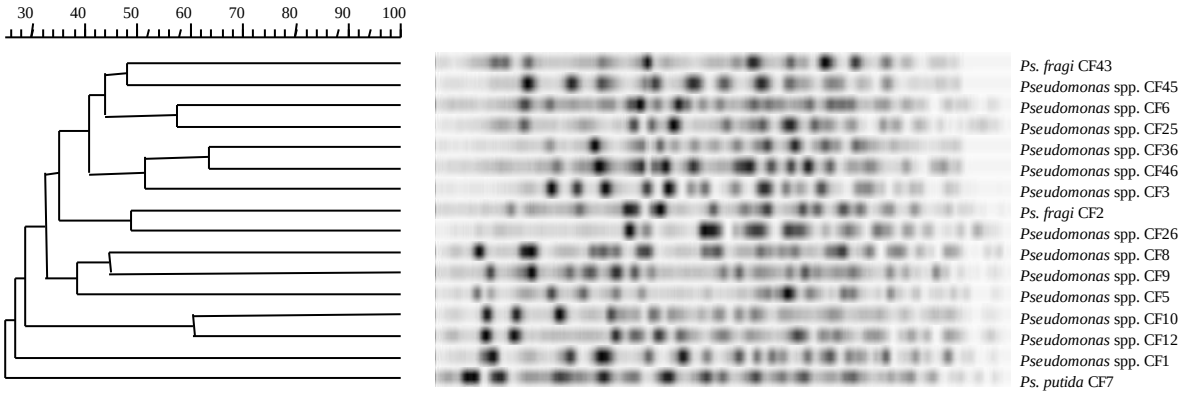


Figure 3.14. Cluster analysis of PFGE *Spe*I digestion fragments of the Pseudomonas agar base isolates recovered from beef fillets calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Table 3.18. Species identification of Pseudomonas agar base medium isolates recovered from beef fillets after sequencing of the variable V1-V3 region of the 16S rRNA genes.

Code	Closest relative	GenBank accession number of closest relative	Identity (%)
CF1	*Pseudomonas* spp.	EF062807	99
CF2	*Ps. fragi*	AM933514	100
CF3	*Pseudomonas* spp.	AM491465	100
CF5	*Pseudomonas* spp.	EF111108	100
CF6	*Pseudomonas* spp.	AY303300	99
CF7	*Ps. putida*	GU060497	99
CF8	*Pseudomonas* spp.	AM491463	99
CF9	*Pseudomonas* spp.	FJ999660	99
CF10	*Pseudomonas* spp.	GU733469	99
CF12	*Pseudomonas* spp.	GU827543	99
CF25	*Pseudomonas* spp.	FJ999660	100
CF26	*Pseudomonas* spp.	AM933515	99
CF36	*Pseudomonas* spp.	FJ999660	100
CF43	*Ps. fragi*	AM933514	100
CF45	*Pseudomonas* spp.	FJ999660	100
CF46	*Pseudomonas* spp.	AY303300	99

Nineteen different fingerprints were detected during storage of beef fillets at 20°C; thirteen and seven fingerprints from middle and final stage of storage respectively. *Ln. mesenteroides* MF5 was the only isolate recovered from the MRS (pH5.7) and MRS (pH5.2) at the final stage of storage (Table 3.19). The aforementioned fingerprint was the most common obtained from MRS (pH5.7) and MRS (pH5.2) at the middle stage of storage (Table 3.19); *Lb. sakei* MF19 was recovered also from MRS (pH5.7); *Lb. sakei* MF19 and MF31 was also recovered from MRS (pH5.2). The unidentified bacterium VF4 (Table 3.17), *Kl. oxytoca* VF5, *Pant. agglomerans* VF6, *Enterobacter* spp. VF7 and *S. grimesii* VF8 were equally recovered from VRBG at the middle stage of storage (Table 3.19). On the other hand, *E. cloacae* VF11 (Table 3.17) was common isolate from the same medium at the final stage

of storage (Table 3.19), while *Kl. oxytoca* VF9 was also recovered. From PAB, at the middle stage of storage, *Pseudomonas* spp. CF5, CF6, CF8 and CF9 as well as *Ps. putida* CF7 were equally recovered (Table 3.19). Similarly, *Pseudomonas* spp. CF1, CF10, CF12 and CF31 were equally obtained from the aforementioned medium at the final stage of storage (Table 3.19).

Table 3.19. Most common isolates recovered from different growth media (MRS, VRBG, PAB) during storage of beef fillets under different temperature conditions (0, 5, 10, 15 and 20°C).

Source			**Growth medium**			
		Storage period	**MRS (pH 5.7)**	**MRS (pH 5.2)**	**VRBG**	**PAB**
Fresh meat			MF3	MF3	VF2	CF3
Tempe-rature (°C)	0	Middle	MF41	MF19	VF18	CF7
		End	MF41/MF44	MF41	VF18	CF6
	5	Middle	MF19/MF5/ MF41/MF44	MF5	VF5	CF3
		End	MF19/MF44	AF26	VF37	CF8/CF12/ CF26/CF36/ CF45
	10	Middle	MF3	MF3/MF5/ MF41	VF24	CF1
		End	MF5	MF5	VF5	CF6
	15	Middle	MF5	MF5	VF14	CF8
		End	MF5	MF5	VF20	CF12
	20	Middle	MF5	MF5	VF4/VF5/ VF6/VF7/ VF8	CF1/CF10/ CF12/CF31
		End	MF5	MF5	VF11	CF5/CF6/ CF7/CF8/ CF9

B. Assessment of microbiota by analysis of DNA extracted directly from beef fillets

The PCR- DGGE of the V6-V8 region of the 16S rRNA gene was applied to DNA directly extracted from beef fillets in all the conditions adopted. Fingerprints obtained from these samples are presented in Figure 3.15, while the results of the band sequencing are shown in Table 3.20. From the fresh sample, the fillets displayed a fingerprint containing one

band (F3) migrating the same distance in DGGE gels with *Ps. fragi* DSM 3456, while the same band was present in all samples. The profiles of beef fillets stored at 10, 15 and 20°C (middle and final stage of storage) as well as at the final stage of storage at 0°C were similar. In the latter case, a second band (F2; migrating the same distance in DGGE gels with *Pseudomonas* spp. CF6, Table 3.18) was obtained. In the addition to the above mentioned band (species), *Staphylococcus* spp. F1 was present in the fingerprints obtained from 0°C (middle stage of storage) and 5°C (middle and final stage of storage) (Table 3.20, Figure 3.15).

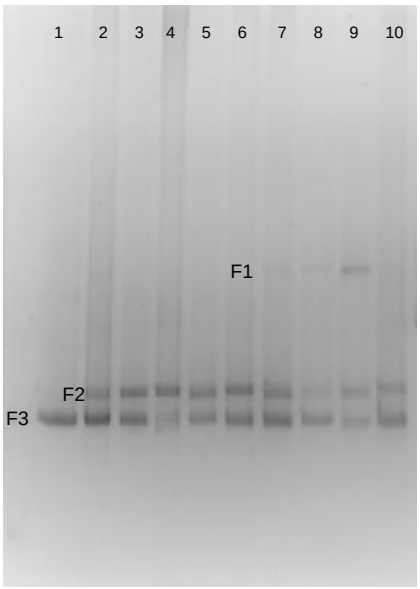


Figure 3.15. PCR DGGE profiles of the V6-V8 amplicons from microbial DNA directly extracted from beef fillets stored at (1) fresh meat, (2) 20°C final, (3) – (4) 15°C middle-final, (5) – (6) 10°C middle - final, (7) – (8) 5°C middle - final and (9) – (10) 0°C middle – final stage of storage.

Table 3.20. Species identification of band obtained from beef fillets after sequencing of the variable V6-V8 region of the 16S rRNA gene.

Code	**Closest relative**	**GenBank accession number of closest relative**	**Identity (%)**
F1	*Staphylococcus* spp.	DQ376925	98

Chapter 4

Discussion

4.1. Survey of microbial levels for minced beef sold in supermarkets

The microbial quality of minced beef was evaluated at a retail level while the effect of packaging systems used and the season during which samples were collected was also studied. Thus, pre-packed minced beef sold in styrofoam trays wrapped with permeable film or MA packaging was obtained from the retail cabinets of supermarkets in Athens. Pseudomonads, *Br. thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria, hydrogen sulfide-producing bacteria as well as yeasts were members of the microbial association of meat. Those results are in agreement with previous studies (Nychas et al. 1991; Tsigarida et al. 2000; Skandamis & Nychas 2001; Skandamis & Nychas 2002; Ercolini et al. 2006; Koutsoumanis et al. 2006; Ercolini et al. 2009).

There were differences in the microbiological loads among different packaging systems, seasons and suppliers. More accurately, higher microbial counts and thus more contamination were found in minced beef sold in styrofoam trays wrapped with permeable film. In contrast, the season did not appear to affect the microbial quality of the samples, although higher counts of *Enterobacteriaceae* and hydrogen sulfide-producing bacteria were observed in the warmer season. It has been reported previously that the microbial association of meat depends on factors that persist during processing, transportation and storage in markets (Nychas et al. 2008). Indeed, in a study where samples from two different countries were collected and examined, the observed differences seemed more likely to be associated with the processing environment and processes in use rather than seasonal variations in bacterial prevalence (Bosilevac et al. 2007).

The observed differences in microbial quality of samples sold in different packaging systems show that the packaging conditions also can play a significant role in extending shelf life of fresh meat, since the growth of aerobic microorganisms was prevented in meat under

modified atmosphere packaging. The lactic acid bacteria and *Br. thermosphacta* are the dominant microorganisms in modified atmosphere packaging and pseudomonads in air (Drosinos & Board 1995; Nychas et al. 2007). In the present study, high levels of pseudomonads were observed in both packaging systems. Giannuzzi et al. (1998) reported that pseudomonads could play a significant role in spoilage of meat stored under MAP with a high permeability film, although in meat samples packed in low gaseous permeability film, lactic acid bacteria grew at the highest rates. Furthermore, the high counts of *Br. thermosphacta* that were observed showed that it was useful spoilage indicator. *Enterobacteriaceae* also contributed to spoilage, although they did not become a numerically dominant part of microbial association on minced beef.

It is well known that temperature is the main factor influencing microbial growth. The refrigerator temperatures are not always kept constant during food handling. Survey studies have shown that temperature conditions higher than 10°C are not unusual during transportation, retail storage and consumer handling (Giannakourou et al. 2001; Gill et al. 2002). The microorganisms that grow in meat during warm periods are different from those that grow in cold periods, based on their temperature requirements (psychrophiles, mesophiles and thermophiles). Gill and Newton (1980) reported that *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. were predominant in the spoilage biota at 30°C. The seasonality of pathogens such as *Salmonella* spp., *Esch. coli*, and *Campylobacter* spp. is well documented (Barkocy-Gallagher et al. 2003; Stanley et al. 1998). Moreover, pathogens increase in the spring, becoming highly prevalent through the summer and into the autumn, and then decreased during the winter (Bosilevac et al. 2007).

The microbial survey of prepacked minced beef samples indicated that quality and safety assurance systems are required to reduce the microbial contamination in meat. Better

quality and safer ground beef will rely on reduction of microbial contamination during slaughtering operations (Eisel et al. 1997; Nychas et al. 2007). Also, effective good manufacturing practice (GMP) programs help to reduce the level of both spoilage and pathogenic microorganisms (Silliker 1980). It is often suggested that retail display is the weakest link in the cold chain for the distribution of raw, chilled meat (James 1996; Nychas et al. 2008). Moreover, Eisel et al. (1997) reported that the most efficient way to reduce microbial contamination and microbial growth in food is to establish in–house food safety programs.

4.2. The effect of oregano essential oil in microbial association of minced beef

The effect of the volatile compounds of oregano essential oil in combination with the use of modified atmosphere packaging and different storage temperatures on the microbial association of minced beef was studied. Also, the effect of these conditions on the survival of *L. monocytogenes* was evaluated. The microbial associations of the minced beef were found to be affected by the different temperature and packaging conditions adopted. Similar results were revealed for the growth/survival of the pathogen.

More specifically, the initial microbial loads presented in this study were in agreement with those reported by Koutsoumanis et al. (2006). Indeed in other studies the initial total viable counts has been found to be lower in beef (Tsigarida et al. 2000; Skandamis & Nychas 2002; Ercolini et al. 2006; 2010) and higher in minced beef (Skandamis & Nychas 2001). The relatively high initial numbers of different groups in minced beef can be attributed to the grinding process, which contributes to the increase of the total viable counts of meat including yeasts (Jay & Margitic 1981; Nychas et al. 1991; Dillon 1998). Especially, the increased level of pseudomonads in minced beef compared to meat cuts reported by Koutsoumanis et al. (2006).

The viable counts showed that the spoilage related microbial groups had different trends depending on the packaging conditions. When the minced beef was stored aerobically all the microbial groups showed viable counts higher than those of the other packaging conditions adopted. Similar results for beef have been described previously (Tsigarida et al. 2000; Skandamis & Nychas 2001; Sakala et al. 2002; Skandamis & Nychas 2002; Ercolini et al. 2006). Aerobic storage accelerated spoilage due to the fast growing of the pseudomonads, while modified atmosphere packaging favoured the dominance of a facultative anaerobic population including lactic acid bacteria and *Br. thermosphacta*. The microbial profile described above has also been reported in other studies for pork and beef (Newton & Gill 1978; Enfors et al. 1979; Lambropoulou et al. 1996; Nychas & Skandamis 2005). Furthermore, the volatile compounds of oregano essential oil were found to be capable of affecting growth of the microbial association of minced beef stored at modified atmospheres. Similar observations have been reported before for beef (Skandamis & Nychas 2002), however such inhibition was not as strong as that due to the contact of pure essential oil with microorganisms when this was added directly on the surface of meat (Tsigarida et al. 2000; Skandamis & Nychas 2001). Overall, other studies with fish and beef showed that MAP acts synergistically with essential oils, since only a selected proportion of the microbiota, compared to aerobic storage, is allowed to develop (Tassou et al. 1995; Tassou et al. 1996; Tsigarida et al. 2000).

In this study growth of *L. monocytogenes* occurred in minced beef stored aerobically, although limited growth was observed under MAP with or without volatile compounds of oregano essential oil. The literature contains conflicting reports on the ability of MAP/VP to control *L. monocytogenes*. For example, growth of *L. monocytogenes* was detected in MAP/VP beef at 5 °C using film of O_2 transmission rate 12 g m^{-2} 24 h^{-1} at 20 °C and 85% r.h. (Hudson and Mott 1993), beef at 3 °C within a film of 2 g m^{-2} 24 h^{-1} at 20 °C and 85% r.h.

(Hudson et al. 1994) and in MAP/VP meat using high permeability film (Tsigarida et al. 2000). Nevertheless, no or limited growth of this pathogen was observed in beef samples stored in MAP/VP within O_2-impermeable film either in the presence or absence of background biota (Tsigarida et al. 2000). In O_2-permeable packs, the dramatic changes in the composition of gaseous atmosphere within VP/MAP (McMullen & Stiles 1991) enhanced growth of pseudomonads (Newton & Rigg 1979) and, as a consequence, stimulation of *L. monocytogenes* could occur. The hydrolysis of proteins, which could provide free amino acids, has been considered as a likely explanation for the stimulus of *L. monocytogenes* growth by pseudomonads in the case of milk (Marshall & Schmidt 1991). Proteolysis caused by the microbial association and *Ps. fragi* was also evident in chicken breast stored under aerobic, VP and MAP conditions (Nychas & Tassou 1997). The fact that pseudomonads did not release such nutrients could be the reason for the lack of stimulation of *L. monocytogenes* (Carlin et al. 1996). Other members of the meat microbial association and/or their metabolic end-products could also influence the growth of *L. monocytogenes* (Thomas et al. 1997; Nychas et al. 1998). Oregano essential oil could also contribute further to inhibition of this pathogen (Tsigarida et al. 2000; Skandamis et al. 2002; Chorianopoulos et al. 2004).

In the present study the sensory evaluation of minced beef was performed in parallel with the microbial analysis. In all conditions adopted, the microbial shelf life was shorter than that estimated by sensory evaluation. It is already known that there is a fundamental difference between shelf life based on microbial criteria and sensory evaluation (Alklint et al. 2004; Nychas et al. 2007). Several studies have shown that the lag phase duration of the specific spoilage microorganisms can be a significant part of the total shelf life of foods (Koutsoumanis & Nychas 2000; Koutsoumanis et al. 2004), although the majority of the mathematical models for spoilage microorganisms have been focused on the effect of the environmental factors on the maximum specific growth rate. A vast number of studies in

meat microbiology have established that spoilage is caused only by the fraction of the initial microbial association that dominates (Nychas et al. 2007). Koutsoumanis et al. (2006) reported that the populations of pseudomonads at the end of shelf life of ground beef was estimated constantly to 9 log cfu g^{-1} using a primary growth model. Other studies have reported that spoilage of aerobically stored chilled meat cuts occurs when pseudomonads reach 7 – 8 log cfu g^{-1} (Gill & Newton 1977; Nychas et al. 2008). Moreover, in a study of beef stored in modified atmosphere packs microbial spoilage occurred when lactic acid bacteria reached 7 log cfu g^{-1}(Nortje & Shaw 1989).

The pH values at the beginning of the storage are within the normal range for fresh beef (Borch et al. 1996). There was a decrease in the pH of all samples stored under MAP without or in the presence of oregano essential oil with the storage time, although an increase was observed in the pH value of all samples stored aerobically. In several studies, it has been reported that meat pH affected the growth kinetics of pseudomonads, *Br. thermosphacta* and *Enterobacteriaceae* (Blixt & Borch 2002; Koutsoumanis et al. 2006). This discrepancy could be attributed to the fact that in meat, small differences in pH can be translated into significant differences in lactate concentration (Blixt & Borch 2002; Lowe et al. 2004) and thus affect the growth of pseudomonads, which are sensitive to lactic acid (Nakai & Siebert 2004). In contrast, the pH value did not affect the growth kinetics of lactic acid bacteria, due to their well establish higher acid tolerance compared to the other spoilage bacteria (Blixt & Borch 2002; Koutsoumanis et al. 2004).

4.3. Study of the microbiota during storage of meat

The present study focused on the evaluation of the microbial diversity of different spoilage related bacteria during storage of beef under different conditions. The diversity of lactic acid bacteria, *Enterobacteriaceae* and the community developed on PAB medium was

determined throughout the storage of minced beef stored under different temperatures (0, 5, 10 and 15°C) and packaging conditions (air, MAP – and MAP +) (see Sections 2.3.1 and 3.3.1) and beef fillets stored aerobically under different temperature conditions (0, 5, 10, 15 and 20°C) (Sections 2.3.2 and 3.3.2). The development of these communities was assessed by different culture dependent approaches. Moreover, the microbiota of beef fillets stored under the different temperatures adopted (Task 2.3.2 and 3.3.2) was also assessed by analysis of DNA extracted directly from the samples.

In general, storage conditions were found to have an important effect on the diversity of the microbial populations, since different strains were recovered during the storage of meat under different conditions; each of the spoilage related bacteria considered in this study will be discussed extensively below. Moreover, the culture independent approach revealed that *Ps. fragi*, *Pseudomonas* spp. and *Staphylococcus* spp. were present dependent on the storage temperature. The latter species was identified from the DGGE fragment analysis; similar results have been observed previously (Cocolin et al. 2004; Ercolini et al. 2006). These findings strengthen the opinion that the storage temperature and modified atmosphere packaging affect the spoilage potential of microbial communities (Stanbringe & Davies 1998; Ercolini et al. 2006; 2009). Moreover, this observation can be explained by the fact that different metabolic activities occur when different species/strains are present and when meat is stored under specific conditions. Not all species belonging to the same bacterial group necessarily grow at the same temperature. It might therefore be misleading to suggest that only selective media should be used for the determination of the spoilage biota. Further characterization of the isolates grown on the selective plates should be done if a better insight and understanding of the phenomenon is required. This is in accordance with the observations of Ercolini et al. (2006) who reported that different species / strains were isolated from beef although similar counts were obtained. Ercolini et al. (2006) also mentioned that the viable

counts alone may not be enough to highlight the shifts of the bacterial communities depending on the environmental changes and species that are actually involved in meat spoilage.

It has to be noted that spoilage and spoilage progress of meat and meat products have been the subject of several studies conducted previously (Borch et al. 1996; Stanbridge & Davies 1998; Labadie 1999; Skandamis & Nychas 2002; Nychas & Skandamis 2005; Nychas et al. 2008). Nevertheless, meat spoilage has only been associated with the physicochemical and microbiological analysis of the bacterial loads ignoring the spoilage potential of a specific bacterial species or strain (Skandamis & Nychas 2002). Only recently did research take into consideration the specific characteristics of the spoilage microbiota of the meat products and its contribution to the deterioration of the product (Cocolin et al. 2004; Rantsiou et al. 2005; Ercolini et al. 2006; 2009; Vasilopoulos et al. 2010).

Lactic acid bacteria

Different lactic acid bacteria were recovered during storage of meat under the different conditions. Within the LAB population obtained from minced beef, *Leuconostoc* spp. and *Lb. sakei* were identified as significant members of the microbiota at abuse and chill temperatures, respectively. Similarly, *Ln. mesenteroides* and *Lb. sakei* dominated the LAB population of beef fillets stored aerobically at abuse and chill temperatures, respectively. Dominance of *Leuconostoc* spp. or *Ln. mesenteroides* at relatively higher temperatures can be partially attributed to the favourable environmental conditions and partially to the shorter generation time (Harris 1998), both of which enabled it to outgrow *Lb. sakei* strains which were indeed detected as a secondary microbiota. On the other hand, dominance of *Lb. sakei* strains at chill temperatures can be attributed partly to its psychrotrophic nature.

More accurately, *Leuconostoc* spp. (B 233) that were initially present at high levels, eventually dominated the microbiota of minced beef stored at abuse temperatures under all packaging conditions. Similarly, *Ln. mesenteroides* (MF5) dominated the LAB population of beef fillets at abuse temperatures. Although, these bacteria were persistent throughout storage at chill temperatures, *Lb. sakei* strains dominated the LAB population only during the final stage of storage in both cases. However, some degree of microbial variability was detected at the final stage of storage of meat at chill temperatures, since different *Lb. sakei* strains were the most prevalent ones at the different packaging conditions and products. Indeed, *Lb. sakei* (B 226), (B 237) and (B 245) dominated the LAB population of minced beef at 0°C under aerobic conditions, at 0 and 5°C under MAP - and at 5°C under MAP +. Similar results were revealed for LAB population recovered from MRS medium (adjusted in two pHs) in the final stage of storage of beef fillets. This finding is of great importance since it shows the intra-species variability of *Lb. sakei* and the ability of certain strains to adapt to the different storage conditions outgrowing the other.

In the case of minced beef, a wide range of strains from the different LAB detected were sporadically present throughout the storage under MAP - and MAP +, especially at chill temperatures. This finding indicates that modified atmosphere packaging resulted in a development of a totally different spoilage ecosystem. It has been previously reported (Jay 2000), that during storage of meat under MAP, the initial heterofermentative microbiota was substituted by a homofermentative one at the end of storage. Moreover, the MAP and the presumed activity of oregano essential oil against heterofermentative LAB species (Axelsson 1998) seem to have provided the latter with an ecological advantage over *leuconostocs*.

In general, among the species recovered throughout the storage of beef, several meat associated ones were identified. Holzapfel (1998) reported that more rarely *Lb. plantarum*

and *Lb. casei* are associated with meat systems and in lower frequency and numbers than *Lb. curvatus* and *Lb. sakei*; the presence of *Ws. viridescens* in raw meat has been also described. Moreover, *Lb. curvatus, Lb. sakei* and *Leuconostoc* spp. have been found to indicate a mixture community of VP beef (Yost & Nattress 2002). *Leuconostocs* have been identified as predominant organisms in beef stored under VP/MAP (Stanbringe & Davis 1998; Yost & Nattress 2002) and their presence in the initial mesophilic bacterial microbiota is very frequent (Borch et al. 1996). *Lb. sakei* has been associated with fresh meat (Champomier – Verges et al. 2001) as well as spoilage of a variety of meat products both under vacuum and modified atmosphere packaging (Ercolini et al. 2006; 2009) and it is known to be among the most psychrotrophic lactobacilli. It has also been found to be the dominant spoilage LAB during storage at chill temperatures (Ercolini et al. 2006; Chenoll et al. 2007).

Enterobacteriaceae

Storage conditions had a profound effect on the diversity of *Enterobacteriaceae* community in accordance to previous studies (Ercolini et al. 2006; 2009; Stanbringe & Davies 1998). Inadequate hygiene, cross – contamination incidents and the psychrotrophic traits of *Enterobacteriaceae* preside over the high levels of their counts in minced beef. It has been reported that the psychrophic nature and simple nutritional requirements of genera enable them to persist and/or multiply in/on water, condensate, soil, equipment surfaces, brine solutions and moist floors (von Holy et al. 1992). However, in meats phychrotrophic *Enterobacteriaceae* can multiply during refrigerated storage and levels can therefore increase so their hygiene significance must be interpreted accordingly (Baylis 2006). Although several members of *Enterobacteriaceae* possess a health – risk potential (Pandey et al. 1999), their spoilage capacity has been only recently indicated (Nychas et al. 2008). Several methods have been proposed to control their growth, including modified atmosphere packaging (Skandamis & Nychas 2001; Skandamis & Nychas 2002; Ercolini et al 2006), utilisation of

nisin containing antimicrobial packaging (Ercolini et al. 2010) and the volatile fraction of oregano essential oil (Skandamis & Nychas 2002). In these studies, the *Enterobacteriaceae* community was regarded as a whole ignoring the potential of the specific bacterial species or strains.

In the present study, the *Enterobacteriaceae* community of fresh minced beef consisted of two *S. liquefaciens* and two *S. proteamaculans* strains; during storage the former prevailed over the latter. Under aerobic conditions the ecosystem was dominated by those *S. liquefaciens* strains that were initially detected in fresh minced beef with an occasional presence of *Serratia* spp. and *C. freundii* strains. This dominance can be attributed partly to the relatively favourable growth conditions and partly to the higher initial population. *S. liquefaciens* represented the dominant isolate of *Enterobacteriaceae* during storage of minced beef for the most conditions adopted, but 10 and 15 °C under MAP + and 10 °C under MAP – (final stage of storage). In the latter case, *Hafnia alvei* represented the dominant fingerprint. This suggests that different strains of *Enterobacteriaceae* occur at different temperatures, possibly because of temperature – induced differences in adaptation and competitiveness, inherent the total population of these species. *S. liquefaciens* has been found to be the most common member of this family on meat stored in atmospheres of different conditions (Stanbridge & Davies 1998). *H. alvei* has been found one of the major spoilage enterobacteria found in meat, in particular due to its psychrotolerant character which gives an adaptation advantage over other microbial members (Borch et al. 1996). *H. alvei* was also the dominant member of *Enterobacteriaceae* on beef steaks stored in modified atmospheres at 5 °C (Stanbridge & Davies 1998). The latter also did not compete well in the high oxygen atmosphere, while it was inhibited by modified atmosphere more at 0 than 5 °C.

Furthermore, packaging under modified atmosphere led to the development of a different *Enterobacteriaceae* consortium since strains of *Proteus vulgaris* were detected at 0 and 5 °C and strains of *H. alvei* were recovered at 10 and 15 °C. Regarding growth preferences of the former, only scarce literature is currently available (Lucia et al. 1993). As far as *H. alvei* is concerned, it is very frequently encountered in minced beef stored under modified atmospheres or vacuum packaging (Borch et al., 1996; Nychas et al. 1998; Drosinos & Board 1995). Thus, storage under MAP without the addition of oregano essential oil was characterised by dominance of *S. liquefaciens* at 5 and 15 °C, a co-existence with *P. vulgaris* at 0 °C and dominance of *H. alvei* at 10 °C. Similarly, *S. liquefaciens* strains dominated at 0 and 5 °C during storage under MAP with the addition of essential oil whereas *Enterobacteriaceae* microbiota consisted almost exclusively by *H. alvei* strains at 10 and 15 °C. The increased diversity observed in both cases can be attributed to the favourable packaging conditions for *Enterobacteriaceae* growth, since they are facultatively anaerobic microorganisms. When oregano essential oil was applied, *S. liquefaciens* and *P. vulgaris* strains seemed to be negatively affected whereas *H. alvei* strain diversity increased. This negative effect of the essential oil on *S. liquefaciens* and *P. vulgaris* diversity and the advance in *H. alvei* diversity could not be explained as limited studies are available for antibacterial activity of essential oils against meat spoilage microorganisms. The only available information provided that *S. liquefaciens* overcome the inhibitory effect of essential oils after 24 hours of exposure (Outtara et al. 1997).

In the case of beef fillets, an *Esc. coli* strain was common in fresh samples. This species is commonly associated with gastrointestinal track and often used as an indicator of faecal contamination (Baylis 2006). At the end of storage, the temperature played a significant role on the selection of the dominated strain. *S. liquefaciens, Serratia* spp., *Kl. oxytoca, E. ludwigii* and *E. cloacae* were common at 0, 5, 10, 15 and 20°C. Several other

species were also recovered i.e. *Pantoea agglomerans*, *Enterobacter* spp., *S. grimesii* and *E. nimipresularis*, while four strains failed to be identified. This wide range of different species could be attributed to the low microbiological levels of *Enterobacteriaceae* community of beef fillets. Nevertheless, those species have to be taken into account, as it is well known that uncommon organisms serve as a reservoir of genetic and functional diversity, often play key roles in ecosystems and can become important if the environmental conditions change (Bent and Forney 2008).

In general, among the species recovered throughout the storage of beef, several meat associated ones were identified. It has been reported that many members of the *Enterobacteriaceae*, belonging to the genera *Serratia, Enterobacter, Pantoea, Klebsiella, Proteus* and *Hafnia*, often contribute to meat spoilage (Borch et al. 1996; Nychas et al. 1998), while high correlations between cadaverine and *Enterobacteriaceae* counts have been obtained (Dainty & Mackey 1992). *S. liquefaciens*, *H. alvei*, *Rahnella aquatilis, C. freundii* were frequently encountered in minced beef, while some of them were found to harbour toxin-encoding genes and other putative virulence factors (Lindberg et al. 1998). Moreover, different members of *Enterobacteriaceae* have been recovered from beef, while *Rahnella* spp. has been shown to play an important role in the spoilage of meat and found as the dominant bacterium in the late phases of refrigerated storage (Ercolini et al. 2006). On the other hand, *S. grimessi* was been shown to be the dominant Gram negative species at the later stage of storage of meat with and without the use of active packaging (Ercolini et al. 2010). *Serratia* and *Proteus* were also the genera most commonly present on working surfaces in the meat processing industry (Stiles 1981). *S. liquefaciens* has been also found by many investigators to be the most common member of this family on meat taken from abattoirs (Stanbridge & Davies 1998). Stiles and Ng (1981) reported that *E. agglomerans* and *S.*

liquefaciens were predominant *Enterobacteriaceae* at the retail level, but they had limited indicator potential for sanitation and hygiene.

Pseudomonas agar base medium community

Two different strategies were used in the present study in the case of minced beef. The traditional method, i.e., pure cultures, were picked randomly from the highest dilution of PAB medium, as well as the direct analysis of the whole cultivable community from the same medium were applied. This approach was used in order to indicate the lack of selectivity. In both cases, members of *Enterobacteriaceae* community and pseudomonads were detected, while at several time points the former community was only obtained. These observations are not in agreement with the results revealed from beef fillets, as the counts of *Enterobacteriaceae* community of the latter were low.

This inability of the medium to select only pseudomonads has been described previously, especially when the number of *Enterobacteriaceae* was high (Stanbridge & Davies 1994; Jeppesen 1995; Tryfinopoulou et al. 2001; Ramalho et al. 2002). A number of researchers use the oxidase reaction to distinguish the pseudomonads from the other bacteria that are able to grow on PAB medium (Shaw & Latty 1982; Stanbridge & Davies 1994; Tryfinopoulou et al. 2002). Nevertheless, this method can be biased by the fact that pseudomonads suggested a variety in oxidase reaction (Jay 2000; Liao 2006).

The outcome of the two strategies was quite different. In the most cases, PCR-DGGE analysis was not able to detect all the bacteria based on the results revealed from the isolation of pure cultures. Similarly, the lack of selectivity was observed at several time points as a higher diversity or different species based on DGGE bands was observed beside the recovered isolates. Moreover, regarding the *Enterobacteriaceae* community detected on the

PAB medium, the results were similar with those from VRBG medium. Thus only the observed results for pseudomonads will be discussed below.

It the present study the PAB community of fresh minced beef consisted of *Ps. fragi*; during the storage the latter prevailed over the other pseudomonads. Under aerobic conditions *Ps. fragi* dominated the pseudomonads community at 15°; equally dominant was *Pseudomonas* spp. CK119 at 5 and 10°C, while with *Pseudomonas* spp. CK73 at 0°C. Furthermore, under modified atmosphere (MAP- and MAP+), *Pseudomonas* spp. CK73 was dominated the pseudomonads community at 0, 5 and 15°C, while *Ps. fragi* and *Ps. fluorescens* was dominated the community at 15°C under MAP- and MAP+, respectively. It has to be noted that *Ps. putida* was recovered / detected only at chill temperatures (0 and 5°C) in all packaging conditions adopted. Similarly, different pseudomonads strains dominated the PAB community of beef fillets. *Ps. fragi* was recovered from the fresh fillets, *Ps. putida* from fillets stored at 0 and 20°C, while *Ps. fluorescens* was not detected. It has been observed by a number of investigators that *Ps. fluorescens* is more abundant on fresh meats than *Ps. fragi* but that the latter becomes dominant over time (Lebert et al. 1998). *Ps. fragi* was reported to be the most frequently dominating species, followed by the *Ps. lundensis* and *Ps. fluorescens* (Dainty & Mackey 1992). Furthermore, *Ps. fluorescens* has been found to combine better at low than at higher temperatures (Olsen & Jezeski 1963; Liao 2006). High concentrations of CO_2 (up to 10%) have been found to inhibit the growth of *Ps. fluorescens* and *Ps. fragi* on red meat (Gill & Tan 1980), whereas *Ps. fragi* was inhibited more so than the other pseudomonads like *Ps. fluorescens* and *Ps. lundensis* (Stanbridge & Davies 1998). *Ps. putida* was detected in lower percentage than the other pseudomonads, this could be attributed to its inability to dominate. Indeed it has been reported that pseudomonads generally dominate on spoiled meats in the order *Ps. fragi* > *Ps. lundesis* > *Ps. fluorescens* > *Ps. putida* (Garcia – Lopez et al. 1998).

In general, among the species recovered throughout the storage of beef, several meat associated ones were identified. Members of the *Ps. fluorescens* group, along with the psychrotrophic *Ps. fragi*, *Ps. lundensis*, and *Ps. putida*, are often isolated from spoiled meat even during storage at low temperatures (Labadie 1999; Stanbridge & Davies 1998). *Ps. fragi* and *Ps. fluorescens* cause deterioration in quality of meat and milk products due to the production of extracellular proteases and lipases at low temperatures (Lebert et al. 1998). On beef, lamb and pork, studies have shown the predominance of *Ps. fluorescens* from the slaughter line to the chilling process (Gustavsson & Borch 1993). Additionally, *Ps. fluorescens* is known to be largely present in the environment (floor, water), on animals (hide, skin) or also in water and surfaces in meat factories (Drosinos & Board 1995). On cutting lines and during storage and retailing, *Ps. fragi* was found as the dominant biota on meat (Molin & Ternstrom 1982; 1986; Prieto et al. 1992). In has to be noted that the species *Ps. aeruginosa*, *Ps. maltophilia*, *Ps. fluorescens*, *Ps. putida*, *Ps. cepacia*, *Ps. stutzeri*, and *Ps. putrefaciens*. *Ps. aeruginosa* which are associated with opportunistic infections are probably the most well-known member of the genus (Lerner & Lerner 2003).

4.4. Dynamic of molecular tools for the study of microbial communities

In this study, several culture dependent methods and one culture independent method have been applied to provide an insight of the spoilage related bacteria of meat in relation to the temperature and packaging conditions. Nevertheless, the main findings were based on the culture – dependent approach, most frequently applied when storage studies are performed.

The different isolates recovered throughout the storage of the products were subjected to PFGE to determine the strain diversity. Comparison of various strains of the same species may reveal specific characteristics of the spoilage strain that aid in growth niche occupation. PFGE is considered the "gold standard" for strain characterization since it is very precise,

reproducible, and reliable. Although, the well known dynamic of the method in case of the *Enterobacteriaceae* community with similar protocols for inter- and intra-species differentiation of *Salmonella* Enterica (Liesegang & Tschape 2002), *Esc. coli* (Izumiya et al. 1997), *Ps. aeruginosa* (Romling & Tummler 2000), *Leptospira* spp. (Ribeiro et al. 2009), the majority of the samples exhibited smeared bands or a smear of high molecular weight DNA entering the gel matrix. Similar results received with the addition of thiourea into Tris-based running buffer (Izumiya et al. 1997; Romling & Tummler 2000; Liesegang & Tschape 2002; Silbert et al. 2003; Alonso et al. 2005) or the replacement of Tris buffer by HEPES (Ray et al. 1992; Koort et al. 2002). Moreover, it has been reported that proper control must be performed, and all components of the digestion mixture (including the slices of plugs), must be checked for the presence of endogenous nuclease activity (Herschleb et al. 2007). Thus, a number of protocols have been developed in order to prevent this DNA degradation. In particularly, culture time should be controlled as some microorganisms display delayed DNA production (Porschen & Sonntag 1974), sodium dodecyl sulfate should be added in the solution used to make the agarose plugs (Hunter et al. 2005) or the incubation time of proteinase K treatment should be increased (Herschleb et al. 2007). However, none of these methods succeeded in obtaining a fine PFGE pattern for the isolates in the present study due to DNA degradation.

Nevertheless, the addition of thiourea after the proteinase K treatment overcomes the problems of the DNA degradation, with all isolates that were previously untypeable now producing high quality fingerprints. Thus, it was possible to differentiate *Enterobacteriaceae* isolates by PFGE fingerprinting after cleavage with restriction enzyme *Xba*I. These findings suggest that a putative nuclease might be present in the agarose blocks and might be responsible for the observed DNA degradation. This is the first time that thiourea was introduced as a step during the preparation of agarose inserts. Since the above modification

enabled the complete typeability within the assayed isolate to be achieved while maintaining both a high degree of discrimination and reproducibility of the technique, a modified PFGE method was established. This modification was successfully used for the differentiation of the isolates from PAB medium, whereas a complex community i.e. large diversity of fingerprints was detected. Thus the succession of the isolates recovered from this medium was presented at species level. In the latter case, the use of a different restriction enzyme (rare cutter) might be useful to overcome the problem of the large number of bands.

Additionally, SDS - PAGE has been applied to determine the species diversity of *Enterobacteriaceae* community of minced beef. Regarding the differentiation between *S. proteamaculans* and *S. liquefaciens* and bearing in mind the [ongoing] scientific debate over their distinction (Grimont et al. 1978), SDS-PAGE of whole-cell proteins proved adequate to supply clear discrimination between strains. Moreover, S. *proteamaculans* was further subdivided into 2 sub-clusters, exhibiting the remarkable intra-species differentiation capacity of this technique. In the present study, an effective differentiation between *C. freundii*, *H. alvei* and *P. vulgaris* has been achieved. It has been reported before that the comparison of whole-cell protein patterns obtained by highly standardised SDS – PAGE has been successfully applied for microbial identification at species or subspecies level, even of closely related species (Pot et al. 1994). The high taxonomic resolution of this technique, regarding inter- and intra-species divergence, that is often the case in the *Enterobacteriaceae* family, has already been exhibited (Hantula et al. 1990; Holmes et al. 1991; Coenye et al. 2001).

The different spoilage bacteria used in this study were recovered from the appropriate selective media. These media have been used for the isolation and subsequent characterization of the microbiota, but the limitations of the method have to be taken into

account. Stressed or injured cells might not have managed to recover and grow, resulting in their non isolation from the plated and giving therefore the impression that they were absent from the studied environment (Liao 2006). Another important limitation is dealing with the possible inability of the selective agents used to inhibit the growth of the rest biota (Corry et al. 1995; 2003). This was the case with the PAB growth medium used as *Enterobacteriaceae* were also isolated from the minced beef samples. Several studies did provide different solutions i.e. modifications of the medium that allows the differentiation between pseudomonads and *Enterobacteriaceae* (Stanbridge & Davies 1994; Jeppesen 1995; Tryfinopoulou et al. 2001; Ramalho et al. 2002). The observations of the present study confirmed that problems occurred when modified medium was inoculated with high inoculum levels (Stanbridge & Davies 1994), as *Enterobacteriaceae* was not recovered from beef fillets and when the levels of *Enterobacteriaceae* community was about 1.5-2 log cfu g^{-1} lower from levels of PBA community. Except from the factors mentioned above, random selection of colonies is required to have a representative sample. This is not always possible because it depends on the person performing the task and it is therefore not objective. Moreover the number of the presumptive colonies selected for the identification is important. Most traditional culture methods for the food require a minimum of five colonies tested, but the probability of the target organisms being correctly identified depends on its proportion amongst the presumptive count (Corry et al. 2007).

An alternative method has been proposed for the characterization of the whole culturable community (Ercolini et al. 2001) as well as the microbiota directly extracted from the samples without previous cultivation. Nowadays, the culture – independent methods like PCR-DGGE are widely applied to analyse the DNA extracted from food (Ercolini et al. 2001;2006;2009;2010, Fontana et al. 2005, Rantsiou et al. 2005, Cocolin et al. 2007). In the present study, the aforementioned method was applied to compare the observations from the

selected isolates and the whole cultivable community of the PAB growth medium. The observed results showed that differences occurred from the different approaches. These differences could be attributed to the limitations of each method used. Similarly, with the culture independent approach the microbiota of beef fillets was studied, but only the predominant bacteria were detected. In the latter case, the inability of the method to detect the rest microbial community recovered from the growth media with the exception of the dominant biota, could be attributed to the several limitations of the method. PCR-DGGE is estimated to detect only community members representing at least 1-2% of the microbial population in an environmental sample (Muyzer et al. 1993). Furthermore, it has been determined that the detection limit for the latter method is in order of 3 - 4 log cfu g^{-1} (Cocolin et al. 2001), is however valuable for communities with a limited number of abundant members (Nocker et al. 2007). Perhaps the most common problematic aspect is the tendency of many investigators to simply ignore the uncommon and draw conclusions regarding microbial community diversity based solely on the number and rank of numerically common organisms (Bent & Forney 2008). The problem of limited sensitivity in applying PCR-DGGE on complex communities might be overcome by limiting the analysis to a specific fraction of the community prior PCR-DGGE (Nocker et al. 2007). Moverover, low amplification efficiency becomes a problem with some environmental templates that are already difficult to amplify because of the presence of inhibitory substances (Nocker et al. 2007). The use of PCR-DGGE for screening communities can further be limited by the small fragment size of the PCR products; amplification of 300-400bp might not contain enough information for the precise taxonomic classification (Ovreas 2000; Nocker et al. 2007). Furthermore, since DNA remains intact in non viable cells, it should be kept in mind that might be enumerate both viable and dead cells; in most cases only viable bacteria are relevant or of interest (Maukonen & Saarela 2009).

A valuable tool in bacterial taxonomy for determining relationships between bacterial groups was found to be the sequencing of 16S rRNA genes (Pace et al. 1986; Hugenholtz et al. 1998; Baylis 2006; Nocker et al. 2007; Maukonen & Saarela 2009). More than 30 million full and partial sequences can be found in public databases, while the sequence databases for other genes contain only limited number of sequences limiting their use in microbial ecological studies (Nocker et al. 2007; Maukonen & Saarela 2009). Nevertheless, 16S rRNA gene – limited resolution of *Pseudomonas* and members of *Enterobacteriaceae* allowed in many cases their identification to species level (Baylis 2006; Ercolini et al. 2006), but besides 16S rRNA gene, other housekeeping or functional genes might be used as targets for PCR. Among the core bacterial genes, the gene for the RNA polymerase beta subunit, *rpo*B has emerged as one of the few potential candidates for bacteria identification, especially when studying closely related isolates (Adekambi et al. 2008). Thus, *rpo*B is suggested to fulfil these criteria and can be used as an alternative to 16s rRNA gene in species identification (Mollet et al. 1997). Moreover, sequencing of the entire *rpo*B gene together with 16S rRNA gene might be necessary when describing new bacterial species or subspecies and refine bacterial community (Adekambi et al. 2008). In this study, the *rpo*B gene was sequenced in order to identify the *Enterobacteriaceae* community that the identification was failed with 16S rRNA gene; nevertheless, four isolates were remained unidentifiable leading to the possibility that new bacterial species were detected. Moreover, the 16S V6-V8 regions used in this study were probably not variable enough among the species of *Pseudomonas*, and this represents a limit in the use of a 16S-based PCR-DGGE approach for the identification of *Pseudomonas* in the meat ecosystem. As a matter of the fact, in the case of pseudomonads characterization *car*A gene has been found capable of simultaneous detection of *Ps. fragi*, *Ps. lundensis* and *Ps. putida* (Ercolini et al. 2007), while the genes *recA*, *gyrB*, *fliC*, and *rpoD* may be supportive for *Pseudomonas* species differentiation (Yamamoto et al. 2000,

Bellingham et al. 2001, Hilario et al. 2004). Similarly, *rec*A gene has been used for the differentiation of *Lb. plantarum, Lb. pentosus* and *Lb*. *paraplantarum* (Torriani et al. 2001). The target gene, *kat*A was used in the present study for the detection *Lb*. *sakei* recovered from minced beef; this gene has been also successfully applied to differentiate *Lb*. *sakei* from the rest lactobacilli even if LAB (Knauf et al. 1992, Hertel et al. 1998). Furthermore, it has to be noted that all PCR methods share limitations mainly caused by inefficient or preferential extraction of community DNA, varying efficiency of different extraction methods in removing inhibitory substances and in maintaining the integrity of DNA and amplification biases during PCR (Nocker et al. 2007). For example, in the present study, the DNA extraction applied in the case of samples subjected to PCR-DGGE was the only method capable of allow the amplification of the target gene.

Chapter 5

Conclusions and future work

5.1. Conclusions

The microbial associations in the present study found to be affected, by factors like storage temperature, packaging, season and different suppliers. The high microbial loads detected on meat at the retail level indicated that strategies have to be considered to improve the microbial quality and safety of the product. Two strategies i.e. modified atmosphere packaging and oregano essential oil, were selected in the present study. The revealed results indicated that volatile compounds of oregano essential oil in combination with MAP can be seen as a means of keeping the microbiological loads and colour change to acceptable levels and as a more effective system for extending the shelf life and increase the safety of meat. Moreover, the volatile compounds of oregano essential oil were found to effect the contribution of the spoilage microorganisms to the microbial association. There was still a need to assess, within each spoilage related microbial group, which species are actually involved in the spoilage of meat.

The assessment of microbial species diversity occurring in meat during storage and the study of the response and adaptability of the species to different antimicrobial conditions could be fundamental for improving and implementing packaging systems aimed at prolonging the shelf life and safety of meat products. Thus, the present study did provide an insight of the population dynamics of bacteria in relation to the temperature and the packaging conditions. The overall outcome has been clearly demonstrated that certain species and/or strains are present or dominant only under certain conditions. This finding is extremely important since studies conducted so far had only taken into consideration the microbiological counts as an indication of the spoilage process, and had ignored the possibility that different species or strains would prevail under different storage and/or packaging conditions. The qualitative information derived from the microbiological analyses and the characterization of the species or even the strains present were not evaluated

previously. It has been shown that storage temperature combined with packaging conditions induced the selectivity of the spoilage microbiota. Moreover, the microbiota recovered from the initial stage of storage was markedly different from that at the final stage of storage at chill temperatures. The above observations are of great importance and fundamental in understanding the spoilage process and in explaining the presence of different products or by-products that occur during the different dynamic storage conditions.

The main findings of the present study were based on the culture dependent approach. PFGE has provided important information in relation to the strain distribution of the microbiota which would have not been acquired if strain typing had not been performed. In the latter case, with the already existing protocols, *Enterobacteriaceae* isolates could not be analysed by PFGE, because a continuous smear of DNA rather than well separated fragments was produced. A modified PFGE protocol i.e. addition of thiourea after the proteinase treatment, was successfully developed with all isolates that were previously untypeable now producing high quality fingerprints; this is the first time that thiourea was introduced in a step during the preparation of agarose inserts. The culture independent approach which applied indicated that it was able to detect the dominant microbiota. Although, the rest bacteria do not form a dominant biota, which may be a result of poor adaptation to meat as an ecological niche, study of their trophic relations as well as growth requirements and capabilities is essential as they are considered opportunistic pathogens. Moreover, another limitation arising from culture independent methods is the mislaid of viable cells. In the latter case, the opportunity to consider the specific characteristics and/or the spoilage potential of isolates is omitted. The above observations are leading to the use of different appropriated molecular approaches, culture – dependent in combination with culture – independent in order to widen the knowledge of the spoilage related bacterial succession during storage of foods.

5.2. Future work

Following the investigations described in this thesis, a number of projects could be taken up:

- It would be interest to study the spoilage potential of the specific spoilage bacteria dominated the microbiota of meat individually or in mixed communities on sterile meat.

- The potential ability of the different lactic acid bacteria strains recovered to control the spoilage and safety of meat could also be studied. If interesting results will be achieved, an antimicrobial film could be developed.

- Monitor the succession of *Brochothrix thermosphacta* strains during storage of meat under different conditions; isolates have been already recovered from the experiments presented in this study.

- Further study is needed to clarify possible discrepancies between culture-dependent and independent methods, and evaluate whether these differences would give a different overview of the ecology of the meat.

- Characterize the four unidentified bacteria recovered from beef fillets; the identification failed with 16S rRNA and *rpo*B gene leading to the possibility that new bacterial species were detected.

Chapter 6

References

Abd El-Rhman, H. A., Marriott, N. G., Wang, H., Yassein, M. M. A. & Ahmed, A. M. (1998). Characteristics of minced beef stored at chilled and abuse temperatures. *J Muscle Foods* **9**, 139-152.

Adams, M. R. & Moss, M. O. (1995). Food Microbiology. Cambridge, The royal society of Chemistry.

Adekambi, T., Drancourt, M. & Raoult, D. (2008). The *rpo*B gene as a tool for clinical microbiologists. *Trends Microbiol* **17**, 37-45.

Aguirre, M. & Collins, M. D. (1993). A review: lactic acid bacteria and human clinical infection. *J Appl Bacteriol* **75**, 95-107.

Aligiannis, A., Kalpoutzakis, E., Mitaku, S. & Chinou, I. B. (2001). Composition and antimicrobial activity of the essential oils of two Origanum species. *J Agr Food Chem* **49**, 4168- 4170.

Alklint, C., Wadso , L. & Sjoholm, I. (2004). Effects of modified atmosphere on shelf-life of carrot juice. *Food Control* **15**, 131-137.

Alonso, R., Martin, A., Pelaez, T., Marin, M., Rodriguez – Creixems, M. & Bouza, E. (2005). An improved protocol for pulsed – field gel electrophoresis typing of *Clostridium difficile*. *J Med Microbiol* **54**, 155-157.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.

Amann, R. I., Ludwig, W. & Schleifer, K-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev **59**, 143-169.

Ampe, F., Ben Omar, N., Moizan, C., Wacher, C. & Guyot, J-P. (1999). Polyphasic study of the spatial distribution of microorganisms in mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Appl Environ Microbiol* **65**, 5464-5473.

Aureli, P., Costantini, A. & Zolea, S. (1992). Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. *J Food Protect* **55**, 344-348.

Avrahami, S., Liesack, W. & Conrad, R. (2003). Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. *Environ Microbiol* **5**, 691-705.

Axelsson, L. T. (1998). Lactic acid bacteria: Classification and physiology. In *Lactic Acid Bacteria Microbiology and Functional Aspects*, pp. 1-72. Edited by S. Salminen, A. Von Wright. NY: Marcel Dekker,

Ayres, J. C. (1955). Microbiological implications in the handling, slaughtering, and dressing of meat animals. *Adv Food Res* **6**, 109-161.

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

Barkocy-Gallagher, G. A., Arthur, T. M., Rivera-Betancourt, M., Nou, X., Shackelford, S. D., Wheeler, T. L. & Koohmaraie, M. (2003). Seasonal prevalence of Shiga toxin–producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *J Food Protect* **66**, 1978-1986.

Baylis, C. L. (2006). *Enterobacteriaceae*. In *Food spoilage microorganisms*, pp. 213-286. Edited by C. de W. Blackburn. Cambridge: Woodhead Publishing Limited, Abington Hall, Abington

Bellingham, N. F., Morgan, J. A. W., Saunders J. R. & Winstanley, C. (2001). Flagellin gene sequence variation in the genus *Pseudomonas*. *System Appl Microbiol* **24**, 157-165.

Ben Amor, K., Vaugham, E. E. & de Vos, W. M. (2007). Advanced molecular tools for identification of lactic acid bacteria. *J Nutr* **137**, 741-747.

Bent, S. J. & Forney, L. J. (2008). The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. *Int Soc Microbial Ecol* **2**, 689-695

Bergey, D. H. (1986). *Bergey's Manual of Systematic Bacteriology*, 2nd edn. NY: Springer.

Betts, G. (2006). Other spoilage bacteria. In *Food spoilage microorganisms*, pp. 213-286. Edited by C. de W. Blackburn. Cambridge, Woodhead Publishing Limited, Abington Hall, Abington.

Blixt, Y. & Borch, E. (2002). Comparison of shelf life of vacuum-packed pork and beef. *Meat Sci* **60**, 371-378.

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

Bosilevac, J. M., Guerini, M. N., Brichta-Harhay, D. M., Arthur, T. M. & Koohmaraie, M. (2007). Microbiological characterization of importer and domestic boneless beef trim used for ground beef. *Int J Food Protect* **70**, 440-449.

Bourget, N., Simonet, J. M. & Decaris, B. (1993). Analysis of the genome of the five *Bifidobacterium breve* strains: plasmid content, pulsed-field gel electrophoresis genome size estimation and rrn loci number. *FEMS Microbiol Lett* **110**, 11-20.

Bracewill, A. J., Reagan, J. O., Carpenter, J. A. & Blankenship, L. C. (1985). Incidence of *Campylobacter jejuni/coli* on pork carcasses the northeast Georgia area. *J Food Protect* **48**, 808-810.

Brody, A. L. (1996). Integrating aspect and modified atmosphere packaging to fulfill a vision of tomorrow. *Food Technol* **50**, 56-66.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *Int J Food Microbiol* **94**, 223-253.

Byun, S. K., Jung, S. C. & Yoo, H. S. (2001). Random amplification of polymorphic DNA typing of *Listeria monocytogenes* isolated from meat. *Int J Food Microbiol* **69**, 227-235.

Carlin, F., Nguyen-The, C. & Morris, C. E. (1996). Influence of background microflora on *Listeria monocytogenes* on minimally processed fresh broad-leaved endive (Cichorium endivia var. latifolia). *J Food Protect* **59**, 698-703.

Castellano, P. H., Holzapfel, W. H. & Vignolo, G. M. (2004). The control of *Listeria innocua* and *Lactobacillus sakei* in broth and meat slurry with the bacteriocinogenic strain *Lactobacillus casei* CRL705. *Food Microbiol* **21**, 291-298.

Champomier-Verges, M.-C., Chaillou, S., Cornet, M. & Zagorec, M. (2001). *Lactobacillus sakei*: recent developments and future prospects. *Res Microbiol* **152**, 839-848.

Chenoll, E., Macian, M. C. & Aznar, R. (2003). Identification of Carnobacterium, *Lactobacillus, Leuconostoc* and *Pediococcus* by rDNA-based techniques. *Syst Appl Microbiol* **26**, 546-556.

Chenoll, E., Macian, M.C., Elizaquivel, P. & Aznar, R. (2007). Lactic acid bacteria associated with vacuum packed cooked meat product spoilage: population analysis by rDNA based methods. *J Appl Microbiol* **102**, 498-508.

Chorianopoulos, N., Kalpoutzakis, E., Aligiannis, N., Mitakou, S., Nychas, G-J. E. & Haroutounian, S. A. (2004). Essential Oils of *Satureja*, *Origanum*, and *Thymus* Species: Chemical Composition and Antibacterial Activities Against Foodborne Pathogens. *J Agr Food Chem* **52**, 8261-8267.

Chorianopoulos, N. G. (2007). PhD Thesis. Agricultural University of Athens

Cocolin, L., Manzano, M., Cantoni, C. & Comi, G. (2001). Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Appl Environ Microbiol* **67**, 5113-5121.

Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C. & Comi, G. (2004). Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. *Appl Environ Microbiol* **70**, 1883-1894.

Cocolin, L., Diez, A., Urso, R., Rantsiou, K., Comi, G., Bergmaier, I. & Beimfohr, C. (2007). Optimization of conditions for profiling bacterial populations in food by culture-independent methods. *Int J Food Microbiol* **120**,100-9.

Cocolin, L., Dolci, P. & Rantsiou, K. (2008). Molecular methods for identification of microorganisms in traditional meat products. In *Meat Biotechology*, pp. 91-127. Edited by F., Toldra. NY, Springer.

Coenye, T., Vandamme, P., Govan, J. R. W. & LiPuma, J. J. (2001). Taxonomy and identification of the Burkholderia cepacia complex. *J Clin Microbiol* **39**, 3427-3436.

Conner, D. E., Beuchat, L. R., Worthington, R. E. & Hitchcock, H. L. (1984). Effects of essential oils and oleoresins of plants on ethanol production, respiration and sporulation of yeasts. *Int J Food Microbiol* **1**, 63-74.

Corry, J. E. L., Baird, R. M. C. & Curtis, G. D. W. (1995). Culture media for Food Microbiology, Progress in Industrial Microbiology Vol 34, Amsterdam, Elsevier.

Corry, J. E. L., Curtis, G. D. W. & Baird, R. M. C. (2003). Handbook of Culture Media for Food Microbiology, Progress in Industrial Microbiology Vol 37, Amsterdam, Elsevier.

Corry, J. E. L., Jarvis, B., Passmore, S. & Hedges, A. (2007). A critical review of measurement uncertainty in th enumeration of food microorganisms. *Food Microbiol* **24**, 230-253.

Corsetti, A., De Angelis, M., Dellaglio, F., Paparella, A., Fox, P. F., Settanni, L. & Gobbetti, M. (2003). Characterization of sourdough lactic acid bacteria based on genotypic and cell-wall protein analyses. *J Appl Microbiol* **94**, 641-654.

Cosentino, C., Tuberoso, C. I. G., Pisan, B., Satta, M., Arzedi, E. & Palmas, F. (1999). *In-Vitro* antimicrobial activity and chemical composition of Sardinian Thymus essential oils. *Lett Appl Microbiol* **29**, 130-135.

Cottin, J., Genthon, H., Bizon, C. & Carbonelle, B. (1985). Study of *Listeria monocytogenes* in meat taken from 514 cattle. *Sci Aliment* **5**, 145-149.

Cutter, C. N. (2000). Antimicrobial effect of herb extracts against *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella typhimurium* associated with beef. *J Food Protect* **63**, 601-607.

Dainty, R. H., Shaw, B. G., de Boer, K. A. & Scheps, E. S. J. (1975). Protein changes caused by bacterial growth on beef. *J Appl Bacteriol* **39**, 73-81.

Dainty, R. H. & MacKey, B. M. (1992). The relationship between the phenotypic properties of bacteria form chill-stored meat and spoilage processes. In *Ecosystems: Microbes: Food*, pp. 103-114. Edited by R.G. Board, D. Jones, R.G. Kroll, G.L. Pettipher. Society for Applied Bacteriology Symposium Series. Vol. 73.

Dalgaard, P., Vancanneyt, M., Euras Vialta, N., Swings, J., Fruekilde, P. & Leisner, J. J. (2003). Identification of lactic acid bacteria from spoilage associations of cooked and brined shrimps stored under modified atmosphere between 0 °C and 25 °C. *J Appl Microbiol* **94**, 80-89.

Daniel, P. (1995). Sizing of the *Lactobacillus plantarum* genome and other lactic acid bacteria species by transverse alternating field electrophoresis. *Curr Microbiol* **30**, 243-246.

Davies, A. R. (1995). Advances in modified-atmospheres packaging. In *New Methods of Food Preservation*, pp. 304-320. Edited by Gould, G.W., London: Blackie Academic and Professional.

Davinson, P. M. (1997). Chemical preservatives and natural antimicrobial compounds. In *Food microbiology: Fundamentals and Frontiers*, pp. 520-556. Edited by M. P. Doyle, L. R. Beuchat, T. J. Montville, NY: ASM Press,

De Angelis, M., Corsetti, A., Tosti, N., Rossi, J., Corbo, M. R. & Cobbetti, M. (2001). Characterization of non-starter lactic acid bacteria from Italian ewe cheeses based on phenotypic, genotypic, and cell wall protein analysis. *Appl Environ Microbiol* **67**, 2011-2020.

Di Maria, S., Basso, A. L., Santoro, E., Grazia, L. & Coppola, R. (2002). Monitoring of *Staphylococcus xylosus* DSM 20266 added as starter during fermentation and ripening of soppressata molisana, a typical Italian sausage. *J Appl Microbiol* **92**, 158-164.

Drosinos, E. H. & Board, R. G. (1995). Microbial and physicochemical attributes of minced lamb: Sources of contamination with pseudomonads. *Food Microbiol* **12**, 189-197.

Edwards, R. A., Dainty, R. H., Hibbard., C. M. & Ramantanis, S. V. (1987). Amines in fresh beef of normal pH and the role of bacteria in changes in concentration observed during storage in vaccum packs at chill temperatures. *J Appl Bacteriol* **63**, 427-434.

Enfors, S.-O., Molin, G. & Ternstroem, A. (1979). Effect of packaging under carbon dioxide, nitrogen or air on the microbial flora of pork stored at 4^{o}C. *J Appl Bacteriol* **47**, 197-208.

Eisel, W. G., Linton, R. H. & Muriana, P. M. (1997). A survey of microbial levels for incoming raw beef, environmental sources, and ground beef in a red meat processing plant. *Food Microbiol* **14**, 273-282.

Engelen, B., Meinken, K., Von Wintzingerode, F., Heuer, H., Malkomes, H.-P. & Backhaus, H. (1998). Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Appl Environ Microbiol* **64**, 2814-2821.

Ercolini, D., Moschetti, G., Blaiotta, G. & Coppola, S. (2001). The Potential of a Polyphasic PCR-DGGE Approach in Evaluating Microbial Diversity of Natural Whey Cultures for Water-Buffalo Mozzarella Cheese Production: Bias of Culture-Dependent and Culture-Independent Analyses. *Syst Appl Microbiol* **24**, 610-617.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food: a review. *J Microbiol Meth* **56**, 297-314.

Ercolini, D., Mauriello, G., Blaiotta, G., Moschetti, G. & Coppola, S. (2004). PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarella cheese. *J Appl Microbiol* **96**, 263-270.

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.

Ercolini, D., Russo, F., Blaiotta, G., Pepe, I., Mauriello, G. & Villani, F. (2007). Simultaneous detection of *Pseudomonas fragi, P. lundensis,* and *P. putida* from meat by use of a multiplex PCR assay targeting the *carA* gene. *Appl Environ Microbiol* **73**, 2354-2359.

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., Ferrocino, I., La Storia, A., Mauriello, G., Gigli, S., Masi, P. & Villani, F. (2010). Development of spoilage microbiota in beef stored in nisin activated packaging. *Food Microbiol* **27**, 137-143.

Ertas, H. B. & Seker, E. (2005). Isolation of *Listeria monocytogenes* from fish intestines and RAPD analysis. *J Vet and Anim Sci* **29**, 1007-1011.

Faber, J. M. (1991). Microbiological aspect of modified atmosphere packaging technology. *J Food Protect* **54**, 58-70.

Fernandes, R. (2009). Chilled and Frozen raw meat, poultry and their products. In *Meat products*, pp. 1-52. Edited by R. Fernandes. Cambridge, Leatherhead Publishing,

Ferris, M. J., Muyzer, G. & Ward, D. M. (1996). Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62**, 340-346.

Ferris, M. M., Yan, X., Habbersett, R. C., Shou, Y., Lemanski, C. L., Jett, J. H., Yoshida, T. M. & Marrone1, B. L. (2004). Performance Assessment of DNA Fragment Sizing by High-Sensitivity Flow Cytometry and Pulsed-Field Gel Electrophoresis. *J Clin Microbiol* **42**, 1965-1976.

Fleet, G. H. (1999). Microorganisms in food ecosystems. *Int J Food Microbiol* **50**, 101-117.

Fontana, C., Cocconcelli, P. & Vignolo, G. (2005). Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. *Int J Food Microbiol* **103**, 131-142.

Fontana, C., Cocconcelli, P. S. & Vignolo, G. 2006. Direct molecular approach to monitoring bacterial colonization on vacuum-packaged beef. *Appl Environ Microbiol* **72**, 5618-5622.

Fung, D. Y. (2010). Microbial Hazards in food: Food-borne infections and intoxications. In *Handbook of meat processing*, pp. 481-500. Edited by F. Toldra, USA: Blackwell Publishing.

Garcıa de Fernando, G. D., Nychas, G-J. E., Peck, M. W. & Ordonez, J. A. (1995). Growth/survival of psychrotrophic pathogens on meat packaged under modified atmospheres. *Int J Food Microbiol* **28**, 221-231.

Garcia-Lopez, M. L., Prieto, M. & Otero, A. (1998). The physiological attributes of Gram-negative bacteria associated with spoilage of meat and meat products. In The Microbiology of Meat and Poultry, pp. 1-34. Edited by A. Davies, R., Board. London: Blackie Academic and Professional.

Gevers, D., Huys, G. & Swings, J. (2001). Applicability of rep-PCR fingerprinting of Lactobacillus species. *FEMS Microbiol Lett* **205**, 31-36.

Giannakourou, M., Koutsoumanis, K., Nychas, G-J. E. & Taoukis, P. S. (2001). Development and assessment of an intelligent shelf life decision system (SLDS) for quality optimization of the food chill chain. *J Food Protect* **64**, 1051-1057.

Giannuzzi, L., Pinotti, A. & Zaritzky, N. (1998). Mathematical modelling of microbial growth in packaged refrigerated beef stored at different temperatures. *Int J Food Microbiol* **39**, 101-110.

Gill, G. O. & Newton, K. G. (1977). The development of aerobic spoilage on meat stored at chill temperatures. *J Appl Bacteriol* **43**, 189-195.

Gill, C. O. & Newton, K. G. (1980). Growth of bacteria on meat at room temperatures. *J Appl Bacteriol* **49**, 315-323.

Gill, C. O. & Tan, K. H. (1980). Effect of carbon dioxide on growth of meat spoilage bacteria. *Appl Environ Microbiol* **39,** 317-319.

Gill, C. O. (1982). Microbial interaction with meats. In *Meat Microbiology*, pp. 225–264. Edited by M. H. Brown, London and NY: Applied Science Publishers.

Gill, G. O. (1983). Meat spoilage and evaluation of the potential storage life of fresh meat. *J Food Protect* **46**, 444-452.

Gill, C. O. (1986). The control of microbial spoilage in fresh meats. In Advances in Meat Research, Meat and Poultry Microbiology, pp. 49-88. Edited by A. M. Pearson, T. R. Dutson, Westport: AVI Publishing Company Inc.

Gill, C. O. & Jeremiah, L. E. (1991). The storage life of non-muscle offals packaged under vacuum or carbon dioxide. *Food Microbiol* **8**, 339-353.

Gill, C. O. & Molin, G. (1991). Modified atmospheres and vacuum packaging. In *Food Preservatives*, pp. 172-199. Edited by N. J. Russell, G. W. Gould, Glasgow and London: Blackie academic and professional.

Gill, C. O. (1996). Extending the storage life of raw chilled meats. *Meat Sci* **43**, 99-109.

Gill, C. O. (1998). Microbiological contamination of meat during slaughter and butchering of cattle, sheep and pigs. In *The microbiology of meat and poultry*, pp. 118-157. Edited by A. Davies, R. Board , London: Blackie academic and professional,

Gill, C. O., Jones, T., Rahn, K., Campbell, S., LeBlank, D. I., Holley, R. A. & Stark, R. (2002). Temperatures and ages of boxed beef packed and distributed in Canada. *Meat Sci* 60, 401-410.

Gill, C. O. (2003). Active packaging in practice: meat, In Novel food packaging technology, pp. 378-396. Edited by H. Ahvenainem, Boca Raton: Woodhead Publishing Limited and CRC Press LLC.

Gill, C. O. (2005). Sources of bacterial contamination at slaughtering plants. In Improving the safety of fresh meat, pp. 231-243. Edited by J. N. Sofos, Cambridge: CRC/Woodhead Publishing Limited.

Gomes, N. C. M., Heuer, H., Schonfeld, J., Costa, R., Mendonca- Hagler, L. & Smalla, K. (2001). Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. *Plant Soil* **232**, 167-180.

Gustavsson, P. & Borch, E. (1993). Contamination of beef carcasses by psychrotrophic *Pseudomonas* and *Enterobacteriaceae* at different stages along the processing line. *Int J Food Microbiol* **20**, 67-83.

Hantula, J., Korhonen, T. K. & Bamford, D. H. (1990). Determination of taxonomic resolution capacity of conventional one-dimensional SDS-polyacrylamide gel electrophoresis of whole-cell proteins using Enterobacteriaceae. *FEMS Microbiol Lett* **70**, 325-330.

Harrigan, W. F. (1998). *Laboratory Methods in Food Microbiology*. California: Elsevier Science and Technology.

Harris, L. J. (1998). The microbiology of vegetable fermentations. In *Microbiology of fermented food*, pp. 45-72. Edited by B. J. B. Wood, London: Blackie Academic Professional.

Hedrick, H. B., Aberle, E. D., Forrest, J. C., Judge, M. D. & Merkel, R. A. (1994). *Principles of Meat Science*, Dubuque: Kendall/Hunt Publishing Co.

Herschleb, J., Anaviev, G. & Schwartz, D. (2007). Pulsed - field gel electrophoresis. *Nat Protoc* **2**, 677-684.

Hertel, C., Schmidt, G., Fischer, M., Oellers, K. & Hammes, W. P. (1998). Oxygendependent regulation of the expression of the catalase gene *kat*A of *Lactobacillus sakei* LTH677. *Appl Environ Microbiol* **64**, 1359-1365.

Hielm, S., Bjorkroth, J., Hyytia, E. & Korkeala, H. (1998). Genomic analysis of *Clostridium botulinum* group II by pulsed-field gel electrophoresis. *Appl Environ Microbiol* **64**, 703-708.

Hilario, E., Buckley, T.R. & Young, J. M. (2004). Improved resolution of the phylogenetic relationships among *Pseudomonas* by the combined analysis of *atpD*, *carA*, *recA* and 16S rDNA. *Anton Leeuw* **86**, 51-64.

Holmes, B., Costas, M. & Wood, A. C. (1991). Typing of *Proteus mirabilis* from clinical sources by computerized analysis of electrophoretic protein patterns. *J Appl Bacteriol* **71**, 46-76.

Holzapfel, W. H. (1998). The Gram-positive bacteria associated with meat and meat products. In *The Microbiology of Meat and Poultry*, pp.35-84. Edited by R. G. Board, A. R. Davies, London: Blackie Academic and Professional.

Hudson, J. A. & Mott, S. J. (1993). Growth of *Listeria monocytogenes*, *Aeromonas hydrophila* and *Yersinia enterocolitica* on cooked beef under refrigeration and mild temperature abuse. *Food Microbiol* **10**, 429-437.

Hudson, J. A., Mott, S. J. & Penney, N. (1994). Growth of *Listeria monocytogenes, Aeromonas hydrophila and Yersinia enterocolitica* on vacuum and saturated carbon dioxide controlled atmosphere-packaged sliced roast beef. *J Food Protect* **57**, 204-208.

Hugenholtz, P. & Pace, N. R. (1996). Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends Biotechnol* **14**, 190-197.

Hunter, S. B., Vauterin, P., Lambert-Fair, M. A., Van Duyne, M. S., Kubota, K., Graves, L., Wrigley, D., Barrett, T. & Ribot, E. (2005). Establishment of a Universal Size Standard Strain for Use with the PulseNet Standardized Pulsed-Field Gel Electrophoresis Protocols: Converting the National Databases to the New Size Standard. *J Clin Microbiol* **43**, 1045-1050.

ICMSF (1980). Microbial Ecology of foods. Volume 1, Factors affecting life and death of microorganisms. NY: Academic press.

Iacumin, L., Comi, G., Cantoni, C. & Cocolin, L. (2006). Molecular and technological characterization of *Staphylococcus xylosus* isolated from Italian naturally fermented sausages by RAPD, Rep-PCR and Sau-PCR analysis. *Meat Sci* **74**, 281-288.

Insausti, K, Beriain, M. J., Purroy, A., Alberti, P., Gorraiz, C. & Alzueta, M. J. (2001). Shelf life of beef from local Spanish cattle breeds stored under modified atmosphere. *Meat Sci* **57**, 273-281.

Izumiya, H., Terajima, J., Wada, A., Inagaki, Y., Itoh, K. I., Tamura, K. & Watanabe, H. (1997). Molecular typing of enterohemorrhagic *Escherichia coli* O157:H7 isolates in Japan by using pulsed-field gel electrophoresis. *J Clin Microbiol* **35**, 1675-1680.

Jay, J. M. & Margitic, S. (1981). Incidence of yeasts in fresh ground beef and their ratios to bacteria. *J Food Sci* **46**, 648-649.

Jay J. M. (2000). Modern food microbiology. Sixth edition. Gaithersburg: An Aspen Publication.

James, S. (1996). The chill chain ‘‘from carcass to consumer’’. *Meat Sci* **43**, 203-216.

Jeremiah, L. E. (2001). Packaging alternatives to deliver fresh meats using short- or long-term distribution. *Food Res Int* **34**, 749-772.

Jeppesen, C. (1995). Media for *Aeromonas* spp., *Plesiomonas shigelloides* and *Pseudomonas* spp. from food and environment. *Int J Food Microbiol* **26**, 25-41.

Jones, R. J. (2004). Observations on the succession dynamics of lactic acid bacteria populations in chill-stored vacuum packaged beef. *Int J Food Microbiol* **90**, 273–282.

Kagkli, D.-M., Vancanneyt, M., Hill, C., Vandamme, P. & Cogan, T. M. (2007). *Enterococcus* and *Lactobacillus* contamination of raw milk in a farm dairy environment. *Int J Food Microbiol* **114**, 243-251.

Kennedy, J., Jackson, V., Blair, I. S., McDowell, D. A., Cowan, C. & Bolton, D. J. (2005). Food safety knowledge of consumers and the microbiological and temperature status of their refrigerator. *J Food Protect* **68**, 1421-1430.

Kersters, K., Pot, B., Dewettinck, D., Torck, U., Vancanneyt, M., Vauterin, L. & Vandamme, P. (1994). Identification and typing of bacteria by protein electrophoresis. In *Bacterial diversity and systematic*, pp. 51-66. Edited by F. G. Priest, A. Ramos-Cormenzana, B. Tyndall, NY: Plenum Press.

Khan, N.H., Ishii, Y., Kimata-Kino, N., Esaki, H., Nishino, T., Nishimura, M. & Kogure, K. (2007). Isolation of *Pseudomonas aeruginosa* from open ocean and comparison with freshwater, clinical, and animal isolates. *Microbial Ecol* 2007 **53,** 173-186.

Klijn, N., Weerkamp, A. H. & de Vos, W. M. (1991). Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. *Appl Environ Microbiol* **57**, 3390-3393.

Knauf, H. J., Vogel, R. F. & Hammes, W. P. 1992. Cloning, sequence, and phenotypic expression of *kat*A, which encodes the catalase of *Lactobacillus sakei* LTH677. *Appl Environ Microbiol* **58**, 832-839.

Konstantinov, S. R., Zhu, W-Y., Williams, B. A., Tamminga, S., de Vos, W. M. & Akkermans, A. D. L. (2003). Effect of fermentable carbohydrates on piglet faecal bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *FEMS Microbiol Ecol* **43**, 225-235.

Koort, J. M., Lukinmaa, S., Rantala, M., Unkila, E. & Siitonen A. (2002). Technical improvement to prevent DNA degardation of enteric pathogens in pulsed – field gel electrophoresis. *J Clin Microbiol* **40**, 3497-3498.

Kostinek, M., Pukall, R., Rooney, A., Schillinger, U., Hertel, C., Holzapfel, W. H. & Franz, C. M. A. P. (2005). *Lactobacillus arizonensis* is a later heterotypic synonym of *Lactobacillus plantarum*. *Int J Syst Evol Microbiol* **55**, 2485-2489.

Koutsoumanis, K., Tassou, C. C., Taoukis, P. S. & Nychas, G-J. E. (1998). Modelling the effectiveness of a natural antimicrobial on *Salmonella enteritidis* as a function of concentration, temperature and pH, using conductance measurements. *J Appl Microbiol* **84**, 981-987.

Koutsoumanis, K., Lambropoulou, K. & Nychas, G-J. E. (1999). A predictive model for the non-thermal inactivation of *Salmonella enteritidis* in a food model system supplemented with a natural antimicrobial. *Int J Food Microbiol* **49**, 67-74.

Koutsoumanis, K. & Nychas, G-J. E. (2000). Application of a systematic experimental procedure to develop a microbial model for rapid fish shelf-life prediction. *Int J Food Microbiol* **60**, 171-184.

Koutsoumanis, K. P., Ashton, L. V., Geornaras, 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 Protect* **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.

Labadie, J. (1999). Consequences of packaging on bacterial growth. Meat is an ecological niche. *Meat Sci* **52**, 299-305.

Labuza, T. P. & Fu, B. (1993). Growth kinetics for shelf life prediction: theory and practice. *J Int Microbiol* **12**, 309-323.

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.

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^{o}C. *Int J Food Microbiol* **30**, 281-291.

Law, B. A. & Kolstad, J. (1983). Proteolytic systems in lactic acid bacteria. *Anton Leeuw* **49**, 225-245.

Lebert, I., Begot, C. & Lebert, A. (1998). Growth of *Pseudomonas fluorescens* and *Pseudomonas fragi* in a meat medium as affected by pH (5.8-7.0), water activity (0.97-1.00) and temperature (7-25c). *Int J Food Microbiol* **39**, 53-60.

Leblond, P., Francou, F. X., Simonet, J-M. & Decaris, B. (1990). Pulsed-field gel electrophoresis analysis of the genome of *Streptomyces ambofaciens* strains. *FEMS Microbiol Lett* **72**, 79-88.

Lee, B. H., Simard, R. E., Layele, L. C. & Holley, R. A. (1983). Microflora, sensory and exudate changes of vacuum- or nitrogen- packed veal chucks under different storage conditions. *J Food Sci* **48**, 1537-1542.

Lee, J., Jang, J., Kim, B., Kim, J., Jeong, G. & Han, H. (2004). Identification of *Lactobacillus sakei* and *Lactobacillus curvatus* by multiplex PCR-based restriction enzyme analysis. *J Microbiol Meth* **59**, 1-6.

Lee, J-S., Heo, G-Y., Lee, J. W., Oha, Y-J., Parka, J. A., Parka, Y-H., Pyunb, Y-R. & Ahna, T. J. S. (2005). Analysis of kimchi microflora using denaturing gradient gel electrophoresis. *Int J Food Microbiol* **102**, 143-150.

Lee, Y.-A., Chen, K.-P. & Hsu, Y.-W. (2006). Characterization of *Erwinia chrysanthemi*, the soft-rot pathogen of white-flowered calla lily, based on pathogenicity and PCR-RFLP and PFGE analyses. *Plant Pathol* **55**, 530-536.

Lerner, K. L. & Lerner, B. W. (2003). World of microbiology and immunology. USA: The Gale Group, Inc.

Liao, C. H. (2006). *Pseudomonas* and related genera. In Food spoilage microorganisms, pp. 213-286. Edited by C. de W. Blackburn, Cambridge: Woodhead Publishing Limited, Abington Hall, Abington.

Liesegang, A. & Tschape, H. (2002). Modified pulsed – field gel electrophoresis method for DNA degradation – sensitive *Salmonella* enterica and *Escherichia coli* strains. *Int J Med Microbiol* **291**, 645-648.

Lindberg, A-M., Ljunghb, A., Ahrne, S., Lofdahlc, S. & Molin, G., (1998). *Enterobacteriaceae* found in high numbers in fish, minced meat and pasteurised milk or cream and the presence of toxin encoding genes. *Int J Food Microbiol* **39**, 11-17.

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.

Lucia, L. M., Vanderzant, C., Acuff, G. R. (1993). Potential role of *Proteus-Providencia-Morganella* species in quality deterioration of beef. *Meat Sci* **35**, 321-330.

Ludwig, W. (2007). Nucleic acid techniques in bacterial systematic and identification. *Int J Food Microbiol* **120**, 225-236.

Lyhs, U., Bjorkroth, J., Hyytia, E. & Korkeala, H. (1998). The spoilage flora in vacuum packed sodium nitrite and potassium nitrate treated cold-smoked rainbow trout stored at 4 °C or 8 °C. *Int J Food Microbiol* **45**, 135-142.

Mangena, T. & Muyima, N. Y. O. (1999). Comparative evaluation of the antimicrobial activities of essential oils of *Artemisia afra, Pteronia incana* and *Rosmarinus officinalis* on selected bacteria and yeast strains. *Lett Appl Microbiol* **28**, 291-296.

Marshall, D. L. & Schmidt, R. H. (1991). Physiological evaluation of stimulated growth of Listeria monocytogenes by Pseudomonas species in milk. *Can J Microbiol* **37**, 694-599.

Mataragas, M., Drosinos, E. H., Vaidanis, A. & Metaxopoulos, I. (2006). Development of a Predictive Model for Spoilage of Cooked Cured Meat Products and Its Validation Under Constant and Dynamic Temperature Storage Conditions. *J Food Sci* **71**, 157-167.

Maukonen, J. & Saarela, M. (2009). Microbial communities in industrial environment. *Curr Opin Microbiol* **12**, 238-243.

Mauriello, G., Ercolini, D., La Storia, A., Casaburi, A. & Villani, F. (2004). Development of polyethylene films for food packaging activated with an antilisterial bacteriocin from *Lactobacillus curvatus* 32Y. *J Appl Microbiol* **97**, 314-322.

McMeekin, T. A. (1981). Microbial spoilage of meats. In *Developments in Food Microbiology*, pp. 1-40. Edited by R. Davies, London: Applied Science.

McMullen, L. M. & Stiles, M. E. (1993). Microbial ecology of fresh pork stored under modified atmosphere at -1, 4,4 and 10°C. *Int J Food Microbiol* **18**, 1-14.

Miller, A., Scanlan, R. A., Lee, J. S. & Libbey, L. M. (1973). Volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas putrefaciens, Pseudomonas fluorescens,* and *Achromobacter* species. *Appl Microbiol* **26**,18-21.

Molin, G. & Ternstrom, A. (1982). Numerical taxonomy of psychrotrophic pseudomonads. *J Gen Microbiol* **128,** 1249-1264.

Molin, G. & Ternstrom, A. (1986). Phenotypically based taxonomy of psychrotrophic Pseudomonas isolated from spoiled meat, water, and soil. *Int J Syst Bacteriol* **36,** 257-274.

Molin, G. (2000). Modified atmospheres. In *The Microbiological Safety and Quality of Food*, pp. 214-234. Edited by B. M. Lund, T. C. Baird-Parker, G. W. Gould, Gaithersburg: MD.

Mollet, C., Drancourt, M. & Raoult, D. (1997). *rpo*B sequence analysis as a novel basis for bacterial identification. *Mol Microbiol* **26**, 1005-1011.

Muyzer, G., De Weal, E. C. & Uitterlinden, A. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695-700.

Muyzer, G., Teske, A., Wirsen, C. O. & Jannasch, H. W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Ach Microbiol* **164**, 165-172.

Muyzer, G. & Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Anton Leeuw Int J G Mol Microbiol* **73**, 127-141.

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.

Narashima Rao, D., Nair, K. K. S. & Sakhare, P. Z. (1998). Meat microbiology and spoilage in tropical countries. In *The microbiology of meat and poultry*, pp. 220-265. Edited by A. Davies, R. Board, London: Blackie academic and professional,

Nattress, F. M. & Jeremiah, L. E. (2000). Bacterial mediated off-flavours in retail-ready beef after storage in controlled atmospheres. *Food Res Int* **33**, 743-748.

Newton, K.G. & Gill, C. O. (1978). The development of anaerobic spoilage flora of meat stored at chill temperatures. *J Appl Microbiol* **44**, 91-95.

Newton, K. G. & Rigg, W. J. (1979). The effect of film permeability on the storage life and microbiology of vacuum-packed meat. *J Appl Bacteriol* **47**, 433-441.

Nocker, A., Burr, M. & Camper, A. K. (2007). Genotypic microbial community profiling: a critical technical review. *Microbial ecol* **54**, 276-289.

Nortje, G. L. & Shaw, B. G. (1989). The effect of ageing treatment on the microbiology and storage characteristics of beef in modified atmosphere packs containing 25% CO_2. plus 75% O_2. *Meat Sci* **25**, 43-58.

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

Nychas, G-J. E. & Tassou, C. C. (1997). Spoilage processes and proteolysis in chicken as detected by HPLC. *J Food Sci Agr* **74**, 199-208.

Nychas, G-J. E., Drosinos, E. & Board, R. G. (1998). Chemical changes in stored meat. In *The Microbiology of Meat and Poultry,* pp. 288-326. Edited by R. G. Board, A. R. Davies, London: Blackie Academic and Professional.

Nychas, G-J. E. & Tassou, C. C. (2000). Preservatives: traditional preservatives – oils and spices In *Encyclopedia of Food Microbiology,* pp. 1717-1722. Edited by R. Robinson, C. Batt, Patel, London: Academic Press.

Nychas, G-J. E., Skandamis, P. N. & Tassou, C. C. (2003). Antimicrobials from herbs and spices. In Natural antimicrobials for the minimal processing of foods, pp. 176-200. Edited by S. Roller, Cambridge: Woodhead publishing in Food Science and Technology.

Nychas, G-J. E. & Skandamis, P. (2005). Fresh meat spoilage and modified atmosphere packaging (MAP). In *Improving the Safety of Fresh Meat*, pp. 461-502. Edited by J. N. Sofos, Cambridge: CRC/Woodhead Publishing Limited.

Nychas, G-J. E., Marshall, D. L. & Sofos, J. N. (2007). Meat, Poultry, and Seafood. In *Food microbiology: fundamentals and frontiers,* pp. 105-140. Edited by M. P. Doyle, L. R. Beuchat, Washington, D.C: ASM Press.

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

Odagami, T., Morita, J., Takama, K. & Suzuki, S. (1994). Substrate specificities of extracellular proteases produced by marine putrefactive bacteria, *Shewanella putrefaciens* and *Alteromonas haloplaanktis. Lett Appl Microbiol* **18**, 50-52.

Olsen, R. H. & Jezeski, J. J. (1963). Some effects of carbon source, aeration, and temperature on growth of a psychrophilic strain of *Pseudomonas fluorescens. J Bacteriol* **86**, 429-433.

O'Sullivan, D. J. (1999). Methods for analysis of the intestinal microflora. In *Probiotics. A Critical Review*, pp. 23-44. Edited by G. W. Tannock, Norfolk, Horizon Scientific Press.

O'Sullivan, D. J. (2000). Methods for analysis of the intestinal microflora. *Curr Iss Intest Microbiol* **1**, 39-50.

Outtara, B., Simard, R. E., Holley, R. A., Piettte, G. J. P. & Begin, A. (1997). Antimicrobial activity of selected fatty acids and essential oils against six meat spoilage organisms. *Int J Food Microbiol* **37**, 155-162.

Ovreas, L. (2000). Population and community level approaches for analysing microbial diversity in natural environments. *Ecol Lett* **3**, 236-251.

Pace, N. R., Stahl, D. A., Lane, D. J. & Olsen, G. J. (1986). The analysis of natural microbial populations by ribosomal-RNA sequences. *Adv Microb Ecol* **9**, 1-55.

Palleroni, N. J. (1993). Pseudomonas classification. A new case history in the taxonomy of Gram-negative bacteria. *Anton Leeuw* **64**, 231-251.

Pandey, A., Joshi, V. K., Nigam, P. & Soccol, C. R. (1999). Enterobacteriaceae coliform and E. coli. In *Encyclopedia of Food Microbiology*, pp. 604-610. Edited by R. Robinson, C. Batt, P. Patel, London: Elsevier.

Pandit, V .A. & Shelef, L. A. (1994). Sensitivity of *Listeria monocytogenes* to rosemary (*Rosmarinus officinalis* L.). *Food Microbiol* **11**, 57-63.

Paramithiotis, S., Mueller, M. R. A., Ehrmann, M. A., Tsakalidou, E., Seiler, H., Vogel, R. & Kalantzopoulos, G. (2000). Polyphasic identification of wild yeast strains isolated from Greek sourdoughs. *Syst Appl Microbiol* **23**, 156-164.

Paramithiotis, S., Kagkli, D.-M., Blana, V. A., Nychas, G-J. E. & Drosinos, E. H. (2008). Identification and characterization of *Enterococcus* spp. in Greek spontaneous sausage fermentation. *J Food Protect* **71**, 1244-1247.

Patarata, L., Pimentel, M. S., Pot, B., Kersters, K. & Mendes-Faia, A. (1994). Identification of lactic acid bacteria isolated from Portuguese wines and musts by SDS-PAGE. *J Appl Bacteriol* **76**, 288-293.

Pennacchia, C., Vaughan, E. E. & Villani, F. (2006). Potential probiotic *Lactobacillus* strains from fermented sausages: Further investigations on their probiotic properties. *Meat Sci* **73**, 90-101.

Porschen, R. K. & Sonntag, S. (1974). Extracellular Dnase production by anaerobic bacteria. *Appl Microbiol* **27**, 1031-1033.

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Modern microbial methods. Chemical methods in prokaryotic systematic,* pp. 493-521. Edited by M. Goodfellow, A. G. O'Donnell, Chichester: John Wiley and Sons Ltd.

Rahkio, T. M. & Korkeala, H. J. (1997). Airborne bacteria and carcass contamination in slaughterhouses. *J Food Protect* **60**, 38-42.

Ramalho, R., Cunha, J., Teixeira, P. & Gibbs, P. A. (2002). Modified Pseudomonas agar: new differential medium for the detection/enumeration of *Pseudomonas aeruginosa* in mineral water. *J Microbiol Meth* **49**, 69-74.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G. & Cocolin, L. (2005). Culture dependent and independent methods to investigate the microbial ecology of Italian fermented sausages. *Appl Environ Microbiol* **71**, 1977-1986.

Rantsiou, K. & Cocolin, L. (2006). New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: A review. *Int J Food Microbiol* **108**, 255-267.

Ray, T., Weaden, J. & Dyson, P. (1992). Tris-dependent site specific cleavage of *Streptomyces lividans* DNA. *FEMS Microbiol Lett* **96**, 247-252.

Ribeiro, R. L., Machry, L., Brazil, J. M. V., Ramos, T. M. V., Avelar, K. E. S. & Pereira, M. M. (2009). Technical improvement to prevent DNA degradation of *Leptospira* spp. in pulsed field gel electrophoresis. *Lett Appl Microbiol* **49**, 289-291.

Romling, U. & Tummler, B. (2000). Achieving 100% typeability of *Pseudomonas aeruginosa* by the pulsed- field gel electrophoresis. J Clin Microbiol **38**, 464-465.

Roussel, Y., Colmin, C., Simonet, J. M. & Decaris B., (1993). Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). *J Appl Bacteriol* **74**, 549-556.

Sakala, R. M., Hayashidani, H., Kato, Y., Hirata, T., Makino, Y., Fukushima, A., Yamada, T., Kaneuchi, C. & Ogawa, M. (2002). Change in the composition of the microflora on vacuum-packaged beef during chiller storage. *Int J Food Microbiol* **74**, 87-99.

Samelis, J. (2006). Managing microbial spoilage in meat industry. In *Food spoilage microorganisms*, pp. 213-286. Edited by C. de W. Blackburn, Cambridge: Woodhead Publishing Limited

Sanders, M. E., Walker, D. C., Walker, K. M., Aoyama, K. & Klaenhammer, T. R. (1996). Performance of commercial cultures in fluid milk applications. *J Dairy Sci* **79**, 943-955.

Schillinger, U. & Holzapfel, W. H. (2006). Lactic acid bacteria. In *Food spoilage microorganisms*, pp. 213-286. Edited by C. de W. Blackburn, Cambridge: Woodhead Publishing Limited.

Schwartz, D. C. & Cantor, C. R. (1984). Separation of yeast chromosomesized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37**, 67-75.

Seal, S. E., Jackson, L. A. & Daniels, M. J. (1992). Use of tRNA consensus primers indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction. *Appl Environ Microbiol* **58**, 3759-3761.

Shaw, B. G. & Latt, J. B. (1982). A numerical taxonomic study of *Pseudomonas* strains from spoiled meat. *J Appl Bacteriol* **52**, 219-228.

Shin, H. S. & Youn, J. H. (2005). Conversion of food waste into hydrogen by thermophilic acidogenesis. *Biodegradation* **16**, 33-44.

Shironi, E. & Labuza, T. P. (2000). Modeling pathogen growth in meat products: future challenges. *Trends Food Sci Tech* **11**, 394-402.

Silbert, S., Boyken, L., Hollis, R. J. & Pfaller, M. A. (2003). Improving typeability of multiple bacterial species using pulsed – field gel electrophoresis and thiourea. *Diagn Micr Infec Dis* **47**,619-621.

Silliker, J. H. (1980). *Microbial Ecology of Foods*. NY: Academic Press.

Silger, W. V., Miniaci, C. & Zeyer, J. (2004). Electrophoresis time impacts the denaturing gradient gel electrophoresis – based assessment of bacterial community structure. *J Microbiol Meth* **57**, 17-22.

Singh, S., Goswami, P., Singh, R. & Heller, K. J. (2009). Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: A review. *Food Sci Tech* **42**, 448-457.

Skandamis, P. N. & Nychas, G-J. E. (2000). Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. *Appl Environ Microbiol* **66**, 1646-1653.

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.

Stanbridge, L. H. & Board, R. G. (1994). A modification of the *Pseudomonas* selective medium, CFC, that allows differentiation between meat pseudomonads and *Enterobacteriaceae*. *Lett Applied Microbiol* **10**, 327-328.

Stanbridge, L. H. & Davies, A. R. (1998). The microbiology of chill stored meat. In *The microbiology of meat and poultry*, pp. 174-219. Edited by A. R. Davies and R.G. Board, London: Blackie Academic and Professional.

Stanley, K. N., Wallace, J. S., Currie, J. E., Diggle, P. J. & Jones, K., (1998). The seasonal variation of thermophilic campylobacters in beef cattle, dairy cattle and calves. *J Appl Microbiol* **85**, 472-480.

Stiles, M. E. (1981). *Enterobacteriaceae* associated with meats and meat handling. *Appl Environ Microbiol* **41**, 867-872.

Stiles, M. E. & Ng, L.-K. (1981). *Enterobacteriaceae* associated with meats and meat handling. *Appl Environ Microbiol* **41**, 867-872.

Swan, B. G. & Latty, J. B. (1982). A numerical taxonomic study of *Pseudomonas* strains from spoiled meat. *J Appl Bacteriol* **52**, 219-228.

Tanskanen, E. I., Tulloch, D. L., Hillier, A. & Davidson, B. E. (1990). Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. *Appl Environ Microbiol* **56**, 3105-3111.

Tassou, C. C., Drosinos, E. H. & Nychas G-J. E. (1995). Effects of essential oil from mint (*Mentha piperita*) on *Salmonella enteritidis* and *Listeria monocytogenes* in model food systems at 4 and 10°C. *J Appl Bacteriol* **78**, 593-600.

Tassou, C. C., Drosinos, E. H. & Nychas, G-J. E. (1996). Inhibition of resident microbial flora and pathogen inocula on cold fresh fillets in olive oil, oregano, and lemon juice under modified atmosphere or air. *J Food Protect* **58**, 31-34.

Temmerman, R., Huys, G. & Swings, J. (2004). Identification of lactic acid bacteria: culture – dependent and culture – independent methods. *Trends Food Sci Tech* **15**, 348-359.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233-2239.

Teske, A., Wawer, C., Muyzer, G. & Ramsing, N. B. (1996). Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probablenumber counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl Environ Microbiol* **62**, 1405-1415.

Thomas, L. V., Wimpenny, W. T. & Barker, G. C. (1997). Spatial interactions between subsurface bacteria colonies in a model system: a territory model describing the inhibition of *Listeria monocytogenes* by a nisin-producing lactic acid bacterium. *Microbiol* **143**, 2575-2582.

Tompkin, R. B. (1973). Refrigeration temperature as an environmental factor influencing the microbial quality of food. A review. *J Food Techn* **27**, 54-58.

Torriani, S., Felis, G. E. & Dellaglio, F. (2001). Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *rec*A gene sequence analysis and multiplex PCR assay with *rec*A gene-derived primers. *Appl Environ Microbiol* **67**, 3450-3454.

Tryfinopoulou, P., Drosinos, E. H. & Nychas, G-J. E. (2001). Performance of Pseudomonas CFC-selective medium in the fish storage ecosystems. *J Microbiol Meth* **47**, 243-247.

Tryfinopoulou, P., Tsakalidou, E. & Nychas, G-J. E. (2002). Characterization of *Pseudomonas* spp. Associated with Spoilage of Gilt-Head Sea Bream Stored under Various Conditions. *Appl Environ Microbiol* **68**, 65-72.

Tsigarida, E., Skandamis, P. N. & Nychas, G-J. E. (2000). Behaviour of *Listeria monocytogenes* and autochthonous flora on meat stored under aerobic, vacuum and modified atmosphere packaging conditions with or without the presence of oregano essential oil at 5°C. *J Appl Microbiol* **89**, 901-909.

Urso, R., Comi, G. & Cocolin, L. (2006). Ecology of lactic acid bacteria in Italian fermented sausages: Isolation, identification and molecular characterization. *Syst Appl Microbiol* **29**, 671-680.

Vasilopoulos, C., De Maere, H., De Mey, E., Paelinck, H., De Vuyst, L. & Leroy, F. (2010). Technology-induced selection towards the spoilage microbiota of artisan-type cooked ham packed under modified atmosphere. *Food Microbiol* **27**, 77-84.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematic. *Microbiol reviews* **60**, 407-438.

Vauterin, L., Hoste, B., Yang, P., Alvarez, A., Kersters, K. & Swings, J. (1993). Taxonomy of the genus *Xanthomonas*. In *Xanthomonas*, pp. 157-192. Edited by J. Swings, E. L. Civerolo, London:Chapman and Hall, Ltd.

Ventura, M., Meylan, V. & Zink, R. (2003). Identification and tracing of *Bifidobacterium* species by use of enterobacterial repetitive intergenic consensus sequences. *Appl Environ Microbiol* **69**, 4296-4301.

Versalovic, J., Koeuth, T. & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823-6931.

Villani, F., Casaburi, A., Pennacchia, C., Filosa, L., Russo, F. & Ercolini, D. (2007). The microbial ecology of the *Soppressata* of Vallo di Diano, a traditional dry fermented sausage from southern Italy, and *in vitro* and *in situ* selection of autochthonous starter cultures. *Appl Environ Microbiol* **73**, 5453-5463.

von Holy, A., Holzapfel, W. H. & Dykes, G. A. (1992). Bacterial populations associated with vienna sausage packaging. *Food Microbiol* **9**, 45-53.

Ward, D. M., Weller, R. & Bateson, M. M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**, 63-65.

Warriss, P. D. (2000). Meat Hygiene, Spoilage and Preservation. *In Meat science: an introductory text*, pp. 182-208. Edited by P. D. Warriss, Wallingford: CABI Publishing.

Welsh, J. & McClelland, M. (1992). PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. *Mol Microbiol* **6**, 1673-1680.

Wildmer, F., Seidler, R. J., Gillevet, P. M., Watrud, L. S. & Di Giovanni, G. D. (1998). A highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas* (*sensu stricto*) in environmental samples. *Appl Environ Microbiol* **64**, 2545-2553.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Viavian, A. & Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiol* **146**, 2385-2394.

Yeung, P. S. M., Kitts, C. L., Cano, R., Tong, P. S & Sanders, M. E. (2004). Application of genotyping and phenotyping analyses to commercial probiotic strain identity and relatedness. *J Appl Microbiol* **97**, 1095-1104.

Yost, C. K. & Nattress, F. M. (2002). Molecular typing techniques to characterize the development of a lactic acid bacteria community on vacuum-packaged beef. *Int J Food Microbiol* **72**, 97-105.

Young, L. L., Reviere, R. D. & Cole, A. B. (1988). Fresh meat red meat: a place to apply modified atmosphere. *Food Techn* **42**, 65-69.

Zoetendal, E. G., Akkermans, A. D. L. & de Vos, W. M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human faecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* **64**, 3854-3859.

Appendix I

The following manuscript has been published in *Food Microbiology*

Food Microbiology 27 (2010) 1028–1034

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions

Agapi I. Doulgeraki [a,c], Spiros Paramithiotis [b], Dafni Maria Kagkli [a], George-John E. Nychas [a,*]

[a] Department of Food Science, Technology and Human Nutrition, Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[b] Department of Food Science, Technology and Human Nutrition, Laboratory of Food Quality Control and Hygiene, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[c] Applied Mycology Group, Cranfield Health, Cranfield University, College Road, Cranfield, Bedfordshire, MK43 0AL, UK

ARTICLE INFO

Article history:
Received 13 November 2009
Received in revised form
27 June 2010
Accepted 6 July 2010
Available online 14 July 2010

Keywords:
Lactic acid bacteria
Meat preservation
PFGE
Essential oils
Microbial ecology

ABSTRACT

A total of 266 lactic acid bacteria (LAB) have been isolated from minced beef stored at 0, 5, 10 and 15 °C aerobically and under modified atmosphere packaging consisting of 40% CO_2–30% O_2–30% N_2 in the presence MAP (+) and absence MAP (−) of oregano essential oil. Sequencing of their 16S rRNA gene along with presence of the *kat*A gene demonstrated dominance of the LAB microbiota by *Leuconostoc* spp. during aerobic storage at 5, 10 and 15 °C, as well as during MAP (−) and MAP (+) storage at 10 and 15 °C; *Lactobacillus sakei* prevailed during aerobic storage at 0 °C, as well as at MAP (−) and MAP (+) storage at 0 and 5 °C. The sporadic presence of other species such as *Leuconostoc mesenteroides*, *Weisella viridescens*, *Lactobacillus casei* and *Lactobacillus curvatus* has also been determined. Pulsed-Field Gel Electrophoresis of high molecular weight genomic DNA revealed the dynamics of the isolated LAB strains. Prevalence of *Leuconostoc* spp. was attributed to one strain only. On the other hand, packaging conditions affected *Lb. sakei* strain spoilage dynamics.

1. Introduction

Food spoilage microbiota has been the subject of several studies conducted so far; the ones focused on meat and meat products were based on the identification and/or characterization of the dominant microbiota at different storage conditions. The concept of 'succession' of spoilage-related microbial groups i.e. ephemeral/specific spoilage organisms (E/SSO), was only recently, taken into consideration (Ercolini et al., 2006; Chenoll et al., 2007; Nychas et al., 2008).

Lactic acid bacteria (LAB) for instance are considered to be the Specific Spoilage Organisms (SSO) that contribute to the meat spoilage stored under packaging conditions in which the concentration of carbon dioxide is increased (Axelsson, 1998; Holzapfel, 1998; Nychas and Skandamis, 2005). *Lactobacillus*, *Leuconostoc* and *Carnobacterium* are among the most frequently encountered genera on vacuum or modified atmosphere packaged meat and play an important role in the spoilage of refrigerated raw meat (Shaw and Harding, 1984; Dainty and Mackey, 1992; Hugas et al., 1993; McMullen and Stiles, 1993; Rovira et al., 1997; Holzapfel, 1998; Labadie, 1999; Parente et al., 2001; Nychas and Skandamis, 2005). Species of *Leuconostoc* sp. and *Lb. sakei* have been associated with the spoilage of vacuum or modified atmosphere packed meat stored at chill temperatures (Champomier-Verges et al., 2001; Yost and Nattress, 2002; Ercolini et al., 2006). The lack of consistency e.g. why these two species were not always found at the end of storage period even if the conditions were similar can be possibly attributed not only to the limitation of the applied methodologies used but also to the potential effect of the man imposed preservation system on the development of the microbial association, e.g. Ephemeral Spoilage Organism (Stanbridge and Davies, 1998; Nychas et al., 2008; Vasilopoulos et al., 2010). In this case the word 'ephemeral' does describe the situation where these specific spoilage bacteria contribute to meat spoilage for a very short period of time till the next climax population is established. The identification and characterization of these ESOs in raw meat under different storage conditions remains still to be elucidated (Jones, 2004; Ercolini et al., 2006, 2009; Fontana et al., 2006; Vasilopoulos et al., 2010).

Oregano essential oil, as a potential 'hurdle', was found to affect the contribution of spoilage microorganisms to the microbial association as well as to the physicochemical changes of the minced meat (Skandamis and Nychas, 2001; Burt, 2004). Skandamis and Nychas (2002) reported that the oregano essential oil effect on microbial population, including LAB, on active packaging conditions. Axelsson

* Corresponding author. Tel./fax: +30 210 5294938.
E-mail address: gjn@aua.gr (G.-J.E. Nychas).

0740-0020/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2010.07.004

(1998) concluded that the addition of oregano essential oil influenced the metabolic activity of LAB. More specifically, the initial heterofermentative microbiota was substituted by a homofermentative one at the end of storage. However, despite the antimicrobial action of essential oil on biota, there is less information about the effect of such compounds on the microbial diversity of the LAB isolated from meat at species and strain level. The only information available relates the essential oil effect on growth of meat spoilage bacteria such as *Lb. sakei*, *Lb. curvatus* and *Carnobacterium piscicola* (Ouattara et al., 1997).

The use of conventional phenotypic methods does not always allow efficient characterization of the microbiota at species level (Holzapfel, 1998; Stanbridge and Davies, 1998). On the contrary, molecular identification and characterization tools are far more consistent, rapid, reliable and reproducible and can discriminate even between closely related groups of species, which are otherwise indistinguishable on the basis of their phenotype. The advances in molecular techniques are expected to widen the knowledge of spoilage-related bacterial succession during storage of foods (Chenoll et al., 2003; Ercolini et al., 2006). Several molecular typing techniques have been developed during the past decade for the identification and classification of bacteria at strain level. Among them, Pulsed-Field Gel Electrophoresis (PFGE) of DNA fragments resulting from the digestion of whole genomic DNAs with rare-cutting restriction endonucleases has proved to be reliable for bacterial typing. This method has been used to differentiate members of several genera including *Lactococcus* (Tanskanen et al., 1990), *Clostridia* (Hielm et al., 1998), *Streptomyces* (Leblond et al., 1990), probiotic lactobacilli (Yeung et al., 2004), and to compare the genomic restriction patterns of five *Bifidobacterium breve* strains (Bourget et al., 1993). It is considered to be a discriminating and reproducible method to differentiate strains of intestinal bacteria (O'Sullivan, 1999) and for chromosome size estimation in *Lb. acidophilus* (Roussel et al., 1993; Sanders et al., 1996), *Lb. plantarum* (Daniel, 1995), and other LAB (Tanskanen et al., 1990). Furthermore, PFGE in association with PCR-based methods are commonly used for strain monitoring (Singh et al., 2009).

The aim of the present study was to systematically monitor the microbial diversity of LAB, isolated from meat stored at different temperatures and under different packaging e.g. aerobic or MAP conditions, at strain level, by using modern molecular tools.

2. Materials and methods

2.1. Sample preparation and storage conditions

Minced beef was purchased from the central market of Athens and prepared according to Argyri et al. (submitted for publication). Briefly, minced beef samples were stored at 0, 5, 10 and 15 °C, aerobically or under modified atmospheres packaging (MAP) consisting of 40% CO_2–30% O_2–30% N_2 with MAP (+) or without MAP (−) the application of volatile compounds of oregano essential oil (2% v/w). The samples were placed on Styrofoam trays; all trays were performed to allow the diffusion of the volatile compounds of the essential oil with both sides of the samples. In the case of the treated samples (MAP+), the essential oil was distributed on a whatman paper that was placed on the bottom side of the tray.

2.2. Sampling of the meat

Minced beef was sampled at appropriate time intervals, depending on storage temperature; the incubation lasted 650, 482, 386 and 220 h at 0, 5, 10 and 15 °C, respectively and all samples were analysed in dublicate. A detailed description of the methodology employed for the enumeration of the total viable counts, *Pseudomonas* spp., *Br. thermosphacta*, LAB, *Enterobacteriaceae*, yeasts and molds in this work is presented elsewhere (Argyri et al. submitted for publication). Briefly, LAB counts were determined on MRS agar (Biolife, Italiana S.r.l., Milano, Italy) (pH = 5.8) overlaid with the same medium and incubated at 30 °C for 72 h. LAB were isolated from the highest dilution from three different time points (initial, middle and final stage of storage) for further analysis; 10% of the colonies (6–10 colonies) derived from plate culture of the highest sample dilution. They were randomly selected and purified by successive subculture on MRS agar at 30 °C. Gram positive, catalase and oxidase negative isolates were stored at −80 °C in MRS broth (Biolife, Milano, Italy) supplemented with 20% (v/v) glycerol (Merck, Darmstadt, Germany) until further use. Before experimental use each strain was grown twice in MRS broth at 30 °C for 24 and 16 h respectively. Purity of the culture was always checked on MRS agar plates before use.

2.3. Pulsed-field Gel Electrophoresis (PFGE)

PFGE was performed according to Kagkli et al. (2007). Briefly, cells were harvested by centrifugation at 10,000×*g* for 5 min and washed with 10 mM Tris–HCl (pH 7.6) containing 1 M NaCl; resuspended in 100 μL of the same solution, heated at 37 °C for 10 min and mixed with an equal volume of 2% (w/v) low melting-point agarose (Bio-Rad, Hercules, CA, USA) in 0.125 M EDTA pH 7.6 before letting them to solidify in moulds (Bio-Rad). The cells were lysed *in situ* in a solution containing 10 mg mL^{-1} of lysozyme in EC buffer (6 mM Tris–HCl, 1 M NaCl, 100 mM EDTA, 1% (w/v) Sarkosyl, pH 7.6) for 16 h at 37 °C. The lytic treatment was repeated with the same solution containing 2 U mL^{-1} mutanolysin. After treatment with proteinase K (0.5 M EDTA containing 1% sarkosyl, pH 8) for 24 h at 55 °C, the agarose blocks were washed twice for 1 h with 1 mM phenylmethylsulfonyl fluoride (PMSF) in 10 mM Tris–HCl containing 1 mM EDTA, (pH 8.0) at 37 °C and then stored at 4 °C in 10 mM Tris–HCl containing 100 mM EDTA (pH 8.0) until further use.

The agarose blocks were cut with sterile coverslips and slices (1–2 mm thick) of the blocks were washed three times at room temperature in 10 mM Tris–HCl containing 0.1 mM EDTA (pH 8.0) for 30 min with gentle agitation. The restriction enzymes *Apa*I and *Sma*I (10 U) (New England Biolabs, Ipswich, MA, USA) were initially selected to digest the slices of a limited number of strains. The enzyme that resulted in the production of clearer and sharper PFGE digestion profile was used for the digestion of all isolates. Digestions were performed according to the recommendations of the manufacturer.

Following digestion, slices were loaded into wells of a 1% PFGE grade agarose gel (Bio-Rad) and the gel was run in 0.5 mM Tris–Borate buffer (45 mM Tris–HCl, 45 mM Boric acid, 1 mM EDTA) using a CHEF-DRII PFGE apparatus and cooling module (Bio-Rad) at 6 V cm^{-1} for 16 h, with a pulse time ramped from 1 to 10 s. Gels were then stained with ethidium bromide (0.5 μg ml^{-1}) in water for 1 h and destained for 2 h before being photographed using a GelDoc system (Bio-Rad). Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Gel compare software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium; kindly provided by E. Tsakalidou, Dairy Laboratory, Agricultural University of Athens).

2.4. DNA extraction and species identification

DNA was extracted according to the protocol described by the manufacturer of GenElute Bacterial Genomic DNA Kit (Sigma, Chemical Co., St. Louis, Mo. USA). Representative number of isolates per distinct PFGE cluster were selected and subjected to species identification by sequencing the V1–V3 variable region of the 16S rRNA gene as described previously (Paramithiotis et al., 2008). PCR

products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (http://www.macrogen.com). The results were aligned with those in GenBank using the BLASTN program in order to determine their closest known relatives of the partial 16S rRNA gene sequence (Altschul et al., 1997). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are GU998850 to GU998881 (Table 2).

2.5. Detection of the heme-dependent catalase (katA) gene

All isolates were screened by PCR for the presence of the *kat*A gene, encoding heme-dependent catalase (Knauf et al., 1992; Hertel et al., 1998). For this purpose the specific primers 702-F (5′-AATTGCCTTCTTCCGTGTA-3′, position 551–536) and 310-R (5′-AGTTGCGCACAATTATTTTC-3′, position 127–139) were used.

3. Results

LAB were found to be the dominant biota in samples stored under MAP supplemented (+) or not (−) with Essential Oil (EO) (results not shown – Argyri et al. submitted for publication). Table 1 summarizes the lactic acid bacterial counts of the initial biota and of the three different time points (initial, middle and final stage of storage) for each of the storage conditions tested. In case of samples stored under MAP (+), the counts of LAB were lower at two time points (middle, end) than the ones stored in air and under MAP (−).

A total of 266 LAB isolates were recovered throughout the storage period; 99 isolates from aerobic storage, 89 isolates from MAP (−) and 78 isolates from MAP (+). The 99 isolates from aerobic storage were subjected to PFGE to determine the strain diversity during storage. For the aforementioned isolates, high molecular weight genomic DNA was digested with two different restriction enzymes (*Apa*I *and Sma*I). *Apa*I restriction generated better distributed bands than *Sma*I allowing a more reliable analysis of the generated profiles (Fig. 1). Therefore, *Apa*I was chosen to digest the 89 and 78 isolates from minced beef stored under MAP (−) and MAP (+), respectively.

A large diversity regarding strain occurrence at the different packaging and temperature conditions was revealed (Fig. 1, Table 3). The dendrogram obtained after image analysis of the different PFGE patterns, resulted into 32 different profiles, nine of which were obtained from aerobic storage, while 15 and 17 from MAP (−) and MAP (+), respectively (Table 3). Each strain present in Fig. 1 was subjected to 16S rRNA gene sequencing. Strains B 225, B 251, B 226, B 236, B 248, B 253, B 228, B 237, B 229, B 255, B 227, B 239, B 230, B 238, B 250, B 252, B 254 and B 249 were assigned to *Lb. sakei*; strains B 245 and B 246 to *Lb. curvatus*; strain B 247 to *Lb. casei*-group; strains B 234 and B 235 to *Ws. viridescens*; strains B 242 and B 243 to *Ln. mesenteroides*-group and strains B 232, B 241, B 258, B 244, B 233, B 240 and B 231 to *Leuconostoc* spp. All isolates were also screened for the presence of the *kat*A gene, specific for *Lb. sakei*. The *kat*A amplification results were in accordance with the ones already described. Two fingerprints (B 232 and B 233 assigned to *Leuconostoc* spp.) were common for all packaging conditions, two (B 226 and B 227 assigned to *Lb. sakei*) were shared between air and MAP (−), one fingerprint (B 230 assigned to *Lb. sakei*) was shared between air and MAP (+), and two fingerprints (B 242 and B 243 assigned to *Ln. mesenteroides*) were shared between MAP (−) and MAP (+).

From the initial stage of storage, two different strains (B 232 and B 233) were recovered, which were assigned to *Leuconostoc* spp. Strain B 233 was the most common isolate, since it was recovered at a percentage of 83.33% of the isolates recovered from the initial stage of storage.

From the 99 isolates from the aerobic storage of minced beef, the largest group was attributed to *Leuconostoc* spp. The corresponding fingerprints were B 231, B 232 and B 233, with the latter being the most common isolate, representing the dominant biota during storage at 5, 10 and 15 °C. The rest of the fingerprints (B 225, B 226, B 227, B 228, B 229 and B 230) were attributed to *Lb. sakei*, which were recovered from 0 and 5 °C, but one (B 228) from 10 °C. At 0 °C, *Lb. sakei* (B 226) was the prevalent one at the final stage of storage. The frequency of isolation and prevalence of the aforementioned isolates obtained from minced beef regarding aerobic storage at 0, 5, 10 and 15 °C is shown in Table 3.

Fifteen different fingerprints were detected during storage of minced beef under MAP (−); they were assigned to *Lb. sakei* (B 226, B 227, B 236, B 237, B 238 and B 239), *Leuconostoc* spp. (B 232, B233, B 258, B 240 and B 241), *Ws. viridescens* (B 234 and B 235), and *Ln. mesenteroides* (B 242 and B 243). Table 3 presents the frequency of isolation and prevalence of the isolates regarding the storage temperature. At 10 and 15 °C, *Leuconostoc* spp. (B 233) represented the dominant biota, whilst *Ws. viridescens* (B 234), *Lb. sakei* (B 237), *Ln. mesenteroides* (B 243) and *Leuconostoc* spp. (B 240) were also recovered. At chill temperatures (0 and 5 °C), the strain diversity was increased, since 11 different fingerprints were recovered. In both temperatures, *Lb. sakei* (B 237) was the prevalent strain at the final stage of storage. Moreover, *Lb. sakei* (B 236, B 238 and B 239), and *Leuconostoc* spp. (B 233 and B 258) were also recovered from storage at 0 °C, whilst *Ws. viridescens* (B 235), *Lb. sakei* (B 226, B 238 and B 227), *Leuconostoc* spp. (B 233 and B 241) and *Ln. mesenteroides* (B 242) were recovered from storage at 5 °C.

Out of 78 isolates, seventeen different fingerprints were obtained during storage of minced beef under MAP (+), indicating the increased diversity of the isolates. The frequency of isolation and prevalence of these isolates regarding storage at 0, 5, 10 and 15 °C is shown in Table 3. At 10 and 15 °C, *Leuconostoc* spp. (B 233) was the most common isolate, representing the dominant strain, whilst *Lb. sakei* (B 252 and B 255) and *Ln. mesenteroides* (B 243) were also recovered. *Lb. sakei* (B 254) was the dominant strain at the final stage of storage at 5 °C, while *Lb. sakei* (B 248 and B 255), and *Leuconostoc* spp. (B 233 and B 244) were also recovered during the storage at 5 °C. At the final stage of storage at 0 °C, *Leuconostoc* spp. (B 233), *Ln. mesenteroides* (B 242), *Lb. curvatus* (B 246) and *Lb. sakei* (B 249 and B 251) were equally contributed. *Lb. curvatus* (B 245), *Lb. casei* and *Lb. sakei* (B 248, B 250 and B 253) were also recovered during storage at 0 °C.

Table 1
Lactic acid bacteria populations in minced meat stored under aerobic, MAP (−) and MAP (+) conditions.

Temperature (°C)	Storage period	Lactic acid bacteria counts (log CFU g^{-1})[a]		
		Air	MAP−	MAP+
	Initial biota	5.26 ± 0.13	5.26 ± 0.13	5.26 ± 0.13
0 °C	Initial	5.33 ± 0.30	5.10 ± 0.11	5.07 ± 0.07
	Middle	6.24 ± 0.12	6.31 ± 0.24	5.48 ± 0.01
	Final	7.30 ± 0.18	7.54 ± 0.11	6.54 ± 0.32
5 °C	Initial	6.04 ± 0.09	5.60 ± 0.39	5.60 ± 0.39
	Middle	7.21 ± 0.09	6.74 ± 0.37	6.63 ± 0.10
	Final	7.66 ± 0.07	7.24 ± 0.08	7.47 ± 0.01
10 °C	Initial	6.01 ± 0.41	5.97 ± 0.42	5.86 ± 0.07
	Middle	7.41 ± 0.05	7.02 ± 0.17	6.52 ± 0.74
	Final	8.50 ± 0.03	8.56 ± 0.15	7.74 ± 0.11
15 °C	Initial	5.86 ± 0.16	6.86 ± 0.08	6.38 ± 0.08
	Middle	7.32 ± 0.02	7.17 ± 0.04	6.70 ± 0.12
	Final	8.62 ± 0.02	8.44 ± 0.01	7.62 ± 0.15

[a] Lactic acid bacteria counts are presented as mean ± standard deviation.

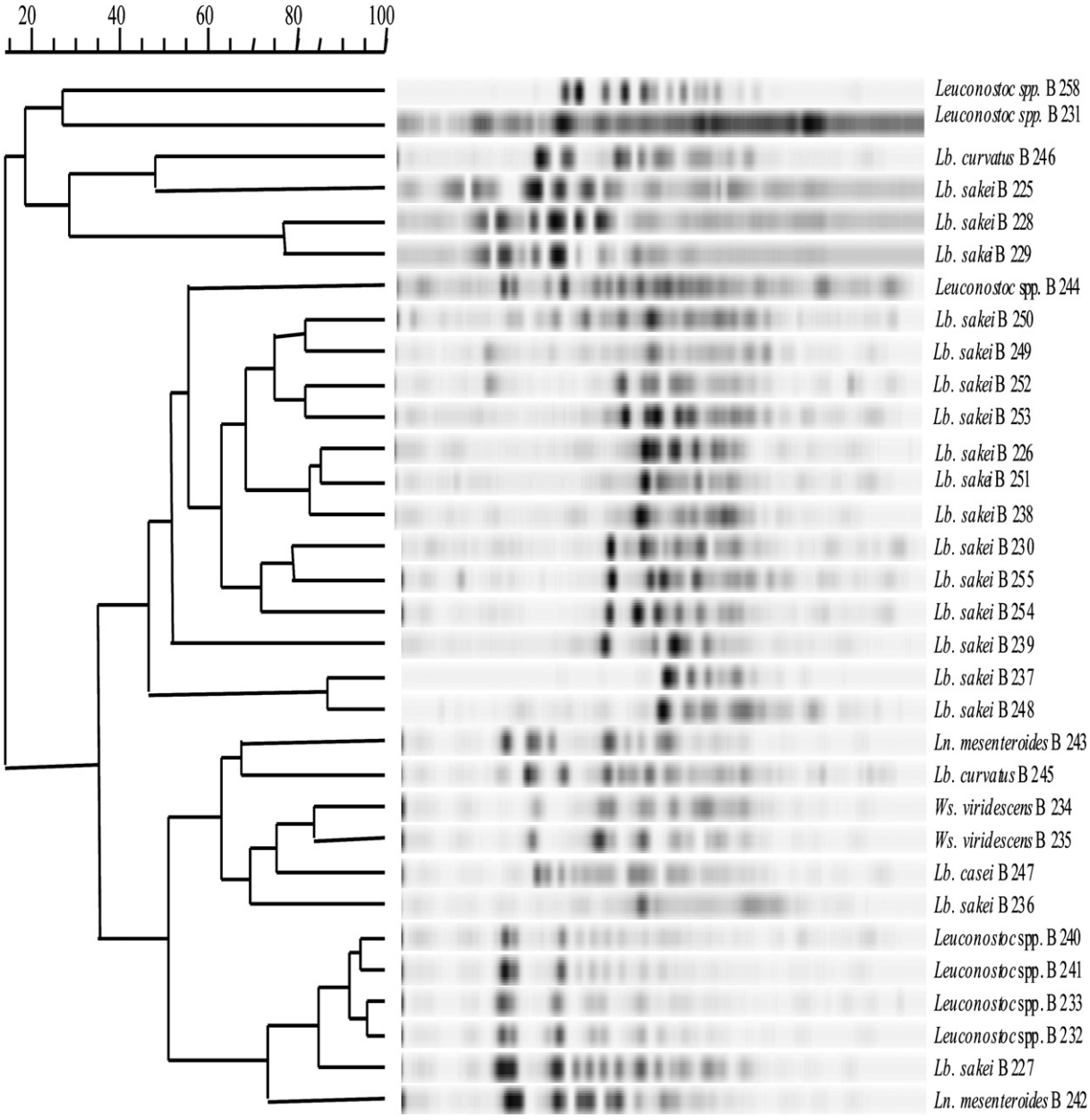


Fig. 1. Cluster analysis of PFGE *Apa*I digestion fragments of the lactic acid bacteria isolates calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%). Strain identity is indicated by the lower and upper case letters.

4. Discussion

Spoilage and spoilage progress of meat and meat products have been the subject of several studies conducted so far (Borch et al., 1996; Stanbridge and Davies, 1998; Labadie, 1999; Skandamis and Nychas, 2002; Nychas and Skandamis, 2005; Nychas et al., 2008). Nevertheless, meat spoilage has only been associated with the physicochemical and microbiological analysis of the bacterial loads ignoring the spoilage potential of a specific bacterial species or strain (Skandamis and Nychas, 2002). Only recently did research took into consideration the specific characteristics of the spoilage microbiota of the meat products and its contribution to the deterioration of the product (Cocolin et al., 2004b; Rantsiou et al., 2005; Ercolini et al., 2006, 2009; Vasilopoulos et al., 2010).

The present study focused on the evaluation of the microbial diversity of LAB isolated from minced beef stored under different storage conditions at strain level. Storage conditions had an important effect on the diversity of the microbial population, since different strains were recovered during the storage of meat under different conditions. These findings strengthen the opinion that the storage temperature and the modified atmosphere packaging affect the spoilage potential of LAB (Stanbridge and Davies, 1998; Ercolini et al., 2006, 2009). Moreover, this observation can be explained by the fact that different metabolic activities occur when different species/strains are present and when meat is stored under specific conditions. Not all species belonging to the same bacterial group, e.g. LAB, necessarily grow at the same temperature. It might therefore be misleading the fact that selective media only are used for the determination of the spoilage biota. Further characterization of the isolates grown on the selective plates should be demanded if a better insight and understanding of the phenomenon is required. This is in accordance with the observations of Ercolini et al. (2006) who reported

Table 2
Identity of isolates obtained from minced beef.

Number of isolates	Closest relative	Selected strain sequenced[a]	Accession Number
1	*Lactobacillus sakei*	B 225	GU998856
6	*Lb. sakei*	B 226	GU998877
2	*Lb. sakei*	B 227	GU998857
1	*Lb. sakei*	B 228	GU998850
1	*Lb. sakei*	B 229	GU998851
2	*Lb. sakei*	B 230	GU998852
1	*Leuconostoc* spp.	B 231	GU998853
9	*Leuconostoc* spp.	B 232	GU998854
205	*Leuconostoc* spp.	B 233	GU998855
1	*Weissella viridescens*	B 234	GU998858
2	*Ws. viridescens*	B 235	GU998859
1	*Lb. sakei*	B 236	GU998860
5	*Lb. sakei*	B 237	GU998861
3	*Lb.sakei*	B 238	GU998862
2	*Lb. sakei*	B 239	GU998863
1	*Leuconostoc* spp.	B 258	GU998864
1	*Leuconostoc* spp.	B240	GU998865
1	*Leuconostoc* spp.	B 241	GU998866
2	*Ln. mesenteroides*	B 242	GU998867
2	*Ln. mesenteroides*	B 243	GU998868
1	*Leuconostoc* spp.	B 244	GU998869
1	*Lb. curvatus*	B 245	GU998870
1	*Lb. curvatus*	B 246	GU998871
1	*Lb. casei*	B 247	GU998872
5	*Lb. sakei*	B 248	GU998873
1	*Lb. sakei*	B 249	GU998874
1	*Lb. sakei*	B 250	GU998875
1	*Lb. sakei*	B 251	GU998876
1	*Lb. sakei*	B 252	GU998878
1	*Lb. sakei*	B 253	GU998879
2	*Lb. sakei*	B 254	GU998880
1	*Lb. sakei*	B 255	GU998881

[a] Code of different PFGE patterns of Fig. 1.

that different species/strains were isolated from beef although similar counts were determined. Ercolini et al. (2006) also mentioned that the viable counts alone may not be enough to highlight the shifts of the bacterial communities depending on the environmental changes and species that are actually involved in meat spoilage.

Among the species listed in Table 2, several meat associated ones were identified. Holzapfel (1998) reported that more rarely *Lb. plantarum* and *Lb. casei* are associated with meat systems and in lower frequency and numbers than *Lb. curvatus* and *Lb. sakei*; the presence of *Ws. viridescens* in raw meat has been also described. Moreover, *Lb. curvatus, Lb. sakei* and *Leuconostoc* spp. have been found to indicate a mixture community of vacuum packed (vp) beef (Yost and Nattress, 2002). *Leuconostocs* have been identified as predominant organisms in beef stored under vp/MAP (Stanbridge and Davies, 1998; Yost and Nattress, 2002) and their presence in the initial mesophilic bacterial microbiota is very frequent (Borch et al., 1996). *Lb. sakei* has been associated with fresh meat (Champomier-Verges et al., 2001) as well as spoilage of a variety of meat products both under vacuum and modified atmosphere packaging (Ercolini et al., 2006, 2009) and it is known to be among the most psychrotrophic lactobacilli. It has also been found to be the dominant spoilage LAB during storage at chill temperatures (Ercolini et al., 2006; Chenoll et al., 2007).

PFGE has also provided important information in relation to the strain distribution of the LAB population which would have not been acquired if strain typing had not been performed. Within the LAB population of the present study, *Leuconostoc* spp. and *Lb. sakei* were identified as significant members of the microbiota at abuse and chill temperatures, respectively. More accurately, *Leuconostoc* spp. (B 233) that was initially present at high levels, dominated eventually the microbiota of the minced beef stored at abuse temperatures at all packaging conditions. Although, it was persistent throughout storage at chill temperatures, *Lb. sakei* strains dominated the LAB population only at the final stage of storage. However, some degree of microbial variability was detected at the final stage of storage of meat at chill temperatures, since different *Lb. sakei* strains were the most prevalent ones at the different packaging conditions. Indeed, *Lb. sakei* (B 226), (B 237) and (B 245) dominated the LAB population at 0 °C under aerobic conditions, at 0 and 5 °C under MAP (−) and at 5 °C under MAP (+). This finding is of great importance since it shows the intraspecies variability of *Lb. sakei* and the ability of certain strains to adapt to the different storage conditions outgrowing the other.

Dominance of *Leuconostoc* spp. at relatively higher temperatures can be partially attributed to the favourable environmental conditions and partially to the shorter generation time (Harris, 1998), both of which enabled it to outgrow *Lb. sakei* strains which were indeed detected as a secondary microbiota. On the other hand, dominance of *Lb. sakei* strains at chill temperatures can be attributed partly to its psychrotrophic nature.

From the different LAB detected throughout the storage under MAP (−) and MAP (+), a wide range of strains were sporadically present, especially at chill temperatures. This finding indicates that modified atmosphere packaging resulted in a development of a totally different spoilage ecosystem. It has been previously reported (Jay, 2000), that during storage of meat under MAP, the initial heterofermentative microbiota was substituted by a homofermentative one at the end of storage. Moreover, the MAP and the presumed activity of oregano essential oil against heterofermentative LAB species (Axelsson, 1998) seem to have provided the latter with an ecological advantage over *leuconostocs*.

The findings of the present study were based on the culture-dependent approach, most frequently applied when storage studies are performed. Selective media have been used for isolation and subsequent characterization of the microbiota; stressed or injured cells might not have managed to recover and grow, resulting in their non isolation from the plates and giving therefore the impression that they were absence from the system under investigation. A bias is therefore inserted which could have an effect on the description of the microbial community present. Except from the factors mentioned above, random selection of colonies is required to have a representative sample. This is not always possible because it depends on the person performing the task and it is therefore not objective.

A culture-independent approach could have been an alternative to the plates used for the characterization of the different microbiota (Cocolin et al., 2004a,b). Nevertheless, even this approach has drawbacks which lie in the fact that species have to be above the detection limit (10^4 cfu g^{-1}) and very frequently, the dominant species prevents evidence of the less abundant ones. Primer affinity to the target has also an effect on the amplification and therefore on the species identified. In order to clarify possible discrepancies between culture-dependent and independent methods, and to evaluate whether these differences would give a different overview of the ecology of the meat stored at the conditions mentioned, a similar study could be performed applying both approaches in the future. This investigation lies beyond the scope of the present study, which was actually focused on elucidating the effect of the different storage conditions with or without the presence of essential oil on the dynamics of LAB strains.

The present study did provide an insight of the population dynamics of LAB strains in relation to the temperature and the packaging conditions. It has been clearly demonstrated that certain species and/or strains are present or dominant only under certain conditions. This finding is extremely important since studies conducted so far had only taken into consideration the microbiological

Table 3
Frequency (%) of isolation and distribution of lactic acid bacteria strains isolates recovered from minced beef stored under aerobic, MAP (−) and MAP (+) conditions.

Closest species	Code	Initial	Air												MAP−												MAP+											
			0 °C			5 °C			10 °C			15 °C			0 °C			5 °C			10 °C			15 °C			0 °C			5 °C			10 °C			15 °C		
			initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end
Lb. sakei	B225							6.3												6.3																		
Lb. sakei	B226			9.1	40.0														50.0																		
Lb. sakei	B227			9.1																12.5																		
Lb. sakei	B228								9.1																														
Lb. sakei	B229				10.0																																	
Lb. sakei	B230							6.3																												11.1		
Leuconostoc spp.	B231					20.0																																
Leuconostoc spp.	B232	16.7		9.1	20.0			31.3																														
Leuconostoc spp.	B233	83.3	100.0	72.7	30.0	80.0	100.0	56.3	90.9	100.0	100.0	100.0	100.0	100.0	00.0	62.5		80.0	50.0	25.0	100.0	100.0	80.0	100.0	100.0	75.0	75.0	42.9	20.0	80.0	44.4	33.3	100.0	100.0	77.8	100.0	100.0	88.9
Ws. viridescens	B234																						10.0															
Ws, viridescens	B235																	10.0		12.5																		
Lb. sakei	B236														20.0																							
Lb. sakei	B237																100.0			25.0						12.5											
Lb. sakei	B238															25.0				12.5																		
Lb. sakei	B239														20.0	12.5																						
Leuconostoc spp.	B240																									12.5												
Leuconostoc spp.	B241																	10.0																				
Ln. mesenteroides	B242																			12.5									20.0									
Ln. mesenteroides	B243																						10.0															11.1
Leuconostoc spp.	B244																													11.1								
Lb. curvatus	B245																											14.3										
Lb. curvatus	B246																												20.0									
Lb. casei	B247																										25.0											
Lb. sakei	B248																											14.3		20.0	33.3							
Lb. sakei	B249																												20.0									
Lb. sakei	B250																											14.3										
Lb. sakei	B251																												20.0									
Lb. sakei	B252																																			11.1		
Lb. sakei	B253																											14.3										
Lb. sakei	B254																															66.7						
Lb. sakei	B255																													11.1								
Leuconostoc spp.	B258														20.0																							

counts as an indication of the spoilage process, and had ignored the possibility that different species or strains would prevail under different storage and/or packaging conditions. The qualitative information derived from the microbiological analyses and the characterization of the species or even the strains present were not evaluated previously. It has been shown that storage temperature combined with packaging conditions induced the selectivity of the spoilage LAB microbiota. Moreover, the microbiota recovered from the initial stage of storage was markedly different from that of the final stage of storage at chill temperatures. The above observations are of great importance and, to our opinion, fundamental in understanding the spoilage process and in explaining the presence of different products or by-products that occur during the different dynamic storage conditions (Skandamis and Nychas, 2002).

Acknowledgements

This work was partly supported by 2 EU Projects ProSafeBeef (Ref. Food-CT-2006-36241) and SYMBIOSIS-EU (No. 211638 Contract) within the 6th and 7th Framework Programme respectively. The information in this document reflects only the authors' views, and the European Community is not liable for any use that may be made of the information contained therein.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Argyri, A.A., Doulgeraki, A.I., Blana, V.A., Panagou, E.Z., Nychas G J.E., Potential of a simple HPLC based approach to quantify spoilage of minced beef stored at different temperatures and packaging systems. Revision FM-S-09–00571R1, submitted for publication.

Axelsson, L.T., 1998. Lactic acid bacteria: classification and physiology. In: Salminen, S., Von Wright, A. (Eds.), Lactic Acid Bacteria Microbiology and Functional Aspects. Marcel Dekker, New York, pp. 1–72.

Borch, E., Kant-Muemans, M.-L., Blixt, Y., 1996. Bacterial spoilage of meat and cured meat products. Int. J. Food Microbiol. 33, 103–120.

Bourget, N., Simonet, J.M., Decaris, B., 1993. Analysis of the genome of the five *Bifidobacterium breve* strains: plasmid content, pulsed-field gel electrophoresis genome size estimation and rrn loci number. FEMS Microbiol. Lett. 110, 11–20.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food Microbiol. 94, 223–253.

Champomier-Verges, M.-C., Chaillou, S., Cornet, M., Zagorec, M., 2001. *Lactobacillus sakei*: recent developments and future prospects. Res. Microbiol. 152, 839–848.

Chenoll, E., Macian, M.C., Aznar, R., 2003. Identification of *Carnobacterium*, *Lactobacillus*, *leuconostoc* and *Pediococcus* by rDNA-based techniques. System. Appl. Microbiol. 26, 546–556.

Chenoll, E., Macian, M.C., Elizaquivel, P., Aznar, R., 2007. Lactic acid bacteria associated with vacuum packed cooked meat product spoilage: population analysis by rDNA based methods. J. Appl. Microbiol. 102, 498–508.

Cocolin, L., Innocente, N., Biasutti, M., Comi, G., 2004a. The late blowing in cheese: a new molecular approach based on PCR and DGGE to study the microbial ecology of the alteration process. Int. J. Food Microbiol. 90, 83–91.

Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C., Comi, G., 2004b. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Appl. Environ. Microbiol. 70, 1883–1894.

Dainty, R.H., Mackey, B.M., 1992. The relationship between the phenotypic properties of bacteria from chill-stored meat and spoilage processes. J. Appl. Bacterial. Symp. Suppl. 73, 103–114.

Daniel, P., 1995. Sizing of the *Lactobacillus plantarum* genome and other lactic acid bacteria species by transverse alternating field electrophoresis. Curr. Microbiol. 30, 243–246.

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.

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.

Fontana, C., Cocconcelli, P.S., Vignolo, G., 2006. Direct molecular approach to monitoring bacterial colonization on vacuum-packaged beef. Appl. Environ. Microbiol. 72, 5618–5622.

Harris, L.J., 1998. The microbiology of vegetable fermentations. In: Wood, B.J.B. (Ed.), Microbiology of Fermented Foods. Blackie Academic Professional, London, pp. 45–72.

Hertel, C., Schmidt, G., Fischer, M., Oellers, K., Hammes, W.P., 1998. Oxygen-dependent regulation of the expression of the catalase gene *kat*A of *Lactobacillus sakei* LTH677. Appl. Environ. Microbiol. 64, 1359–1365.

Hielm, S., Bjorkroth, J., Hyytia, E., Korkeala, H., 1998. Genomic analysis of *Clostridium botulinum* group II by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 64, 703–708.

Holzapfel, W.H., 1998. The Gram-positive bacteria associated with meat and meat products. In: Board, R.G., Davies, A.R. (Eds.), The Microbiology of Meat and Poultry. Blackie Academic and Professional, London, U.K, pp. 35–84.

Hugas, M., Garriga, M., Aymerich, T., Monfort, J.M., 1993. Biochemical characterization of lactobacilli from dry fermented sausages. Int. J. Food Microbiol. 18, 107–113.

Jay, J.M., 2000. Modern Food Microbiology, sixth ed. An Aspen Publication, Gaithersburg, Maryland, pp 288.

Jones, R.J., 2004. Observations on the succession dynamics of lactic acid bacteria populations in chill-stored vacuum packaged beef. Int. J. Food Microbiol. 90, 273–282.

Kagkli, D.-M., Vancanneyt, M., Hill, C., Vandamme, P., Cogan, T.M., 2007. *Enterococcus* and *Lactobacillus* contamination of raw milk in a farm dairy environment. Int. J. Food Microbiol. 114, 243–251.

Knauf, H.J., Vogel, R.F., Hammes, W.P., 1992. Cloning, sequence, and phenotypic expression of *kat*A, which encodes the catalase of *Lactobacillus sakei* LTH677. Appl. Environ. Microbiol. 58, 832–839.

Labadie, J., 1999. Consequences of packaging on bacterial growth. Meat is an ecological niche. Meat Sci. 52, 299–305.

Leblond, P., Francou, F.X., Simonet, J.-M., Decaris, B., 1990. Pulsed-field gel electrophoresis analysis of the genome of *Streptomyces ambofaciens* strains. FEMS Microbiol. Lett. 72, 79–88.

McMullen, L., Stiles, M.E., 1993. Microbial ecology of fresh pork stored under modified atmosphere at − 1, 4.4 and 10 °C. Int. J. Food Microbiol. 18, l–14.

Nychas, G.-J.E., Skandamis, P., 2005. Fresh meat spoilage and modified atmosphere packaging (MAP). In: Sofos, J.N. (Ed.), Improving the Safety of Fresh Meat. CRC/Woodhead Publishing Limited, Cambridge, UK, pp. 461–502.

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

O'Sullivan, D.J., 1999. Methods for analysis of the intestinal microflora. In: Tannock, G.W. (Ed.), Probiotics. A Critical Review. Horizon Scientific Press, Norfolk, UK., pp. 23–44.

Ouattara, B., Simard, R.E., Holley, R.A., Piettte, G.J.P., Begin, A., 1997. Antimicrobial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int. J. Food Microbiol. 37, 155–162.

Paramithiotis, S., Kagkli, D.-M., Blana, V.A., Nychas, G.-J.E., Drosinos, E.H., 2008. Identification and characterization of *Enterococcus* spp. in Greek spontaneous sausage fermentation. J. Food Prot. 71, 1244–1247.

Parente, E., Grieco, S., Crudele, M.A., 2001. Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). J. Appl. Microbiol. 90, 943–952.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L., 2005. Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. Appl. Environ. Microbiol. 71, 1977–1986.

Roussel, Y., Colmin, C., Simonet, J.M., Decaris, B., 1993. Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). J. Appl. Bacteriol. 74, 549–556.

Rovira, A., Nieto, J.C., Rodriguez, A., Reguera, J.I., Gonzalez, Z., 1997. Characterization and selection of lactobacilli isolated from Spanish fermented sausages. Microbiol 13, 201–208.

Sanders, M.E., Walker, D.C., Walker, K.M., Aoyama, K., Klaenhammer, T.R., 1996. Performance of commercial cultures in fluid milk applications. J. Dairy Sci. 79, 943–955.

Shaw, B.G., Harding, C.D., 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J. Appl. Bacterial 56, 25–40.

Singh, S., Goswami, P., Singh, R., Heller, K.J., 2009. Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: a review. Food Sci. Techn 42, 448–457.

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.

Stanbridge, L.H., Davies, A.R., 1998. The microbiology of chill stored meat. In: Davies, A., Board, R. (Eds.), The Microbiology of Meat and Poultry. Blackie Aademic & Professional, London, UK, pp. 174–219.

Tanskanen, E.I., Tulloch, D.L., Hillier, A., Davidson, B.E., 1990. Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. Appl. Environ. Microbiol. 56, 3105–3111.

Vasilopoulos, C., De Maere, H., De Mey, E., Paelinck, H., De Vuyst, L., Leroy, F., 2010. Technology-induced selection towards the spoilage microbiota of artisan-type cooked ham packed under modified atmosphere. Food Microbiol. 27, 77–84.

Yeung, P.S.M., Kitts, C.L., Cano, R., Tong, P.S., Sanders, M.E., 2004. Application of genotyping and phenotyping analyses to commercial probiotic strain identity and relatedness. J.Appl. Microbiol. 97, 1095–1104.

Yost, C.K., Nattress, F.M., 2002. Molecular typing techniques to characterize the development of a lactic acid bacteria community on vacuum-packaged beef. Int. J. Food Microbiol. 72, 97–105.

Appendix II

The following manuscript has been published in *International Journal of Food Microbiology*

International Journal of Food Microbiology 145 (2011) 77–83

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Characterization of the *Enterobacteriaceae* community that developed during storage of minced beef under aerobic or modified atmosphere packaging conditions

Agapi I. Doulgeraki [a,c], Spiros Paramithiotis [b], George-John E. Nychas [a,*]

[a] Department of Food Science, Technology and Human Nutrition, Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[b] Department of Food Science, Technology and Human Nutrition, Laboratory of Food Quality Control and Hygiene, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[c] Applied Mycology Group, Cranfield Health, Cranfield University, Bedford MK43 0AL, UK

ARTICLE INFO

Article history:
Received 4 August 2010
Received in revised form 13 November 2010
Accepted 17 November 2010
Available online 29 November 2010

Keywords:
Enterobacteriaceae
Meat
PFGE
SDS–PAGE
DNA degradation

ABSTRACT

The whole cell protein and macrorestriction analysis of DNA of *Enterobacteriaceae* isolates recovered from minced beef stored at 0, 5, 10 and 15 °C aerobically and under modified atmosphere packaging consisting of 40% CO_2–30% O_2–30% N_2 in the presence (MAP+) and absence (MAP−) of oregano essential oil were studied. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) profiles obtained from whole cell protein analysis of the *Enterobacteriaceae* isolates revealed seven groups. Moreover, application of a modified PFGE protocol with *Xba*I restriction, resulted into 19 different fingerprints. The *Enterobacteriaceae* community of fresh meat consisted of *Serratia liquefaciens* and *Serratia proteamaculans*. *S. liquefaciens* strain VK23 was the dominant isolate of *Enterobacteriaceae* for the most conditions adopted, except 10 °C and 15 °C under MAP + and 10 °C under MAP−. In the latter cases, *Hafnia alvei* represented the dominant fingerprint. *Citrobacter freundii* was recovered from minced beef stored aerobically, while *H. alvei* and *Proteus vulgaris* were recovered under MAP. Storage conditions affected the *Enterobacteriaceae* community; modified atmosphere packaging increased both species and strain diversity.

1. Introduction

The microbial quality of meat depends on the physiological status of the animal at slaughter, the spread of contamination during slaughter and processing, the temperature and other conditions of storage and distribution. A wide spectrum of Gram-negative bacteria (*Pseudomonas* spp., *Acinetobacter* spp., *Serratia* spp., *Enterobacter* spp., *Proteus* spp. and *Vibrio* spp.) have been recovered from hides and work surfaces within abattoirs, from carcasses, butchered meat as well as from environmental samples in meat processing plants (von Holy et al., 1992; Gill, 2005; Nychas et al., 2008). Members of the family *Enterobacteriaceae* are successful colonizers of wet environments in the structural and work surfaces within abattoirs (Newton and Gill, 1978). This group is very common in fresh and frozen beef, pork and related meats (Jay, 2000), while cold tolerant *Enterobacteriaceae* also occur on chilled meat stored aerobically but in low numbers (Nychas et al., 1998, 2008). Although more attention is generally paid to the pathogenic properties of particular genera of *Enterobacteriaceae* (e.g. *Salmonella*), some members of the family constitute an important spoilage group when conditions favour their growth (Stanbridge and Davies, 1998; Nychas et al., 2008). *Hafnia alvei* and *Serratia liquefaciens* produce malodorous diamines (putrescine and cadaverine), while a green discoloration of the meat was associated with the growth of these two organisms (Stanbridge and Davies, 1998). The presence of these members in large numbers in meat is, therefore, of commercial importance.

Different treatments such as addition of preservatives, vacuum and modified atmosphere packaging affect the microbial association, such as Ephemeral Spoilage Organisms (Stanbridge and Davies, 1998; Nychas et al., 2008; Vasilopoulos et al., 2010). Product stored unpacked or packed in air permeable films tends to develop a spoilage biota dominated by *Pseudomonas* spp. (chill temperatures) or environmental *Enterobacteriaceae* (higher temperatures) (Stanbridge and Davies, 1998). In the case of meat stored in vacuum or modified atmospheres at abused temperatures *Enterobacteriaceae* may become a significant portion of the spoilage microbiota (Penney and Bell, 1993).

Differences in the microbial association, at species level, were also observed during storage of meat under different conditions. However, research only recently has taken into consideration the specific characteristics of microbiota and its contribution to the deterioration of the product. Changes in the spoilage related microbiota (Ercolini et al., 2006, 2010a), in specific microbiota such as lactic acid bacteria (Doulgeraki et al., 2010) and *Pseudomonas fragi* (Ercolini et al., 2010b) during storage of meat under different conditions have been

* Corresponding author. Tel.: +30 210 5294938; fax: +30 210 5294938.
E-mail address: gjn@aua.gr (G.-J.E. Nychas).

0168-1605/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2010.11.030

monitored. Moreover, the microbial ecology of fresh sausages (Cocolin et al., 2004), Italian fermented sausages (Rantsiou et al., 2005) and artisan-type cooked ham packed under modified atmosphere (Vasilopoulos et al., 2010) has been also studied. Regarding, the diversity of *Enterobacteriaceae* community the data available indicated that, *S. liquefaciens* was the most common member of the *Enterobacteriaceae* family on meat stored in atmospheres of different composition, while *H. alvei* was dominant in vacuum packed pork and beef steaks stored in modified atmospheres (Stanbridge and Davies, 1998). Although the effect of natural preservatives such as oregano essential oil on the microbial population has already been exhibited (Skandamis and Nychas, 2002), limited information is currently available regarding the effect of antimicrobial compounds on the microbial diversity of the *Enterobacteriaceae* on meat at the species and strain level.

Thus the aim of the present study was to determine the diversity of *Enterobacteriaceae* that were isolated from minced beef stored under different packaging and temperature conditions. The comparison of whole-cell protein patterns obtained by SDS–PAGE as well as macrorestriction analysis of DNA by a PFGE modified protocol have been used for classification at species and strain level.

2. Materials and methods

2.1. Bacterial cultures and growth

Two hundred and thirty two *Enterobacteriaceae* were isolated from minced beef during storage according to Doulgeraki et al. (2010). In brief, isolates were recovered from minced beef stored at 0, 5, 10 and 15 °C aerobically and under modified atmosphere packaging consisting of 40% CO_2–30% O_2–30% N_2 in the presence (MAP+) and absence (MAP−) of oregano essential oil. Minced beef was sampled at appropriate time intervals, depending on storage temperature; the incubation lasted 650, 482, 386 and 220 hours at 0, 5, 10 and 15 °C, respectively. Colonies (approximately 10) were selected randomly (Harrigan, 1998) from the highest dilution of Violet Red Bile Glucose agar (VRBG, Biolife, Italiana S.r.l., Milano, Italy) from different time points (fresh meat, middle and final stage of storage). Pure cultures included in this study (Table 1), were stored at −80 °C in Brain Heart Infusion Broth (BHI, Merck, Darmstadt, Germany) supplemented with 20% (v/v) glycerol (Serva, Heidelberg, Germany). Before experimental use each isolate was subcultured twice in BHI at 37 °C for 16 h and 6 h respectively.

2.2. Whole cell protein profiling

The whole cell proteins were analysed by SDS–PAGE in 12% polyacrylamide gel according to Paramithiotis et al. (2000). Briefly, cells were collected and washed with sodium phosphate buffer (per liter bidistilled water: 40.5 mL 0.2 M $Na_2HPO_4.12H_2O$, 9.5 mL 0.2 M $NaH_2PO_4.H_2O$, 8 g NaCl, pH 7.3). Cell extracts were prepared by sonicating (3 min, 50 W) 5 mL of bacterial culture resuspended in 800 μL sample treatment buffer (62.5 mM Tris–HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulphate, 5% β-mercaptoethanol, 0.025% bromophenol blue). The lysate was heated at 95 ° C for 10 min and centrifuged for 10 min at 14,000 rpm. The supernatant (protein extract) was stored at −20 °C until SDS PAGE analysis. Protein bands were visualized by using brilliant blue R-250 staining before being photographed using a Model GS-800 Calibrated Imaging Densitometer (Biorad Hercules, CA, USA). All chemicals were of high purity grade and obtained from Sigma-Aldrich (Sigma, Chemical Co., St. Louis, Mo. USA). Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Gel compare software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium.

Table 1
Distribution of *Enterobacteriaceae* isolates according to their whole-cell protein profiling and the specific storage conditions of minced beef.

Source	Temperature (°C)	SDS–PAGE profile							Total isolates
		A	B	C	D	E	F	G	
Fresh meat		6[a]	6						12
Meat stored aerobically	0	6 (3,3)[b]			4 (2,2)				70
	5	16 (8,8)			4 (2,2)				
	10	18 (8,10)				2 (2,–[c])			
	15	14 (4,10)		2 (2,–)	4 (4,–)				
Meat stored under MAP−[d]	0	14 (10,4)					2 (–,2)	4 (–,4)	74
	5	12 (2,10)						4 (4,–)	
	10	10 (10,–)			2 (–,2)		8 (–,8)		
	15	14 (6,8)		2 (2,–)			2 (–,2)		
Meat stored under MAP+[e]	0	16 (8,8)	2 (–,2)		2 (2,–)			2 (2,–)	76
	5	18 (10,8)			2 (–,2)				
	10						20 (10,10)		
	15		2 (2,–)				12 (2,10)		
Total isolates		144	10	4	18	2	44	10	232

[a] Number of isolates.
[b] Number of isolates from different time points (middle, final).
[c] None isolated.
[d] Modified atmosphere packaging (40% CO_2/30% O_2/30% N_2).
[e] Volatile compounds of 2% v/w oregano essential oil.

2.3. PFGE

PFGE was performed by a modification of the method proposed by Herschleb et al. (2007). Briefly, cells were harvested by centrifugation at 10,000×*g* for 5 min and washed with 10 mM Tris–HCl (pH 7.6) containing 1 M NaCl; resuspended in 100 μL of the same solution, heated at 37 °C for 10 min and mixed with an equal volume of 2% (w/v) low melting-point agarose (Biorad) in 0.125 M EDTA pH 7.6 (Applichem, GmbH, Darmstadt, Germany) before leaving them to solidify in moulds (Biorad). The cells were lysed *in situ* in a solution containing 10 mg mL^{-1} of lysozyme (Applichem) in EC buffer (6 mM Tris–HCl, 1 M NaCl, 100 mM EDTA, 1% (w/v) Sarkosyl, pH 7.6) for 16 h at 37 °C. A treatment with proteinase K (Sigma) (0.5M EDTA containing 1% Sarkosyl, pH 8) for 24 h at 55 °C followed the lytic treatment. After the proteinase K treatment, the plugs were incubated for 1 h at room temperature in TE solution, containing 50 μM thiourea (Applichem), with gentle agitation. They were subsequently washed with 500 μL of TE solution with gentle agitation for 30 min. This step was repeated 3 times. The restriction enzyme *Xba*I (10U) (New England Biolabs, Ipswich, MA, USA) was applied according to the manufacturer's recommendation for 16 h. Restriction fragments were separated in 1% PFGE grade agarose gel (Biorad) in 0.5 mM Tris–Borate buffer containing 100 μM thiourea on CHEF-DRII equipment (Biorad) with the following running parameters: 6 V/cm, 2.2 s initial switching time, 54.2 s final switching time and a 20 h of total run at 14 ° (Ferris et al., 2004). Gels were then stained with ethidium bromide (0.5 μg/mL) (Sigma) in water for 1 h and destained for 2 h before being photographed using a GelDoc system (Biorad). Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Gel compare software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium.

2.4. DNA extraction and species identification

DNA was extracted with a modification of the enzymatic method according to Ercolini et al. (2001). One milliliter of overnight culture was centrifuged at 14,000 rpm for 5 min at 4 °C. The pellet was resuspended in 0.5 mL buffer solution (1 M sorbitol, 0.1 M EDTA, pH 7.5) containing 25 mg/mL lysozyme, incubated for 2 h at 37 °C and centrifuged at 14,000 rpm for 10 min at 4 °C. After centrifugation, the pellet was resuspended in 0.5 mL of buffer (50 mM Tris–HCl, 20 mM EDTA, pH 7.4) and incubated for 30 min at 65 °C after the addition of 50 μL 10% SDS solution. Then, the sample was mixed with 0.2 mL potassium acetate (5M) (Merck), placed on ice for 30 min and centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was precipitated with 1 mL ice-cold isopropanol (Applichem) and centrifuged 14,000 rpm for 10 min at 4 °C. Finally the pellet was dried and resuspended in 50 μL sterile ddH_20.

A representative number of isolates per distinct PFGE cluster were selected and subjected to species identification by sequencing the V1–V3 variable region of the 16S rRNA gene as described previously (Doulgeraki et al., 2010). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (http://www.macrogen.com). The results were aligned with those in GenBank using the BLASTN programme in order to determine their closest known relatives of the partial 16S rRNA gene sequence (Altschul et al., 1997). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are exhibited in Table 2.

3. Results

A total of 232 *Enterobacteriaceae* isolates recovered from different storage times and conditions according to Doulgeraki et al. (2010) were subjected to SDS–PAGE of whole-cell proteins and PFGE in order to determine the species and strain diversity, respectively.

Table 2
Species identification after sequencing of the variable V1–V3 region of the 16S rRNA genes.

Strain	Closest relative	GenBank accession number of closest relative	Identity (%)	GenBank accession number of sequence
VK5	*S. proteamaculans*	AJ508694	99	HM242268
VK6	*S. proteamaculans*	AJ508694	100	HM242269
VK17	*S. liquefaciens*	FJ811866	99	HM242270
VK19	*Citrobacter freundii*	AB548826	100	HM242271
VK20	*Hafnia alvei*	AJ508360	100	HM242272
VK23	*S. liquefaciens*	EU880537	100	HM242273
VK25	*S. proteamaculans*	EU627690	100	HM242274
VK27	*H. alvei*	AJ508360	99	HM242275
VK32	*S. proteamaculans*	EU627690	99	HM242276
VK40	*S. liquefaciens*	EU880537	99	HM242277
VK53	*H. alvei*	AJ508360	100	HM242278
VK60	*H. alvei*	AB244473	99	HM242279
VK74	*S. liquefaciens*	AJ306725	100	HM242280
VK75	*S. liquefaciens*	AJ306725	99	HM242281
VK90	*Serratia* spp.	AJ545753	99	HM242282
VK101	*Proteus vulgaris*	AY870320	99	HM242283
VK103	*P. vulgaris*	GQ292550	99	HM242284
VK108	*Serratia* spp.	EF491959	99	HM242285
VK113	*S. proteamaculans*	AJ508694	100	HM242286

3.1. Whole cell protein profiling

Enterobacteriaceae isolates were clustered into seven groups on the basis of their SDS–PAGE profile obtained from whole-cell proteins. The protein profile of each group is shown in Fig. 1 whereas the number of the isolates as well as the storage condition and time points of isolation is presented in Table 1. Profile A was common for all packaging and temperature conditions, except for 10 °C and 15 °C under MAP +.

In fresh meat, two different profiles (A and B) were detected, which were equally recovered. Moreover, profile A was the most common during aerobic storage, representing the dominant biota of *Enterobacteriaceae* community in all time points adopted, except for the middle stage of storage at 15 °C, where profiles C and D were also recovered. Furthermore, profiles D and E were detected at 0, 5 and 10 °C respectively.

Five profiles were detected during storage of minced beef under MAP−, with profile A dominating the *Enterobacteriaceae* community at the middle stage of storage at 0, 10 and 15 °C. The same group dominated the aforementioned population at the final stage of storage at 5 and 15 °C. On the other hand, profile G and F dominated the *Enterobacteriaceae* community at the middle stage of storage at 5 °C and the final stage of storage at 10 °C, respectively. At the final stage of storage at 0 °C, profiles A and G were equally contributed, while profile F was also detected. Moreover, profiles C and D were obtained from 15 and 10 °C.

In the middle and final stages of storage of minced beef stored under MAP +, profile A dominated the *Enterobacteriaceae* community at 0 and 5 °C, while profiles B, D and G were also recovered. On the other hand, profile F dominated the aforementioned community at 10 and 15 °C, except for middle stage of storage at 15 °C where profile B was equally represented.

3.2. Genotypic analysis

In the present study, macrorestriction analysis by PFGE was used for strain differentiation of *Enterobacteriaceae* isolates. However, most of the *Enterobacteriaceae* isolates (groups A, B, D, E, and G based on SDS–PAGE analysis) could not be analysed by PFGE when isolation of the intact chromosomal DNA was performed according to Herschleb

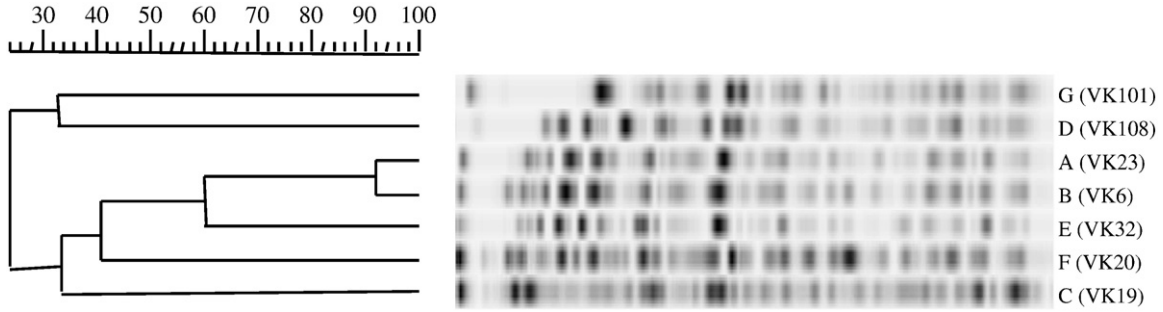


Fig. 1. Cluster analysis of SDS–PAGE whole-cell protein profiles of representative strains. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%) and clustering was performed by UPGMA analysis. The letters (A to G) indicate the respective profile; in the parenthesis the specific strains are given.

et al. (2007) as a continuous smear of DNA rather than well separated fragments was produced. Similar results were observed when sodium dodecyl sulphate was added in the solution used to make the agarose plugs (Hunter et al., 2005) and when the incubation time for proteinase K treatment was increased from 24 to 48 h (Herschleb et al., 2007) (data not shown). Neither thiourea addition into running buffer (Romling and Tummler, 2000; Silbert et al., 2003; Lee et al., 2006; Liesegang and Tschape, 2002) nor the use of HEPES buffer as a running buffer (Koort et al., 2002; Ray et al., 1992) were able to prevent DNA degradation (data not shown). On the other hand, all isolates yielded well-separated DNA fragments with a modification of protocol i.e. the addition of 50 μM thiourea after the proteinase K treatment described in this study. Even better results were observed when the addition of thiourea (50 μM) after the proteinase treatment was combined with the addition of thiourea (100 μM) to the running buffer. Then, macrorestriction profiles with no background smearing were produced and at the same time, the pattern of the control strain, which was typeable without thiourea addition and was clustered to profile C (based on SDS–PAGE analysis), was not affected. Therefore, the modified protocol was chosen to analyse the 232 isolates.

The dendrogram obtained after image analysis of the different PFGE patterns from *Xba*I restriction, resulted in 19 different profiles (Fig. 2). Each fingerprint present in Fig. 2 was subjected to 16S rRNA gene sequencing. Five fingerprints (VK17, VK23, VK40, VK74 and VK75), which belonged to SDS–PAGE profile A, were assigned to *S. liquefaciens* (Table 2). SDS–PAGE profile B was represented by 4 different PFGE fingerprints, namely VK6, VK25, VK113 and VK5; all of them being assigned to *Serratia proteamaculans*. Similarly, fingerprints VK90 and VK108, members of SDS–PAGE profiles group D, were assigned to *Serratia* spp. Fingerprints VK19 and VK32, belonged to SDS–PAGE profiles C and E and were attributed to *Citrobacter freundii* and *S. proteamaculans*, respectively. The four fingerprints, namely VK20, VK27, VK53 and VK60 (SDS–PAGE profile F) were identified as *H. alvei*, while the two fingerprints, namely VK101 and VK103 (SDS–PAGE profile G) were assigned to *Proteus vulgaris*.

In Table 3 the prevalence of the different *Enterobacteriaceae* PFGE fingerprints related to the different storage conditions is summarized. *S. proteamaculans* (VK5, VK6) and *S. liquefaciens* (VK17, VK23) constituted the *Enterobacteriaceae* community of fresh meat. Regarding the five different PFGE fingerprints assigned to *S. liquefaciens*, VK17 and VK23 were the most common, whereas the rest (VK40, VK74 and VK75) were only sporadically recovered. *S. liquefaciens* VK17 was the dominant isolate in the middle stage of storage at 5 and 10 °C under aerobic conditions, at 15 °C under MAP− and in the final stage of storage at 0 °C under aerobic conditions. On the other hand, *S. liquefaciens* VK23 was the dominant isolate for the rest of the storage conditions, except for 10 and 15 °C under MAP+. *S. proteamaculans* VK113 and VK25 were recovered during storage

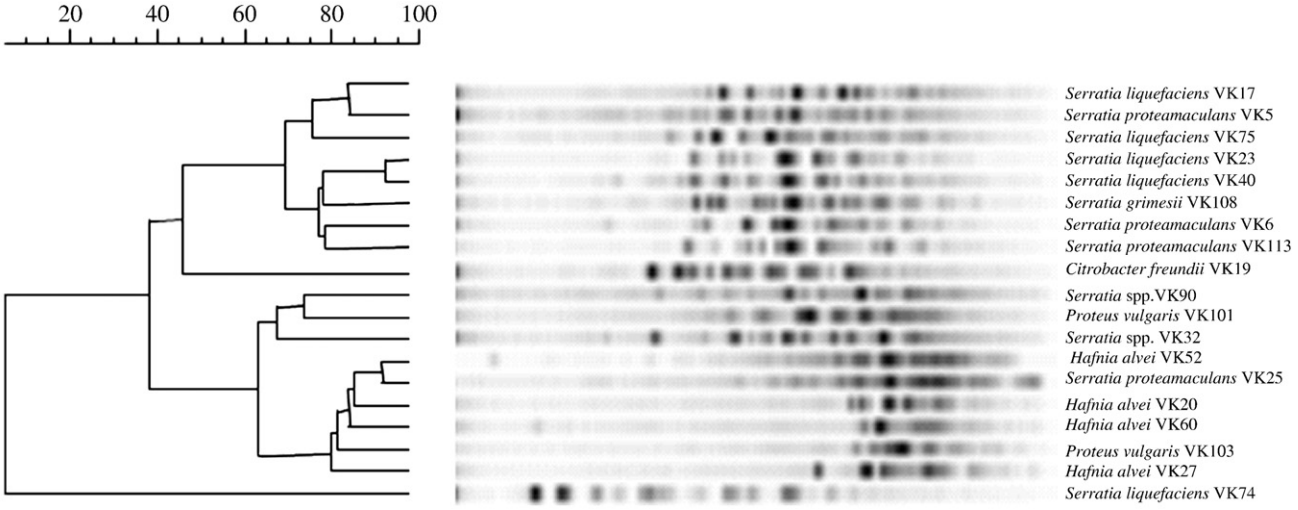


Fig. 2. Cluster analysis of PFGE *Xba*I digestion fragments of the *Enterobacteriaceae* isolates calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Table 3
Distribution of *Enterobacteriaceae* isolates according to their whole-cell protein profiling, PFGE fingerprinting and the specific storage conditions of minced beef.

Closest species	Source PFGE fingerprint (SDS-PAGE profile)	Fresh meat	Meat stored aerobically				Meat stored under MAP−[a]				Meat stored under MAP +[b]			
			0[c]	5	10	15	0	5	10	15	0	5	10	15
Serratia liquefaciens	VK17 (A)	33	60/60[d]	80/40	80/20		40/		20/	75/	/20	40/		
	VK23 (A)	17		/40	/60	40/100	60/40	33/60	80/	/23	67/60	60/80		
	VK40 (A)				/20									
	VK74 (A)							/20						
	VK75 (A)							/20						
S. proteamaculans	VK5 (B)	17												
	VK6 (B)	33												
	VK25 (B)													50/
	VK113 (B)										/20			
Citrobacter freundii	VK19 (C)					20/				25/				
Serratia spp.	VK90 (D)		20/20											
	VK108 (D)		20/20	20/20		40/			/20		17/	/20		
S. proteamaculans	VK32 (E)				20/									
Hafnia alvei	VK20 (F)								/60	/20			40/20	50/80
	VK27 (F)												20/40	50/20
	VK53 (F)												20/	
	VK60 (F)						/20		/20				20/40	
Proteus vulgaris	VK101 (G)						/20	33/						
	VK103 (G)						/20	33/			17/			

[a] Modified atmosphere packaging (40% CO_2/30% O_2/30% N_2).
[b] Volatile compounds of 2% v/w oregano essential oil.
[c] Storage temperature.
[d] Percentage (%) of isolates from different time points (middle/final stage of storage).

under MAP + at 0 and 15 °C respectively, while VK32 was recovered in the middle stage of storage under aerobic conditions at 10 °C. PFGE fingerprint VK20 was the most common among the 42 *H. alvei* isolates and represented the dominant fingerprint under MAP− at 10 °C (final stage of storage), under MAP+ at 10 °C (middle stage of storage) and at 15 °C (middle and final stage of storage). Additionally, *P. vulgaris* VK101 and VK103 were both isolated during storage under MAP− at 0 and 5 °C, with VK101 also isolated during storage under MAP + at 0 °C.

4. Discussion

The diversity of the microbial population has been reported in numerous studies dealing with meat stored under various conditions (Ercolini et al., 2006, 2010a; Stanbridge and Davies, 1998). This was evident with *Enterobacteriaceae* where different genera and species are reported in different studies. This wide range of findings can be attributed to the different material for the enumeration of this group, to different methods or to different preservation conditions such as packaging and storage temperature used in these studies. The different methods that have been applied to monitor the microbiota of meat and meat products, under different storage conditions, were based on culture dependent and independent approaches. In the case of culture dependent approaches, a bias is inserted due to the limitations of the methods that could have an effect on the description of the microbial community present (Doulgeraki et al., 2010). Nowadays, culture independent approaches such as DGGE (Cocolin et al., 2004; Ercolini et al., 2001, 2006, 2010a) and pyrosequencing (Ronaghi, 2001; Roesch et al., 2007) were found to be efficient for monitoring and profiling microbial populations. These new technologies are very promising and might one day be widely applied in this field (King et al., 2008). Nevertheless, all the above mentioned approaches have certain drawbacks that could be overcome by applying both approaches (Doulgeraki et al., 2010). This investigation lies beyond the scope of the present study which was actually focused on determining the diversity of *Enterobacteriaceae* that were isolated from minced beef stored under different packaging and temperature conditions. To our knowledge this is the first report of the diversity of the *Enterobacteriaceae* community present during storage of minced beef under different temperatures (0, 5, 10 and 15 °C) and packaging conditions (air, MAP− and MAP+) with a systematic approach.

In the present study, the development of the *Enterobacteriaceae* community during storage of minced beef under aerobic and modified atmosphere packaging conditions was assessed by a culture-dependent approach using SDS–PAGE analysis, PFGE fingerprinting and sequencing of the V1–V3 variable region of the 16S rRNA gene.

Comparison of whole-cell protein patterns obtained by highly standardised SDS–PAGE has been successfully applied for microbial identification at species or subspecies level, even of closely related species (Pot et al., 1994). The high taxonomic resolution of this technique, regarding inter- and intra-species divergence, that is often the case in the *Enterobacteriaceae* family, has already been exhibited (Hantula et al., 1990; Holmes et al., 1991; Coenye et al., 2001). In the present study, an effective differentiation between *C. freundii*, *H. alvei* and *P. vulgaris* has been achieved. Regarding the differentiation between *S. proteamaculans* and *S. liquefaciens* and having in mind the scientific debate over their distinction (Grimont et al., 1978), SDS–PAGE of whole-cell proteins proved adequate to supply clear discrimination. Moreover, S. *proteamaculans* was further subdivided into 2 sub-clusters, namely B and E demonstrating the remarkable intra-species differentiation capacity of this technique. As far as differentiation of profile D was concerned, further study is required for accurate classification and determination of a possible intra- or inter-species variability.

On the other hand, Pulsed-field gel electrophoresis is considered the “gold standard” for strain differentiation since it is very precise, reproducible, and reliable. Current PFGE protocols for typing of Gram-positive or-negative microorganisms require embedding intact cells in agarose, cell lysis, restriction digestion of DNA and gel electrophoresis. However, when the protocols for inter- and intra-species differentiation of *Salmonella* Enterica (Liesegang and Tschape, 2002), *Escherichia coli* (Izumiya et al., 1997), *Pseudomonas aeruginosa* (Romling and Tummler, 2000), and *Leptospira* spp. (Ribeiro et al., 2009) were applied, the majority of the *Enterobacteriaceae* isolates under study exhibited smeared bands or a smear of high molecular weight DNA entering the gel matrix. Similar results were observed when thiourea was added into Tris-based running buffer (Izumiya et al., 1997; Romling and Tummler, 2000; Liesegang and Tschape,

2002; Silbert et al., 2003; Alonso et al., 2005) or when Tris buffer was replaced by HEPES (Ray et al., 1992; Koort et al., 2002). Moreover, it has been reported that proper control must be performed, and all components of the digestion mixture (including the slices of plugs), must be checked for the presence of endogenous nuclease activity (Herschleb et al., 2007). Thus, a number of protocols have been developed in order to prevent this DNA degradation. More particularly, culture time should be controlled as some microorganisms display delayed nuclease production (Porschen and Sonntag, 1974), sodium dodecyl sulphate should be added to the solution used to make the agarose plugs (Hunter et al., 2005) or the incubation time of proteinase K treatment should be increased (Herschleb et al., 2007). However, none of these methods were successful in obtaining a fine PFGE pattern for the isolates in the present study due to DNA degradation. In contrast, a complete PFGE pattern was achieved when the addition of thiourea took place after proteinase K treatment. These findings suggest that a putative nuclease might be present in the agarose blocks and might be responsible for the observed DNA degradation. After this modification it was possible to differentiate the isolates that were obtained by PFGE fingerprinting after cleavage with the restriction enzyme *Xba*I.

In the present study, the *Enterobacteriaceae* community of fresh minced beef consisted of 2 *S. liquefaciens* and 2 *S. proteamaculans* strains but during storage *S. liquefaciens* prevailed over *S. proteamaculans*. The *Enterobacteriaceae* community of minced beef stored under aerobic conditions was dominated by those *S. liquefaciens* strains that were initially detected in fresh minced beef with an occasional presence of *Serratia* spp. and *C. freundii* strains. This dominance can be attributed partly to the relatively favourable growth conditions. *S. liquefaciens* represented the dominant isolate of *Enterobacteriaceae* for most conditions adopted, except 10 and 15 °C under MAP+ and 10 °C under MAP− (final stage of storage). In the latter cases, *H. alvei* represented the dominant fingerprint. This suggests that different strains of *Enterobacteriaceae* occur at different temperatures, possibly because of temperature-induced differences in adaptation and competitiveness, inherent in the total population of these species. *S. liquefaciens* has been found to be the most common member of this family on meat stored in atmospheres of different conditions (Stanbridge and Davies, 1998). *H. alvei* has been found to be one of the major spoilage enterobacteria found in meat, in particular due to its ability to grow at low temperatures which gives an adaptation advantage over other microbial members (Borch et al., 1996). *H. alvei* was also the dominant member of *Enterobacteriaceae* on beef steaks stored in modified atmospheres at 5 °C (Stanbridge and Davies, 1998). Stanbridge and Davies (1998) also show that *H. alvei* did not compete well in a high oxygen atmosphere, while it was inhibited by modified atmosphere more at 0 than at 5 °C.

Packaging under modified atmosphere led to the development of a different *Enterobacteriaceae* consortium since strains of *P. vulgaris* were detected at 0 and 5 °C and strains of *H. alvei* were recovered at 10 and 15 °C. Regarding growth preferences of *P. vulgaris*, only scarce literature is currently available (Lucia et al., 1993). As far as *H. alvei* is concerned, it is very frequently encountered in minced beef stored under modified atmospheres or in vacuum packages (Borch et al., 1996; Nychas et al., 1998; Drosinos and Board, 1995). Thus, storage under MAP without the addition of oregano essential oil was characterised by dominance of *S. liquefaciens* at 5 and 15 °C, a co-existence with *P. vulgaris* at 0 °C and dominance by *H. alvei* at 10 °C. The increased diversity can be attributed to the favourable packaging conditions for *Enterobacteriaceae* growth, due to their facultatively anaerobic character. When oregano essential oil was applied, *S. liquefaciens* and *P. vulgaris* strains seemed to be negatively affected whereas *H. alvei* strain diversity increased. Thus, *S. liquefaciens* strains dominated at 0 and 5 °C whereas *Enterobacteriaceae* microbiota consisted almost exclusively by *H. alvei* strains at 10 and 15 °C. These results suggest a temperature-dependent effect on the specific species. The negative effect of the essential oil on *S. liquefaciens* and *P. vulgaris* diversity and the advance in *H. alvei* diversity could not be explained as limited studies are available for antibacterial activity of essential oils against meat spoilage microorganisms. The only available information showed that *S. liquefaciens* overcomes the inhibitory effect of essential oils after 24 h of exposure (Outtara et al., 1997).

Among the species recovered throughout the storage of beef, several meat associated ones were identified. It has been reported that many members of the *Enterobacteriaceae*, belonging to the genera *Serratia, Enterobacter, Pantoea, Proteus* and *Hafnia*, often contribute to meat spoilage (Borch et al., 1996; Nychas et al., 1998), while high correlations between cadaverine and *Enterobacteriaceae* counts have been observed (Dainty and Mackey, 1992). *S. liquefaciens*, *H. alvei*, *Rahnella aquatilis* and *C. freundii* were frequently encountered in minced beef and some of them were found to harbour toxin-encoding genes and other putative virulence factors (Lindberg et al., 1998). Moreover, different members of *Enterobacteriaceae* have been recovered from beef, while *Rahnella* spp. has been shown to play an important role in the spoilage of meat and has been shown to be the dominant bacterium in the late phases of refrigerated storage (Ercolini et al., 2006). On the other hand, *S. grimesii* was been shown to be the dominant Gram negative species at the later stage of storage of meat with and without the use of active packaging (Ercolini et al., 2010a). *Serratia* and *Proteus* were the genera most commonly present on working surfaces in the meat processing industry (Stiles and Ng, 1981). *S. liquefaciens* has been also found by many investigators to be the most common member of this family on meat taken from abattoirs (Stanbridge and Davies, 1998). Stiles and Ng (1981) reported that *Enterobacter agglomerans* and *S. liquefaciens* were predominant *Enterobacteriaceae* at the retail level, but they had limited indicator potential for sanitation and hygiene. It has been reported that the psychrotrophic nature and simple nutritional requirements of these genera enable them to persist and/or multiply in/on water, condensate, soil, equipment surfaces, brine solutions and moist floors (von Holy et al., 1992). However, in meats, phychrotrophic *Enterobacteriaceae* can multiply during refrigerated storage so their levels can therefore increase so their hygiene significance must be interpreted accordingly (Baylis, 2006).

In this study, two different methods (SDS–PAGE and PFGE) have been applied to provide insight into the *Enterobacteriaceae* community of meat in relation to the temperature, packaging conditions and the presence or absence of oregano essential oil. A modification of the current PFGE protocols has been developed that enable typeability within the assayed isolates while maintaining both a high degree of discrimination and reproducibility of the technique. Both techniques were found to be able to ascribe the succession of the community studied at species and strain level. It was found that storage conditions induced the selectivity of the *Enterobacteriaceae* community, while modified atmosphere packaging increased both species and strain diversity. The overall outcome of the present study was that certain species and/or strains are present or dominate only under certain conditions. These observations are of great importance and are fundamental in widening the knowledge of spoilage related bacterial succession and consequently understanding the meat spoilage process.

Acknowledgments

The present study was funded by the European Commission under the 6th Framework Programme for RTD (contract no. FOOD-CT-2006-016264) and partly the SYMBIOSIS-EU Project No. 211638 of the 7th FP. The information in this document reflects only the authors' views, and the European Community is not liable for any use that may be made of the information contained therein.

References

Alonso, R., Martin, A., Pelaez, T., Marin, M., Rodriguez–Creixems, M., Bouza, E., 2005. An improved protocol for pulsed-field gel electrophoresis typing of *Clostridium difficile*. Journal of Medical Microbiology 54, 155–157.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Baylis, C.L., 2006. Enterobacteriaceae. In: Blackburn, C.W. (Ed.), Food Spoilage Microorganisms. Woodhead Publishing In Food Science, Technology And Nutrition, New York, pp. 624–667.

Borch, E., Kant-Muemans, M.-L., Blixt, Y., 1996. Bacterial spoilage of meat and cured meat products. International Journal of Food Microbiology 33, 103–120.

Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C., Comi, G., 2004. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Applied and Environmental Microbiology 70, 1883–1894.

Coenye, T., Vandamme, P., Govan, J.R.W., LiPuma, J.J., 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. Journal of Clinical Microbiology 39, 3427–3436.

Dainty, R.H., MacKey, B.M., 1992. The relationship between the phenotypic properties of bacteria from chill-stored meat and spoilage processes. In: Board, R.G., Jones, D., Kroll, R.G., Pettipher, G.L. (Eds.), Ecosystems: Microbes: Food: Society for Applied Bacteriology Symposium Series, vol. 73, pp. 103–114.

Doulgeraki, A.I., Paramithiotis, S., 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 Microbiology 27, 1028–1034.

Drosinos, E.H., Board, R.G., 1995. Microbial and physicochemical attributes of minced lamb: Sources of contamination with pseudomonads. Food Microbiology 12, 189–197.

Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: Bias of culture-dependent and culture-independent analyses. Systematic and Applied Microbiology 24, 610–617.

Ercolini, D., Russo, F., Torrieri, E., Masi, P., Villani, F., 2006. Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. Applied and Environmental Microbiology 72, 4663–4671.

Ercolini, D., Ferrocino, I., La Storia, A., Mauriello, G., Gigli, S., Masi, P., Villani, F., 2010a. Development of spoilage microbiota in beef stored in nisin activated packaging. Food Microbiology 27, 137–143.

Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Ferranti, P., Mauriello, G., Villani, F., 2010b. Different molecular types of *Pseudomonas fragi* have the overall behaviour as meat spoilers. International Journal of Food Microbiology 142, 120–131.

Ferris, M.M., Yan, X., Habbersett, R.C., Shou, Y., Lemanski, C.L., Jett, J.H., Yoshida, T.M., Marrone, B.L., 2004. Performance assessment of DNA fragment sizing by high sensitivity flow cytometry and pulsed field gel electrophoresis. Journal of Clinical Microbiology 42, 1965–1976.

Gill, C.O., 2005. Sources of bacterial contamination at slaughtering plants. In: Sofos, J.N. (Ed.), Improving the Safety of Fresh Meat. CRC/Woodhead Publishing Limited, Cambridge, pp. 231–243.

Grimont, P.A.D., Grimont, F., Starr, M.P., 1978. *Serratia proteamaculans* (Paine and Stansfield) comb. nov., a senior heterotypic synonym of *Serratia liquefaciens* (Grimes and Hennerty) Bascomb et al. International Journal of Systematic Bacteriology 28, 503–510.

Hantula, J., Korhonen, T.K., Bamford, D.H., 1990. Determination of taxonomic resolution capacity of conventional one-dimensional SDS–polyacrylamide gel electrophoresis of whole-cell proteins using *Enterobacteriaceae*. FEMS Microbiology Letters 70, 325–330.

Harrigan, W.F., 1998. Laboratory Methods in Food Microbiology. Elsevier Science and Technology, California, pp. 89–91.

Herschleb, J., Anaviev, G., Schwartz, D., 2007. Pulsed-field gel electrophoresis. Nature Protocols 2, 677–684.

Holmes, B., Costas, M., Wood, A.C., 1991. Typing of *Proteus mirabilis* from clinical sources by computerized analysis of electrophoretic protein patterns. The Journal of Applied Bacteriology 71, 46–76.

Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves, L., Wrigley, D., Barrett, T., Ribot, E., 2005. Establishment of a universal size standard strain for use with the PulseNet standardized Pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. Journal of Clinical Microbiology 43, 1045–1050.

Izumiya, H., Terajima, J., Wada, A., Inagaki, Y., Itoh, K.I., Tamura, K., Watanabe, H., 1997. Molecular typing of enterohemorrhagic *Escherichia coli* O157:H7 isolates in Japan by using pulsed-field gel electrophoresis. Journal of Clinical Microbiology 35, 1675–1680.

Jay, J.M., 2000. Modern Food Microbiology, Sixth edition. An Aspen Publication, Gaithersburg, Maryland, p. 288.

King, R.A., Read, D.S., Traugott, M., Symondson, W.O.C., 2008. Molecular analysis of predation: a review of best practice for DNA-based approaches. Molecular Ecology 17, 947–963.

Koort, J.M., Lukinmaa, S., Rantala, M., Unkila, E., Siitonen, A., 2002. Technical improvement to prevent DNA degradation of enteric pathogens in pulsed-field gel electrophoresis. Journal of Clinical Microbiology 40, 3497–3498.

Lee, Y.-A., Chen, K.-P., Hsu, Y.-W., 2006. Characterization of *Erwinia chrysanthemi*, the soft-rot pathogen of white-flowered calla lily, based on pathogenicity and PCR-RFLP and PFGE analyses. Plant Pathology 55, 530–536.

Liesegang, A., Tschape, H., 2002. Modified pulsed-field gel electrophoresis method for DNA degradation-sensitive *Salmonella* enterica and *Escherichia coli* strains. International Journal of Medical Microbiology 291, 645–648.

Lindberg, A.-M., Ljunghb, A., Ahrne, S., Lofdahlc, S., Molin, G., 1998. *Enterobacteriaceae* found in high numbers in fish, minced meat and pasteurised milk or cream and the presence of toxin encoding genes. International Journal of Food Microbiology 39, 11–17.

Lucia, L.M., Vanderzant, C., Acuff, G.R., 1993. Potential role of *Proteus–Providencia–Morganella* species in quality deterioration of beef. Meat Science 35, 321–330.

Newton, K.G., Gill, C.O., 1978. The development of anaerobic spoilage flora of meat stored at chill temperatures. Journal of Applied Microbiology 44, 91–95.

Nychas, G.J.E., Drosinos, E., Board, R.G., 1998. Chemical changes in stored meat. In: Davies, A.R., Board, R.G. (Eds.), The Microbiology of Meat and Poultry. Blackie Academic and Professional, London, pp. 288–326.

Nychas, G.-J.E., Skandamis, P.N., Tassou, C.C., Koutsoumanis, K.P., 2008. Meat spoilage during distribution. Meat Science 78, 77–89.

Outtara, B., Simard, R.E., Holley, R.A., Piettte, G.J.P., Begin, A., 1997. Antimicrobial activity of selected fatty acids and essential oils against six meat spoilage organisms. International Journal of Food Microbiology 37, 155–162.

Paramithiotis, S., Mueller, M.R.A., Ehrmann, M.A., Tsakalidou, E., Seiler, H., Vogel, R., Kalantzopoulos, G., 2000. Polyphasic identification of wild yeast strains isolated from Greek sourdoughs. Systematic and Applied Microbiology 23, 156–164.

Penney, N., Bell, R.G., 1993. Effect of residual oxygen on the colour, odour and taste of carbon dioxide-packaged beef, lamb and pork during short term storage at chill temperatures. Meat Science 33, 245–252.

Porschen, R.K., Sonntag, S., 1974. Extracellular Dnase production by anaerobic bacteria. Applied Microbiology 27, 1031–1033.

Pot, B., Vandamme, P., Kersters, K., 1994. Analysis of the electrophoretic whole-organism protein fingerprints. In: Goodfellow, M., O'Donnell, A.G.N. (Eds.), Chemical Methods in Prokaryotic Systematics. Wiley, New York, pp. 493–521.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L., 2005. Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. Applied and Environmental Microbiology 71, 1977–1986.

Ray, T., Weaden, J., Dyson, P., 1992. Tris-dependent site specific cleavage of *Streptomyces lividans* DNA. FEMS Microbiology Letters 96, 247–252.

Ribeiro, R.L., Machry, L., Brazil, J.M.V., Ramos, T.M.V., Avelar, K.E.S., Pereira, M.M., 2009. Technical improvement to prevent DNA degradation of *Leptospira* spp. in pulsed field gel electrophoresis. Letters in Applied Microbiology 49, 289–291.

Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub, S.H., Camargo, F.A.O., Farmerie, W.G., Triplett, E.W., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. International Society for Microbial Ecology 1, 283–290.

Romling, U., Tummler, B., 2000. Achieving 100% typeability of *Pseudomonas aeruginosa* by the pulsed-field gel electrophoresis. Journal of Clinical Microbiology 38, 464–465.

Ronaghi, M., 2001. Pyrosequencing sheds light on DNA sequencing. Genome Research 11, 3–11.

Silbert, S., Boyken, L., Hollis, R.J., Pfaller, M.A., 2003. Improving typeability of multiple bacterial species using pulsed-field gel electrophoresis and thiourea. Diagnostic Microbiology and Infectious Disease 47, 619–621.

Skandamis, P.N., Nychas, G.-J.E., 2002. Preservation of fresh meat with active and modified atmosphere packaging conditions. International Journal of Food Microbiology 79, 35–45.

Stanbridge, L.H., Davies, A.R., 1998. The Microbiology of Chill Stored Meat. In: Davies, A. R., Board, R.G. (Eds.), The microbiology of meat and poultry. Blackie Academic and Professional, London, pp. 174–219.

Stiles, M.E., Ng, L.-K., 1981. *Enterobacteriaceae* associated with meats and meat handling. Applied and Environmental Microbiology 41, 867–872.

Vasilopoulos, C., De Maere, H., De Mey, E., Paelinck, H., De Vuyst, L., Leroy, F., 2010. Technology-induced selection towards the spoilage microbiota of artisan-type cooked ham packed under modified atmosphere. Food Microbiology 27, 77–84.

von Holy, A., Holzapfel, W.H., Dykes, G.A., 1992. Bacterial populations associated with Vienna sausage packaging. Food Microbiology 9, 45–53.

Appendix III

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

Reviewing status: Revised and resubmitted

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

Anthoula A. Argyri [a,b], Agapi I. Doulgeraki [a,b], Vasiliki A. Blana [a,b], Efstathios Z. Panagou [a], George-John E. Nychas [a,*]

[a] *Laboratory of Microbiology and Biotechnology of Foods, Department of Food Science and Technology, Agricultural University of Athens, Iera Odos 75, Athens, Greece, GR-11855*

[b] *Applied Mycology Group, Cranfield Health, Cranfield University, Bedford MK43 0AL, UK*

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.

Appendix IV

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

Journal of Food Protection, Vol. 000, No. 000, 0000, Pages 000–000
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

VASILIKI A. BLANA,[1,2] AGAPI I. DOULGERAKI,[1,2] AND GEORGE-JOHN E. NYCHAS[1]*

[1]*Laboratory of Microbiology and Biotechnology of Foods, Department of Food Science, Technology and Human Nutrition, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece; and* [2]*Applied Mycology Group, Cranfield Health, Cranfield University, College Road, Bedford MK43 0AL, United Kingdom*

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

ABSTRACT

1 2 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 also was 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).*

* Author for correspondence. Tel: +30-210-5294938; Fax: +30-210-5294938; E-mail: gjn@aua.gr.

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 deMan 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*::Tn5 sensor 1^- sensor 2^+) biosensor strain, which only senses the AI-2 molecule, and the AI-2–producing *V. harveyi* BAA-1119 (*luxL*::Tn5 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)*.

As exogenous source of AI-2–like molecules in the inhibition assays was used. This 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 employed 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* (B243) 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 232). 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. Revision FM-S-09-00571R1. 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.

Authors Queries

Journal: **Journal of Food Protection**
Paper: **food-74-04-21**
Title: **Autoinducer-2–Like Activity in Lactic Acid Bacteria Isolated from Minced Beef Packaged under Modified Atmospheres**

Dear Author
During the preparation of your manuscript for publication, the questions listed below have arisen. Please attend to these matters and return this form with your proof. Many thanks for your assistance

Query Reference	Query	Remarks
1	Author: This article has been edited for grammar, style, and usage. Please compare it with your original document and make corrections on these pages. Please limit your corrections to substantive changes that affect meaning. If no change is required in response to a question, please write ''OK as set'' in the margin. Copy editor	
2	Author: Key words: Autoinducer-2–like activity, lactic acid bacteria, meat, quorum sensing, spoilage. Key words are used for indexing; please approve or edit as needed. Copy editor	

Proceedings

Oral Presentations

- **Doulgeraki, A. I.** & Nychas, G-J. E. **(2010)**. Monitoring the succession of microbiota during storage of beef fillets under different temperature conditions. 2nd Hellenic Congress on meat and meat products thereof «from stable to table», 24-26 September, Athens, Greece.
- **Doulgeraki, A. I.** & Nychas, G-J. E. **(2010)**. A modified pulsed - field gel electrophoresis method to prevent DNA degradation of *Enterobacteriaceae*. 3rd Pan – Hellenic of Interdisciplinary Society of Food Hygiene assurance, 4- 6 of June, 2010, Thessaloniki, Greece.
- **Doulgeraki, A. I.,** Paramithiotis, S. & Nychas, G-J. E. **(2010).** Identification with molecular tools of *Enterobacteriaceae* isolates recovered from meat. 3rd Pan – Hellenic of Interdisciplinary Society of Food Hygiene assurance, 4- 6 of June, 2010, Thessaloniki, Greece.
- Argyri**, A. A**., **Doulgeraki, A. I**., Blana, V. A, Panagou, E. Z., & 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.
- **Doulgeraki, A. I.,** Paramithiotis, S. & Nychas, G-J. E. **(2009).** Genomic analysis of lactic acid bacteria isolated during the spoilage process of minced beef stored under different conditions. 3rd Pan-Hellenic conference of Biotechnology and Technology of foods, 15 – 17 October, Rethymno, Crete, Greece.
- **Doulgeraki, A. I.** Nisiotou, A. & Nychas, G-J. **(2008).** The influence of package and storage temperature of meat on lactic acid bacteria population. Meat and meat products, 10-12 October, Athens, Greece.
- **Doulgeraki, A. I.,** Blana, V., Panagou, E. & Nychas G-J. E. **(2008).** The role of lactic acid bacteria and Quorum Sensing phenomenon in beef spoilage. Meat and meat products, 10-12 October, Athens, Greece.

Poster presentations

- **Doulgeraki, A. I.,** Paramithiotis, S., Tassou, C.C. and Nychas, G- J. E. **(2010)**. Contribution of *Enterobacteriaceae* Community to Minced Beef Spoilage. 22nd International ICFMH Symposium Food Micro 2010, Copenhagen 30th August- 3rd September, Copenhagen, Denmark
- **Doulgeraki, A. I.,** Paramithiotis, S. & Nychas, G-J. E. **(2010).** Spoilage dynamics of lactic acid bacteria strains during minced beef storage under aerobic or modified atmosphere packaging conditions. 3rd Pan – Hellenic of Interdisciplinary Society of Food Hygiene assurance, 4- 6 of June, 2010, Thessaloniki, Greece.
- **Doulgeraki, A. I.,** Blana, V. A., Argyri, A. A., Kuriakopoulou, A-V., Panagou, E. & Nychas, G-J. E. **(2010).** Effectiveness of volatile compounds of oregano essential oil on *Listeria monocytogenes in vitro* and in meat. 3rd Pan – Hellenic of Interdisciplinary Society of Food Hygiene assurance, 4- 6 of June, 2010, Thessaloniki, 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
- **Doulgeraki, A.** Nisiotou, A., & Nychas, G-J. **(2009)** Contribution of lactic acid bacteria population in meat stored under modified atmospheres, Ashtown Food Research Centre
- 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 shelf 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
- **Doulgeraki, A.I.**. Nisiotou, A., & Nychas, G-J. **(2008)** The influence of package and storage temperature of meat on lactic acid bacteria population. Microbiokosmos
- **Doulgeraki, A.I.,** Blana, V., Panagou, E. & Nychas G-J (2008) The role of lactic acid bacteria and Quorum Sensing phenomenon in beef spoilage, Microbiokosmos
- **Doulgeraki A**.I., Hayes J., Kagkli D.-M., Jordan K. and Nychas G.-J. E**. (2008)** Dynamics of Lactic Acid Bacteria Population in minced beef. Food Microbiology 2008, 1-3 September, Aberdeen, Scotland
- **Doulgeraki A.I.,** 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. Food Microbiology 2008, 1-3 September, Aberdeen, Scotland

- **Doulgeraki A.I.,** Argyri A, Blana V. and Nychas G.-J.E**. (2008)** Potential use of Essential oils for Beef preservation, Natural Products, Athens Greece
- Ammor M.S., Michaelidis C.**, Doulgeraki A.I**., 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, France