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Study of spoilage microbial populations during storage of beef

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Introduction

1.1 Food packaging for storage of beef

Food packaging serves to protect products against deteriorative effects, contain the product, communicate to the consumer as a marketing tool, and provide consumers with ease of use and convenience (Yam, et al. 2005). The display of meat in plastic materials allows consumer evaluation of the product in an attractive, hygienic and convenient package (Renerre et al. 1993). The properties of meat that are important in determining shelf life include water binding (or holding) capacity, color, microbial quality, lipid stability, and palatability (Renerre et al., 1993; Zhao et al., 1994). The quality of packaged food is directly related to the food and packaging material attributes (Han, 2005) so packaging materials have been developed to maintain the desired properties of meat during storage and display. Materials used in food packaging are glass, metal, paper, and plastic (Marsh and Bugusu, 2007). Properties of plastic make them highly suitable for food packaging (Jenkins and Harrington, 1991). Plastics have properties of low density, resistance to breaking, no sharp edges, ready sealability, fabrication flexibility, environmental durability, barrier and permeability properties, print and metal coating receptivity, resistance to tear and puncture, and flexibility at low temperatures that make them suited for food packaging. The most commonly used polymers for food packaging are show in table 1. The common methods to stored fresh meat are vacuum packaging, modified atmosphere packaging (MAP) and by using an active packaging.

1.2 Vacuum packaging for raw chilled meat

Vacuum packaging extends the storage life of chilled meats by maintaining an oxygen deficient environment within the pack. Using high oxygen impermeable films can inhibit the growth of aerobic microorganism such as *Pseudomonas* but it does not affect the growth of anaerobic microorganism such as lactic acid bacteria (LAB) and *B. thermosphacta*, which are facultative anaerobes able to growth in VP meats. LAB have two particular advantages; firstly, they develop at a slower rate than aerobic Gram negative flora, resulting in a longer shelf-life. Second, the off odors which can be detected on opening of the packs, is far less offensive than putrid odours. The most frequently types of bacterial spoilage in VP are a sweet odour caused by lactobacilli, leuconostocs and streptococci (Mol et al., 1971); a cheesy odour caused by *B. thermosphacta* (Egan et al., 1980) a sulphide odour caused by *Enterobacteriaceae* and greening caused by hydrogen peroxide producing lactobacilli. The preservative effect of VP is achieved by maintaining an oxygen depleted atmosphere since potent spoilage bacteria are inhibited in normal pH of meat under optimum vacuum packaged condition. When meat is first vacuum packaged any residual of oxygen remaining in the pack is consumed by meat and muscle pigments and CO_2 is produced as the end product of tissue and microbial respiration. However, VP is considered unsuitable for red meats for retail display purposes since the oxygen depleted atmosphere causes the meat in these packages to be the purplish colour of deoxymyoglobin and therefore not acceptable to consumers (Gill, 1991).

1.3 MAP packaging for raw chilled meat

Modified atmosphere packaging (MAP) is the removal and/or replacement of the atmosphere surrounding the product before sealing in vapor-barrier materials (McMillin, 2008). MAP can be vacuum packaging, which removes most of the air before the product is enclosed in barrier materials, or forms of gas replacement, where air is removed by vacuum or flushing and replaced with another gas mixture before packaging sealing in barrier materials. The purpose of MAP is to maintain the desired properties of meat for the desired period of storage and display. The major packaging types by using MAP are show in table 2. Aerobic microorganisms like *Pseudomonas* spp. and *Achromobacter* spp. are commonly found on meat and reduce the O_2 tension and increase discoloration of raw meat in ambient air environments (Robach and Costilow, 1961). Bacterial numbers lower than the level of log 10^6 colony forming units (CFU) associated with spoilage may affect color by reducing the O_2 tension at the meat surface and excreting oxidizing agents (Siegel, 2001). Increased levels of CO_2 inhibit microbial growth in refrigerated storage, with 20–40% CO_2 used in MAP (Clark and Lentz, 1969), and high levels raise the possibility of establishing conditions where pathogenic organisms may survive (Daniels et al., 1985). The CO_2 in MAP is absorbed by water and lipid portions of meat until saturation or equilibration is reached (Jakobsen and Bertelsen, 2002), with the full preservative effect of CO_2 achieved only with excess of CO_2 above saturation levels (Gill and Penney, 1988). Gram-negative bacteria are generally more sensitive to CO_2 than Gram-positive bacteria (Church, 1994) because most Gram-positive bacteria are facultative or strict anaerobes (Gill and Tan, 1980), but individual bacteria vary in sensitivity to CO_2 (Farber,

1991). Levels of 20–60% CO_2 are required for effectiveness against aerobic spoilage organisms by penetrating membranes and lowering intracellular pH (Smith et al., 1990), but little or no effect is observed with CO2 above 50–60% (Gill and Tan 1980). A major decision in choosing a MAP system is color of meat desired during transit and subsequent display. The packaging systems that provide meat for retail display with a red color are more highly used because consumers will discriminate against beef that is not red during display (Carpenter et al., 2001) and will avoid purchasing meat with 20% or more metmyoglobin (MacDougall, 1982).

1.4 Antimicrobial food packaging

Antimicrobial packaging is the packaging system that is able to inhibit or retard growth of spoilage and pathogenic microorganisms that are contaminating foods. Adding antimicrobial agents can achieve the new antimicrobial function in the packaging system and/or using antimicrobial polymers that satisfy conventional packaging requirements. Antimicrobial packaging materials have to extend the lag phase and reduce the growth rate of microorganisms to prolong the shelf life and maintain food quality and safety (Han, 2000). Most food packaging systems represent either a package/food system or a package/headspace/food system (Fig.1). A package/food system is a solid food product in contact with the packaging material, or a low-viscosity or liquid food without headspace. Antimicrobial agents may be incorporated into the packaging materials initially and migrate into the food through diffusion and partitioning (Han, 2000). Antimicrobial packaging can take several forms including: addition of sachets/pads

containing volatile antimicrobial agents into packages; incorporation of volatile and non volatile antimicrobial agents directly into polymers; coating and adsorbing antimicrobials onto polymers surfaces; immobilization of antimicrobials to polymers by ion or covalent linkages; use of polymers that are inherently antimicrobial (table 3) (Appendini et al., 2002). All antimicrobial agents have different activities which affect microorganisms differently. Bacteriocins are antimicrobial peptides or small proteins which inhibit, by a bactericidal or bacteriostatic mode of action, micro-organisms that are usually closely related to the producer strain (De Vuyst and Vandamme, 1994). Bacteriocins produced by lactic acid bacteria are of great interest to the food fermentation industry as natural preservatives because of their ability to inhibit the growth of many food spoilage and pathogenic bacteria, including *Listeria monocytogenes, Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum*. Nisin is a bacteriocin produced by *Lactococcus lactis subsp. lactis* is active against Gram positive organisms including bacterial spores, but it is not generally active against Gram negative bacteria, yeasts and fungi. Nisin is currently the only one whose employment in food as pure substance is allowed (Deegan et al. 2006; Galvez et al., 2007; Settanni and Corsetti, 2008) and it is also approved by the US Food and Drug Administration (Federal Register, 1988). Treatment with chelators such us EDTA, can alter the outer membrane permeability of Gram negatives (Delves-Broughton 1993). Studies have been published by a number of authors on the use of nisin as an antimicrobial in a wide variety of food products (Delves-Broughton 1993, Mauriello et al. 2005, 2004, Ercolini et al. 2006). Application of antimicrobial treatments using nisin and EDTA to raw poultry products in combination with

modified atmosphere packaging (MAP) or vacuum packaging (VP) has received little attention (Economou et al. 2009, Ercolini et al. 2010).

1.5 Spoilage of raw meat

Food spoilage usually refers to the deterioration of quality in food products due to the growth of contaminating microorganisms, although non-microbial activity, such as the activity of endogenous enzymes, can also contribute to food spoilage. The main defects of spoilage are sensory changes, such as off-odours and off-flavours, slime production, texture change, discoloration and gas production. Food spoilage processes determine the shelf life of food products, as the products can only be stored until a maximum unacceptable level of off-odour/off-flavours develop (Borch et al. 1996). Shelf life is the period of time between packaging of the product and its use that the product properties remain acceptable to the product user, with shelf life properties being appearance, texture, flavor, color, and nutritive value (Singh & Singh, 2005, Chap. 3). The development of microbial flora in food systems during storage is affected by many intrinsic and extrinsic factors. Examples include the characteristics of the food itself (nutrient composition, water activity, pH, natural antimicrobial substances, etc.), mode of processing and preservation, initial composition of the contaminants, storage temperature and packaging atmosphere. Packaging influences the extension of raw chilled meat shelf life (Renerre & Labadie, 1993). The properties of meat that are important in determining shelf life include water binding (or holding) capacity, color, microbial quality, lipid stability, and palatability (Renerre & Labadie, 1993; Zhao et al., 1994). The variables that influence the shelf life properties of packaged fresh meat are the product, gas mixture, package and headspace, packaging equipment, storage temperature, and additives (Hotchkiss, 1989).

Deteriorative changes during meat storage are affected by metabolic reactions from biological membrane disruption (Stanley, 1991) and biochemical oxidative processes (Xiong & Decker, 1995). Deterioration of quality may include discoloration, off-flavor and off-odor development, nutrient loss, texture changes, pathogenicity, and progression of spoilage factors (Skibsted et al., 1994). Meat is a good support for bacterial growth as shown by the numerous reports dealing with the influence of microorganisms on the storage life of meat products. The main property, which explains rapid microbial growth on meats, is its composition: 75% water and many metabolites such as amino acids, peptides, nucleotides, and sugars. After slaughter, microbial contamination of carcasses is the consequence of the processing applied from skinning to conditioning. Processing influences not only the quantity of microrganisms/cm^2 but also the type of microorganisms present. Spoilage is characterised by any change in a food product that renders it unacceptable to the consumer from a sensory point of view. Microbial numbers are not always related to degree of spoilage, but microbial activity is considered to be of great importance for the manifestation of spoilage (Nychas et al. 1998). The species and population of microorganisms on meat are influenced by animal species, state of health, and handling of live animals; slaughter practices, plant and personnel sanitation, and carcass chilling; fabrication sanitation, type of packaging, storage time, and storage temperature (Nottingham, 1982, Chap. 2; Grau, 1986). Discoloration, off-odors, and slime production are among the deteriorative factors caused by bacterial growth (Butler et al.1953). The predominant organisms on the surface of raw meat are *Brochothrix thermosphacta*, *Lactobacillus* spp.,

Leuconostoc spp., *Carnobacterium* spp., *Pseudomonas* spp. and *Enterobacteriaceae* (Dainty 1992; Borch et al. 1996; Huis in't Veld 1996; Jay et al. 2003; Nychas et al. 2008). Despite the heterogeneity of the initial contaminating microbial flora, aerobic refrigerated meat storage selects *Pseudomonas* spp., particularly *P. fluorescens, P. putida* and *P. fragi*, as the dominant spoilage flora in proteinaceous raw foods (Gennari and Dragotto 1992; Ternstrom et al. 1993). *Pseudomonas fragi* is recognized as one of the principal agents of meat spoilage (Labadie, 1999) and very frequently isolated from fresh and spoiled meat products (Ercolini *et al*., 2007; Ercolini *et al*., 2009; Arnaut-Rollier *et al*., 1999). The fresh meat environment is a particularly suitable substrate for the growth of *P. fragi*. Beyond the nutritional value of meat components, the strict chill chain applied to the fresh meat production from slaughtering through portioning until distribution is completed advantages this psychrophilic species. *P. fragi* is by far one of the most threatening species in the spoilage of meat. Several interactions between spoilage flora have been proposed (Gram et al. 2002). The antagonistic interaction between bacteria can happen through the competition for nutrients. For example, the depletion of glucose (Nychas et al. 1988; Lambropoulou et al. 1996), or the siderophore mediated competition for iron by pseudomonads (Gram 1993; Gram and Melchiorsen 1996) will effectively inhibit other bacteria. Microbial interactions could also occur through cell-cell signalling, or quorum sensing, which is a mechanism intensively studied in the past decade (Whitehead et al. 2001; Miller and Bassler 2001). Quorum sensing-mediated microbial interactions could rely on communication via specific signalling molecules between different bacterial species, so that gene expression can

be modulated within the population for enhanced survival. Bacterial interaction through quorum sensing has been reported to take place in several ecological systems, such as biofilms (Eberl and Tummler 2004), or in the rhizosphere (Steidle et al. 2001). in several food spoilage systems, such as cold-smoked salmon, vacuum-packed meat, and bean sprouts, certain cell-cell signaling molecules have been isolated (Gram et al. 1999; Gram et al. 2002; Bruhn et al. 2004). It was hypothesized, therefore, that bacterial interactions through quorum sensing could also take place in food spoilage systems (Gram et al. 2002). The significance of quorum sensing in bacterial ecological interactions during food spoilage, especially the involvement of quorum sensing in the systems which *P. fragi* dominate, however, remains to be elucidated. As with other *Pseudomonas* species in different natural habitats, the dominance and activities of *P. fragi* in meat may be regulated by QS mechanisms.

1.6 Quorum sensing

Bacteria are capable of modulating gene expression or other cellular functions in response to a variety of extracellular signals called autoinducers to regulate their behaviour according to population density. Several categories of signal molecules are involved in Quorum Sensing (QS) *(*Ammor et al. 1999; Gram et al. 1999; Shauder et al. 2001). *N*-acyl homoserine lactones (AHLs), composed of a fatty acyl chain ligated to a lactonized homoserine through an amide bond *(*Parsek et al. 2005), generically called autoinducer-1 (AI-1), are produced and used by gram negative bacteria mainly for intraspecies communication (see table 4) (Fuqua et al 2001; Miller et al. 2001). There are now over 50 species recognized to produce AHLs, and these signals regulate a diverse range of bacterial processes. Moreover, 2(5H)-furanones,

chemically similar to AHL but presumably coming from different precursors, are released by *Lactobacillus helveticus* exposed to oxidative and heat stresses (Ndagijimana et al. 2006). *Pseudomonas* spp. are found in many ecosystems. Some species are plant (*P. syringae*) and human (*P. aeruginosa*) opportunistic pathogens, whereas others (*P. fluorescens*, *P. putida*, *P. chlororaphis*) promote plant growth and act as antagonists of plant pathogens through their ability to colonize the rhizosphere. In all the above species, quorum sensing (QS) has been found to regulate their activities in natural habitats, through the production of different AHLs and regulated by different couples of LuxI-LuxR proteins and related genes (McGrath et al. 2004; Tavender et al. 2008; Tryfinopoulou et al. 2002). The QS of *P. aeruginosa* is relatively well understood, but surprisingly little is known about the QS systems of other members of the genus *Pseudomonas* (Jay et al. 2003). Furanosyl borate diesters, known as Autoinducer-2 (AI-2), were discovered for the first time in the marine bacterium *Vibrio harveyi* (Bassler et al. 1993; 1994). Although different AI-2 structures were discovered in *V. harveyi*, *S.* Typhimurium and *Escherichia coli*, the same *luxS* gene was found to be responsible for the synthesis of AI-2 molecules (see table 5) (Staidle et al. 2002). Among 89 fully sequenced bacterial genomes, 35 have *luxS* homologs (Winson et al 1998). This suggests that AI-2 functions as a unique, universal signal, which could be shared by Gram-positive and Gram-negative bacteria, and can therefore be used not only in intraspecies but also in interspecies communication (Shaw et al. 1997).

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1.8 Tables

Table 1 individual polymer materials, common abbreviation and associated properties (Walsh and Kerry, 2002)

Polymer materials	Abbreviations	Associated properties
Low density polyethylene	LPDE	Sealability, formability, moisture barrier, low cost
Linear low density polyethylene	LLDPE	Sealability, abuse resistance, formability, moisture barrier
Polypropylene	PP	Thermal resistance, dimensional stability, moisture barrier,
Ethylene vinyl acetate copolymer	EVOH	Sealability, improved abuse resistance over LDPE, clarity
Polyesters	PET	Mechanical resistance, heat resistance, medium O_2 barrier
Ethylene vinyl alcohol	EVA	High O_2 barrier, good co-extrusion, processability, clarity
Polyamides	PA	Mechanical strength, O_2 barrier, formability
Polyvinylidene chloride	PVDC	High O_2 barrier, grease and fat barrier
Polystirene	PS	Excellent clarity, low cost, readily thermoformed and injection moulded
Ionomer	/	Heat sealability, produce films of unusual toughness and clarity
Polyvinyl chloride	PVC	Versatile, shrink properties, sparkling clear, low cost
High density polyethylene	HDPE	More gas impermeable than LPDE, low cost, strong, reduced clarity
Polycarbonate	PC	High clarity, strong, impact resistance, dimensional rigidity

Table 2 Packaging types and characteristics for fresh retail meat (McMillin 2008)

Package	Air-permeable overwrap	Air-permeable in master pack	Vacuum packaging (VP)	Low O_2 with CO_2 and N_2	Peelable VP or low O_2 with CO_2 :N_2	Low O2 with CO2	High O2
System description	Air-permeable film overwrap of product on tray	Barrier bag with single or multiple trays of product in air-permeable packaging	Flexible film shrunk around product on a rigid base web	Thermoformed or preformed trays with lidding film	VP or barrier tray with 2 layer lidding film	VP; product displayed in package	Thermoformed or preformed tray with lidding
Gases in headspaces	Atmosphere air	Usually CO_2 and or N_2	No gas	CO_2 and/or N_2	No headspace with VP; CO_2 and/or N_2	CO_2 and/or N_2; No headspace with VP	O_2 and CO_2; often 80%:20% CO_2
Meat color in storage	Red	Purple	Purple	Purple	Purple	Red	Red
Muscle shelf life, days at 4°C	5-7	10-14	60-90	30-60	30-45	35	12-16
Advantages	Consumers familiar with packaging; low cost	Storage life extended before display	Long storage life before display	Long storage life before display	Long storage life before display	Long red color stability and no lipid oxidation	Moderate red color stability
Disadvantages	Short display life	Double packaging costs	Display with purple color	Purple display color increase cost with masterpack	Film peeling at retail store; inconsistent bloomed color after air exposure, short display life	Negative image by consumers; concern red product may be spoiled in other factors	Lipid oxidation; maybe bone decresed tenderness; may be premature browning of cooked meat

Antimicrobials	packaging materials[a]	foods
benzoic acids	PE, IONOMER	culture media
benzoic e sorbic acids	PE	culture media
Sorbates	LDPE,PE,PET	cheese, chicken,water
acetic,propionic acid	chitosan	water
lysozime	PVOH, NYLON,CELLULOSE ACETATE	culture media
Pediocin	Cellulose	Cooked meat
nisin	Silicon coating SPI, corn zein film LDPE, nylon PE HPMC SPI, WPI, WG, EA	Beef tissue Culture media Broiler drumstick
Glucose-oxidase	ALGINATE	fish
Lactic acid	ALGINATE	Lean beef muscle
Benomyl	ionomer	culture media
Chitosan	chitosan paper	strowberry
UV/ecsicimer laser	nylon	culture media
grapefruit seed	LDPE	ground beef

Table 3 - Applications of antimicrobial food packaging (Han, 2000).

[a] :LDPE, low-density polyethylene; HPMC, hydroxypropyl MC; PE, polyethylene; PVOH, polyvinyl alcohol; SPI, soy protein isolate; WPI, whey protein isolate; WG, wheat gluten

Species	R/I gene	AHL	Phenotype
Vibrio fischeri	luxR, luxI, ainS	3O-C6	Bioluminescence, colonization factors
Agrobacterium tumefaciens	traR,traI, trlR	3O-C8	Virulence factors
Erwinia carotovora	carR, carI	3O-C6	Production of antibiotic
Pseudomonas aeruginosa	luxR, luxI, rhlR, rhlI	3O-C12; C4	Biofilm formation; alkaline protease; virulence factors
Pantoea stewartii	esaR, esaI	3O-C6	Exopolysaccharide production
Cromobacterium violaceum	cviR, cviI	C6	Exoenzimes, antibiotics, violacein
Burkholderia capacia	cepR,cepI	C8, C6	Protease, siderophores, biofilm, swarming
Serratia liquifaciens	swrR, swrI	C4	Extracellular protease, swarming

Table 4 Phenotipes controlled by AHL based R-I quorum sensing system (Federle and Bassler 2003; Lazdunski et al. 2004)

Species	Phenotype
Salmonella typhi	Biofilm formation
Salmonella typhimurium	ABC transporter expression
E. coli w3110	Cell division, motility, metabolism
E. coli, EHEC and EPEC	Virulence, type III secretion
Clostridium perfringes	Toxin production
Campylobacter jejuni	Motility
Streptococcus mutans	Biofilm formation
Streptococcus pneumoniae	Virulence
Borrelia burgdorferi	Protein expression
Vibrio harveyi	Luminescence, protease production, siderophore production
Vibrio cholerae	Virulence factors
Vibrio vulnificus	virulence

Table 5 Phenotipes controlled by LuxS/AI-2 based quorum sensing system (Federle and Bassler 2003; Lazdunski et al. 2004)

Chapter 2
Study the spoilage microbial populations of beef during storage under vacuum condition

2.1 Introduction

Vacuum packaging extends the storage life of chilled meat by maintaining an oxygen deficient environment within the pack. Active packaging is one of the innovative food packaging concepts that has been introduced as a response to demands of consumers for high quality, safety and extended shelf-life of food products (Vermeiren et al. 1999; Quintavalla and Vicini 2002; Cagri et al. 2004). Bacteriocin activated plastic films for food packaging have been developed for the storage of milk (Mauriello et al. 2005), hamburgers (Mauriello et al. 2004), hot dogs (Franklin et al. 2004) frankfurters (Ercolini et al. 2006a), cooked ham (Marcos et al. 2007) and cold smoked salmon (Neetoo et al. 2008). In all the cases the activation of plastic films with bacteriocin solutions was helpful to retard the growth of pathogenic and/or spoilage bacteria. Among the known bacteriocins, nisin is currently the only one whose employment in food as pure substance is allowed (Deegan et al. 2006; Galvez et al., 2007; Settanni and Corsetti, 2008) and it is also approved by the US Food and Drug Administration (Federal Register, 1988).

The aim of the first activities of PhD thesis project was to study the spoilage microbial populations of beef during storage under vacuum condition using a nisin activated antimicrobial packaging.

2.2 Materials and Methods

2.2.1 Nisin antimicrobial solution (NAS) and plastic bags activation.

Two different antimicrobial solution were obtained:

NAS-1 was obtained as follows: Nisin 0.012g/mL (Nisin 2.5%, Sigma, Milano, Italy) was dissolved in 0.02 mol l^{-1} HCl and the mixture was centrifuged at 6.500 x g for 10 min. The pellet was dissolved in the same volume of 0.02 mol l^{-1} HCl, centrifuged at 6.500 x g for 10 min and resuspended in the same volume of a solution containing 0.02 mol l^{-1} HCl and EDTA (0.071g/mL, pH 4.05) in 2:1 ratio. The antimicrobial activity was expressed in arbitrary units per mL (AU/mL) and it was determined by an agar diffusion assay as described by Villani et al. (1993).

NAS-2 was obtained as follows: 0.012g/mL of nisin (Nisin 2.5%, Sigma, Milano, Italy) was dissolved in a solution containing ascorbic acid 1%, citric acid 1% and $CaCl_2$ 1% The mixture was centrifuged at 6.500 x g for 10 min. The pellet was dissolved in the same volume of the acid solution, centrifuged at 6.500 x g for 10 min and resuspended in the same volume of the same solution containing 0.071g/mL of EDTA The antagonistic activity was determined by the agar diffusion assay (Villani et al. 1994).

Bags (200 x 300 mm) of plastic barrier film (co-extruded, copolymer of vinylidene chloride (VDC) and Ethyl Vinyl Alcohol (EVA) as barrier layer, and low density polyethylene (LDPE) on the nisin-treated surface, CRYOVAC BB3050, oxygen transmission 0.83 cm^3 m^{-2} h^{-1} at 23°C, provided by CRYOVAC Sealed Air S.r.l., Milano, Italy) were used for the development of the antimicrobial packaging in two different condition using one time the NAS-1 and the second time NAS-2 for the activation of the bags. Four ml of the

antimicrobial solution were poured into the bags and the contact was allowed for 1 h at room temperature. The internal part of the bag was then air-dried at 50°C. The antimicrobial activity of pieces of plastic film from the bags after activation were checked in agar assays as previously described (Mauriello et al. 2004). For the film antimicrobial activity assay, samples (2 x 2 cm) of the treated films were located onto the surface of a TSA (Oxoid) soft (0.75%) agar plates seeded with 2.5% of an overnight culture of different spoilage bacteria (Table2.1). The treated face of the film was in contact with the agar; untreated films were also assayed as negative controls. The antagonistic activity was evaluated by observing a clear zone of growth inhibition in correspondence of the active film.

2.2.2 NAS-1 activated plastic film for storage of meat under vacuum condition at 1°C for 32 days

Bone less beef cuts of about 40 g each were obtained from a single meat muscle 24 h after slaughter (*longissimus dorsi*) and vacuum-packed using the above mentioned bags after thermal sealing. NAS-1 as a antimicrobial solution (see paragraph 2.2.1) was used for the activation of the internal surface of the bags. Meat cuts were singly packed in antimicrobial and in non activated (control) plastic bags and stored at 1°C. Four single samples for both antimicrobial and control bags were taken after 0, 5, 11, 22 and 32 days of storage for microbial analysis and microbial population assessment.

2.2.2.1Microbial analysis

Samples (25 g) were cut from the chops arising from each bag and were aseptically weighed, homogenized in 225 ml of quarter strength Ringer's solution (Oxoid) for 2 min in a stomacher (LAB Blender 400, PBI, Italy; stomacher bags: Sto-circul-bag, PBI, Italy) at room temperature. Decimal dilutions in quarter strength Ringer's solution (Oxoid) were prepared and aliquots of 0.1 ml of the appropriate dilutions were spread in triplicate on the following media: Plate Count Agar (PCA, Oxoid) incubated at 20°C for 42 h; Violet Red Bile Glucose Agar (VRBGA, Oxoid) for the *Enterobacteriaceae*, incubated at 30°C for 24-48 h; MRS agar (Oxoid) for LAB, incubated at 30°C for 48 h; STAA medium (Oxoid) for *Brochothrix thermosphacta*, incubated at 20°C for 48 h and CTSI agar medium (Wasney et al. 2001) for *Carnobacterium* incubated for 7 days at 20°C. MRS and VRBGA plates were incubated under anaerobic conditions by using an Anaerogen kit (Oxoid). Results were calculated as the means of Log counts for three determinations.

2.2.2.2 DNA extraction from beef and bulk cells

After microbial counts, the plates were used for bulk formation as previously described (Ercolini et al., 2001; Ercolini 2004). Briefly, all the colonies present on the surface of each countable plate were suspended in a volume of quarter strength Ringer's solution (Oxoid) to reach 1 unit of optical density (600 nm), harvested with a sterile pipette and stored by freezing at –20°C. When necessary, 100 μl of the bulk was used for DNA extraction.

For DNA extraction from beef and bulk cells, the protocol described by the manufacturer of the Wizard DNA purification kit (Promega, Madison, Wiscon)

was applied (Ercolini et al. 2006b). Where not specified, the chemicals were from Sigma (Milan, Italy). Beef samples (10 g) treated or untreated with the antimicrobial packaging, were homogenized in a stomacher bag with 20 ml of quarter strength Ringer's solution (Oxoid) for 1 min; a deposit was allowed to set for 1 min and the supernatant was used for the DNA extraction. One ml of the beef homogenate suspension or 100 µl of bulk cells sample were centrifuged at 17000 x *g* for 5 min at 4°C and the resulting pellet was resuspended in 100 μl of TE buffer (100 mmol l^{-1} TRIS, 10 mmol l^{-1} EDTA); then 160 μl of 0.5 mol l^{-1} EDTA/ Nuclei Lysis Solution (Wizard DNA purification kit, Promega) in 1/4.16 ratio and 20 μl of pronase E (20 mg ml^{-1}, Sigma) were added, and the mixture was incubated for 60 min at 35°C. After incubation, 1 vol. of ammonium acetate 5 mol l^{-1} was added to the sample that was then centrifuged at 17000 x *g* for 5 min at 4°C. The supernatant was precipitated with 0.7 vol. of isopropanol and centrifuged at 29000 x *g* for 5 min. Finally, the pellet was dried for 15 min and resuspended in 45 μl of DNA Rehydration Solution (Promega) by incubation at 55°C for 45 min. Finally, 5 µl of 10 X RNAse buffer (Promega) and 0.5 µl of RNAse (Promega) were added and the DNA solution was incubated at 37°C for 30 min before storage at –20°C.

2.2.2.3 PCR-DGGE analysis

The primers U968 and L1401 were used (Zoetendal et al. 1998) amplifying the variable V6-V8 region of the 16S rRNA gene, giving PCR products of about 450 bp. To the forward primers, a GC clamp was added according to Muyzer et al. (1993). Amplifications were performed in a programmable heating incubator (Techne, Progene, Italy). Each mixture (final volume, 50 μl) contained 20 ng of template DNA, each primer at a concentration of 0.2 μM, each deoxynucleoside triphosphate at a concentration of 0.25 mmol l^{-1}, 2.5 mmol l^{-1} $MgCl_2$, 2.5 μl of 10 X PCR buffer (Invitrogen, Milano, Italy) and 2.5 U of *Taq* polymerase (Invitrogen). Template DNA was denatured for 5 min at 94°C. A "touchdown" PCR was performed as previously described (Ercolini et al. 2001). The initial annealing temperature was 66°C and this was decreased 1°C every cycle for 10 cycles, finally 20 cycles were performed at 56°C. The extension for each cycle was carried out at 72°C for 3 min while the final extension was at 72°C for 10 min. Aliquots (2μl) of PCR products were routinely checked on 1.5% agarose gels. PCR products were analyzed by DGGE by using a Bio-Rad Dcode apparatus. Samples were applied to 7% (wt vol^{-1}) polyacrylamide gels in 1 X TAE buffer. Parallel electrophoresis experiments were performed at 60°C by using gels containing a 25 to 55% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% (wt vol^{-1}) formamide). The gels were run for 10 min at 50 V, followed by 4 h at 200 V. They were then stained with ethidium bromide for 5 min, rinsed for 15 min in distilled water, observed and photographed by Bio-Rad Gel Doc system (Bio-Rad, Milano, Italy).

2.2.2.4 Sequencing of DGGE fragments

DGGE bands to be sequenced were purified in water according to Ampe et al. (1999). One μl of the eluted DNA of each DGGE band was re-amplified by using the primers and the conditions described above. PCR products that gave a single band co-migrating with the original band were then purified by QIaex PCR purification kit (Qiagen, Milano, Italy) according to the manufacturer's instructions and sequenced. Sequencing was performed by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) using the primer L1401. To determine the closest known relatives of the partial 16S rRNA gene sequences obtained, searches were performed in public data libraries (GenBank) with the Blast search program (http://www.ncbi.nlm.nih.gov/blast/).

2.2.3 NAS-1 activated plastic film for storage of meat under vacuum condition at 1°C for 46 days

Bone less beef cuts of about 500 g each were obtained from a single meat muscle 24 h after slaughter (*longissimus dorsi*) and vacuum-packed using NAS-1 as a antimicrobial solution for the activation of the internal surface of the bags. Meat cuts were singly packed in antimicrobial and in non activated (control) plastic bags and stored at 1°C. Four single samples for both antimicrobial and control bags were taken after 0, 9, 20, 36 and 46 days of storage. From each sample 10 chops of about 1g (size 2 cm of diameters) were aseptically cut and a total of 31,4 cm^2 of meat were used for microbial analysis. The populations monitored were *Enterobacteriaceae, Brochothrix thermosphacta, Carnobacterium* spp. and LAB (see paragraph 2.2.2.1), in addition Pseudomonas Agar with cetrimide-fucidin-cephaloridine (CFC) selective supplement (Oxoid) were used for *Pseudomonas*, incubated at 20°C for 48. To perform DNA extracion directly from meat, 1 mL of the first decimal diluition prepered from standard plate count was used. The protocol for DNA extraction and the protocol for PCR-DGGE analysis were describe in paragraph 2.2.2.2, 2.2.2.3 and 2.2.2.4

2.2.4. NAS-2 activated plastic film for storage of meat under vacuum condition at 4°C for 45 days

Beef chops of about 20 g each were obtained from single meat muscle 24 h after slaughter (bone less tender beef) and vacuum-packed using the above mentioned bags after thermal sealing.

NAS-2 as a antimicrobial solution was used for the activation of the internal surface of the bags. Three chops for each bags were packed in antimicrobial

and in non activated (control) plastic bags and stored at 4°C. Samples for both antimicrobial and control bags were taken after 0, 7, 14, 21,30, 35 and 45 days of storage for microbial analysis as describe in paragraph 2.2.3.1.

2.3 Results

2.3.1 Microbial analysis of meat stored under vacuum condition by using NAS-1 for 32 days

The NAS-1 could be evenly distributed in the internal face of the bags and the plastic film kept its overall transparency. The even distribution of the antimicrobial solution on the surface of the bags was appreciated by the presence of homogeneous antimicrobial activity in agar assays (Mauriello et al. 2004; La Storia et al. 2008). NAS-1 shown to be active against Gram-positive bacteria and the best activity was obtained against *B. thermosphacta* with an activity of 102400 AU/ml (Table 2.1). The best and homogeneous antimicrobial activity of the activated plastic film was obtained only against *B. thermosphacta*, for the other indicator strains the plastic film showed not activity (Table 2.1).

The results of the viable counts on specific media during the storage of beef in antimicrobial and control plastic films are shown in Table 2.3. All the targeted microbial populations showed to be affected by the use of the antimicrobial bags even though the effect was shown with different reduction of viable counts and different kinetics; the most affected were the Gram positive populations (Table 2.3). The total viable counts seemed to be initially unaffected by the use of the antimicrobial film at least for the first five days; however, after 22 days until the end of the storage time the total viable counts

of the beef stored in antimicrobial bags was 2 log cycles lower than the control. The load of LAB in control samples increased from 10^2 to about 10^6 CFU g^{-1} during the whole storage time while the LAB population was dramatically reduced with the use of NAS activated bags in the first 11 days of storage and the load was kept to 10^2 CFU g^{-1} at the end of the storage. *B. thermosphacta* had an initial load of 10^2 CFU g^{-1} in control samples and it increased to 10^5 CFU g^{-1} from the 11^{th} day of storage keeping constant until the end. By contrast, in cuts stored in activated bags, *B. thermosphacta* resulted uncountable for 22 days and displayed a final load lower than 10^3 CFU g^{-1} after 32 days. *Carnobacterium* spp. in control samples started to grow only after the first week of storage and reached values of 10^4 CFU g^{-1} after 32 days while the same population in beef stored in active packaging was uncountable for the first 11 days and displayed a final load of 10^2 CFU g^{-1} after 32 days at the end of storage. Enterobacteria grew in control meat samples showing viable counts higher than 10^6 CFU g^{-1} after 22 days of storage. However, when the activated bags were used, enterobacteria were kept 1 to almost 3 log cycles lower than the control during the whole storage time with a final load of about 10^4 CFU g^{-1} after 32 days.

2.3.2 Identification of microbial species

The PCR-DGGE fingerprints obtained from DNA directly extracted from meat cuts is presented in Figure 2.1, while the results of the band sequencing are shown in Table 2.4. Fragments arising from different profiles and migrating with the same distance in DGGE gels were repeatedly sequenced giving the same results in terms of closest relative species and percent of identity. Repeated DNA extraction and PCR-DGGE analysis of the samples confirmed

the fingerprinting obtained; therefore, the band sequencing was performed on one set of samples only. For the first five days of storage the microbial profiles of the meat did not show significant changes (Fig. 2.1). The profiles of control and treated samples were similar and showed the presence of bands identified as *Pseudomonas* spp., *Carnobacterium* spp., *Carnobacterium divergens*, *B. thermosphacta* and *Serratia grimesii* (Table 2.4). In particular, *Carnobacterium* spp. and *Serratia grimesii* (bands 2 and 4, respectively) were present in all the fingerprints up to 22 days of storage. Occurrence of other taxa belonging to *Photobacterium* spp. and *Staphylococcus* spp. was observed (Fig. 2.1, Table 2.4).

The cultivable microbial populations were identified by PCR-DGGE and sequencing of fragments obtained from bulk cells from selective media used for viable counts (Ercolini et al. 2001). The PCR-DGGE fingerprints obtained from bulk cells from MRS agar plates showed an initial contamination by *C. divergens* in the control samples at time zero (Fig. 2.2, Table 2.5). The control beef cuts appeared contaminated also by *Weissella* spp. after five days and then by *Leuconostoc mesenteroides* and *Lactobacillus* spp. until the end of the storage. In the beef stored in the antimicrobial bags, viable counts on MRS agar were detectable only at times 22 and 32 and consisted of *C. divergens*, *L. mesenteroides*, and *Lactobacillus* spp. (Fig. 2.2, Table 2.5). The MRS agar plates were found to be also occasionally contaminated by colonies of *Staphylococcus* spp. (Table 2.5). The bulk cells from STAA in all the control samples and in the treated meat after 32 days showed a profile with a single band that proved to be *B. thermosphacta* (100%, AY543029) after sequencing (data not shown). The bulk cells from CSTI were analyzed only from the 22th

day and displayed identical results in treated and untreated samples: a single band resulting as uncultured *bacterium* (99%, AB32615) at 22 days and a single band identified as *C. maltaromaticum* (99%, AY543034) in treated samples after 32 days (data not shown).

The PCR-DGGE analysis of bulk cells from VRBGA showed that the meat cuts stored in active and non active (control) bags were initially dominated by *Pseudomonas* spp. (Fig. 2.3, Table 2.6). However, after 22 days of storage in both conditions the only species found was *Serratia grimesii* that kept growing in the control samples whereas it was replaced by *Rhanella* spp. in the treated samples after 32 days of storage.

2.3.3 Microbial analysis and meat storage life by using NAS-1 for 46 days

The results of the viable counts on specific media during the storage of beef in antimicrobial and control plastic films are shown in Table 2.7. Only the Gram positive populations seem to be affected by the use of the antimicrobial films. The total viable counts seemed to be unaffected by the use of the antimicrobial condition, the counts start to increase from the 9^{th} day of storage in both control and treated and display a final load of about 10^6 CFU cm^{-2}. Load of LAB in control sample start to increase form 10^2 CFU cm^{-2} to 10^5 CFU cm^{-2} at the end of the storage, by the way the LAB population in treated sample were uncountable for the first 36^{th} days of storage and display a final load of 10^4 CFU cm^{-2}. *B. thermosphacta* population was the only unable to growth for all the storage time in treated samples, however the load in control sample start to increase from 10^2 to 10^4 CFU cm^{-2} at the end of the storage.

Carnobacterium spp. in control sample start to increase to 10^5 CFU cm^{-1} from the 9th day of storage keeping constant until the end. By contrast in treated sample the populations was uncountable for the first 9th day and reached values lower then 10^5 CFU g^{-1} after 46 days. Enterobacteria grew in both samples at the same level and displayed a final load of about 10^4 CFU cm^{-1}. The same results was obtained for *Pseudomonas* spp., the population were not affected for the use of the antimicrobial bags and display a final load of about 10^4 CFU cm^{-1} in both control and treated samples.

2.3.4 Identification of microbial species

The PCR-DGGE fingerprints obtained from DNA directly extracted from meat are presented in Figure 2.4, while the results of the band sequencing are shown in Table 2.8. For the first 9th days of storage the microbial profiles of the meat did not show significant changes (Fig. 2.4). The profiles of control and treated samples after 36th days of storage were similar and showed the presence of bands identified as *Pseudomonas* spp., *Carnobacterium* spp., *Carnobacterium divergens* and *Rahnella aquatilis* (Table 2.8). In particular, *Pseudomonas* spp. and *Carnobacterium divergens* (bands 2 and 5, respectively) were present in all the fingerprints from the beginning of the storage.

2.3.5 Microbial analysis and meat storage life by using NAS-2

The development of the microbial flora for the meat samples packed by using NAS-2 is shown in table 2.9.

Total viable counts in control sample start to increase from the first week of storage reached value of about 10^7 CFU g^{-1} while in treated sample the total viable count start to increase only from the 30th days of storage and display the same value then the control at the end of the storage.

B. thermosphacta counts in treated samples started to grow only after 35th days of storage and reached values of 10^5 CFU g^{-1} after 45 days. By contrast in non activate package the load start to increase from 10^2 to 10^5 CFU g^{-1} at the end of the storage. LAB counts were affected from the beginning by using active packaging. LAB population were uncountable until the 21st days of storage and displayed a final load of 10^4 CFU g^{-1}. In control samples the counts were kept constant to about 10^6 CFU g^{-1} from the 21st days of storage until the end. Unexpected results came from the counts of enterobacteria, in treated samples. The population were uncountable for whole the storage time, while in non activated packaging started to grow only after 21st days of storage and display a final load of lower than 10^2 CFU g^{-1}. *Pseudomonas* spp. in control samples start to growth only after the first week of storage and reached a final value of 10^4 CFU g^{-1}. However in active packaging the same population start to growth after 21st days of storage and display a final load lower than 1 log cycle compared with the control. *Carnobacterium* spp. in control samples started to grow only after 14th days of storage and reached values of 10^6 CFU g^{-1} after 45 days while the same population in beef stored in active packaging was uncountable for the first 14th days and displayed a final load of about 10^4 CFU g^{-1}.

2.4 Discussion

The inhibitory spectra of antimicrobial nisin solutions (NAS1 and NAS2) were evaluated against a range of Gram positive and Gram-negative spoilage bacteria, the results are showed in Table 2.2 and 2.3. Both solutions were able to inhibit the Gram positive strains tested and in some cases to inhibit Gram-negative bacteria. The bacteriocins are generally only active against Gram-positive cells (Stevens, K.A. et al., 1991). Coupling nisin and EDTA an improved antimicrobial effect could be obtained (Cutter et al. 2001, Gill and Holley 2002) and, in some cases, also an enhancement of nisin efficacy against Gram negative bacteria (Stevens et al. 1991; Stevens et al. 1992; Delves-Broughton, 1993). EDTA alter the out membrane of the cell by chelating the magnesium ions that stabilize the membrane (Hanckock 1984).

For active antimicrobial packaging to be effective, an adequate procedure of activation is necessary in order to assure that the antimicrobial is linked to the film and to keep the antimicrobial activity during the film shelf life. Moreover, the activated film has to exert its preservative antimicrobial potential during packed food storage.

We succeeded in obtaining two nisin solution to be suitably spread on the internal surface of plastic bags to be used for meat packaging and employed the solution in order to develop an active antimicrobial packaging that we used for the storage of meat at chilled condition. Moreover, the antimicrobial activity of the plastic films tested in agar plates (Mauriello et al. 2004) against spoilage indicator strains (Table 2.1 and 2.2) proved that the NAS1 and NAS2, and their antimicrobial activity, were homogeneously distributed on the surface of the plastic films. By contrast only against *B. thermosphacta* there

was an antimicrobial effect using activated plastic film. A homogeneous distribution of an antimicrobial solution on the surface of a plastic film to be activated is fundamental for its efficacy. In fact, the antimicrobial power is mainly exerted by the contact between active film and food surface as recently demonstrated by an epifluorescence microscopy study (Ercolini et al. 2006a) and a proper distribution can avoid the development of microenvironments of microbial growth. This is the reason why we opted for the vacuum packaging with the nisin activated bags, in order to make sure to have a contact between beef surface and the activated antimicrobial bag. Alternatively, doubtful efficacy could be obtained by adding nisin solutions directly to meat samples with no assurance that the antimicrobial effect would affect the entire surface microbiota. Other nisin activated plastic films have been developed and proved to be useful to inhibit undesired food related bacteria (Cutter et al. 2001; Lee et al. 2003; Franklin et al. 2004; Grower et al. 2004; Mauriello et al. 2005; Neeto et al. 2008).

The use of the active packaging, with NAS-1, clearly showed its antimicrobial power from the beginning. In fact, compared to the non active control, the antimicrobial bags retarded the growth of the populations of LAB, carnobacteria and *B. thermosphacta* for at least two weeks. However, in spite of the overall reduction of the viable counts as result of the antimicrobial storage, the sensory acceptability of the meat stored in active packaging did not differ from the control. This could be due to the release of even little amounts of volatile organic compounds with a low odor treshold (Ercolini et al. 2009) or to other factors that do not depend on the microbial modifications of

meat such as colour change, which caused a darkening of the meat in treated as well as untreated samples as result of the vacuum packaging.

However, in spite of the overall reduction of the viable counts as result of the antimicrobial storage, the sensory acceptability of the meat stored in active packaging did not differ from the control. This could be due to the release of even little amounts of volatile organic compounds with a low odor treshold (Ercolini et al. 2009) or to other factors that do not depend on the microbial modifications of meat such as colour change, which caused a darkening of the meat in treated as well as untreated samples as result of the vacuum packaging. Using NAS2 the main population affected were LAB and carnobacteria, the active packaging retarded the growth at least for 21 days of storage. *B. thermosphacta* and Enterobacteria were uncountable for whole the storage time.

The species diversity within the spoilage microbial groups developing during the storage of meat was also assessed in this study. The PCR-DGGE approach used has been also employed with good results in other studies to assess the spoilage microbiota of beef during storage in modified atmosphere packaging (Ercolini et al. 2006b), the LAB populations developing during the storage of pork under vacuum (Fontana et al. 2006) and the spoilage microbial populations of *morcilla* blood sausages treated with high hydrostatic pressure (Diez et al. 2008).

In this study, the overall outcome arising from PCR-DGGE analysis of DNA extracted from meat and bulk cells from media was that the species diversity occurring with and without the use of the active packaging did not change significantly. Although there was a clear influence in the use of the

antimicrobial packaging on the loads of the spoilage populations during storage, this influence did not affect the species diversity developing during the storage at 1°C. The constant species occurrence not only between treated and control samples, but also within samples during storage, is not in agreement with our recent studies where we showed that the species diversity occurring during MAP storage of beef was extremely and unpredictably variable (Ercolini et al. 2006b). All the microbial species identified in the first two set of experiment, by sequencing proved to belong to *Bacteria* and their closest relatives were usually capable to grow at low temperatures. *Pseudomonas* spp., *Carnobacterium* spp. and, at a later stage *Serratia grimesii,* were shown to be the dominant species within the Gram negative populations. LAB are recognized as causative agents of meat spoilage (Huis in't Veld et al. 1996; Labadie 1999; Gill 2003; Skandamis and Nychas 2005). *Lb. sakei* and *Lb. curvatus* were found to be the main LAB responsible of the spoilage of meat stored under vacuum by using a molecular approach (Fontana et al. 2006). Species of LAB such as *Lactobacillus* spp., *Weissella* spp. and *Leuconostoc mesenteroides* were found during the storage of meat (Table 2.6). Their growth was probably favoured by our specific storage conditions; however, they were replaced by carnobacteria when an active packaging was used showing a higher sensitivity to the antimicrobial solution employed. A DGGE fragment directly extracted from a control meat fingerprint (Table 2.6) at the end of storage proved to be *Photobacterium kishitaniiclade*. Members of this genus are not often isolated from food environments but they have been recently associated to the spoilage of atlantic cod stored in modified atmosphere packaging (Hovda et al. 2007).

In conclusion, the combination of chill temperature and antimicrobial packaging proved to be effective in enhancing the microbiological quality of beef cuts by inhibiting LAB, carnobacteria and *B. thermosphacta* in the early stages of storage and by reducing the loads of *Enterobacteriaceae*. The best antimicrobial activity were showed using NAS-2, the presence of ascorbic acid and citric acid could improve the powerfull of the antimicrobial solution. Development is needed of further improved storage systems to prevent the spoilage, in order to keep the quality of chilled meat for longer storage times.

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2.6 Table

Table 2.1: Antimicrobial activity of NAS-1 and activated plastic films.

Indicator strains	Origin	Growth conditions	AU/ml	Antimicrobial activity of plastic film
Listeria innocua 1770	Milk	TSB[a] 24h at 30°C	3200	-
Pseudomonas fragi 25P	Meat	TSB 24h at 20°C	100	-
Pseudomonas fragi 6P2	Meat	TSB 24h at 20°C	200	-
Carnobacterium sp. 9P	Meat	TSB 24h at 20°C	6400	-
Brochothrix thermosphacta 3R2	Meat	TSB 24h at 20°C	25600	+
Brochothrix thermosphacta 1R2	Meat	TSB 24h at 20°C	102400	+
Brochothrix thermosphacta 7R1	Meat	TSB 24h at 20°C	102400	+
Escherichia coli 32	Meat	TSB 24h at 37°C	200	-
Lactobacillus plantarum 18M	Fruit	MRS[b] 24h at 30°C	6400	-
Hafnia alvei 53M	Meat	TSB 24h at 30°C	< 100	-
Salmonella Kadar	Poultry	TSB 24h at 30°C	< 100	-
Salmonella Thompson MCV1	Poultry	TSB 24h at 30°C	< 100	-
Serratia proteamaculans 42M	Meat	TSB 24h at 30°C	< 100	-
Staphylococcus 3S	Meat	TSB 24h at 30°C	12800	-

[a]TSB = Tryptone soya broth (Oxoid, Milan, Italy) supplemented with 0.5% yeast extract;
[b]MRS = de Man Rogosa Sharp (Oxoid).

Table 2.2: Antimicrobial activity of NAS-2 and activated plastic films.

Indicator strains	Origin	Growth conditions	AU/ml	Antimicrobial activity of plastic film
Listeria innocua 1770	Milk	TSB[a] 24h at 30°C	3200	-
Pseudomonas fragi 25P	Meat	TSB 24h at 20°C	800	-
Pseudomonas fragi 6P2	Meat	TSB 24h at 20°C	6400	-
Carnobacterium sp. 9P	Meat	TSB 24h at 20°C	6400	-
Brochothrix thermosphacta 3R2	Meat	TSB 24h at 20°C	409600	+
Brochothrix thermosphacta 1R2	Meat	TSB 24h at 20°C	409600	+
Brochothrix thermosphacta 7R1	Meat	TSB 24h at 20°C	102400	+
Escherichia coli 32	Meat	TSB 24h at 37°C	100	-
Lactobacillus plantarum 18M	Fruit	MRS[b] 24h at 30°C	6400	-
Hafnia alvei 53M	Meat	TSB 24h at 30°C	100	-
Salmonella Kadar	Poultry	TSB 24h at 30°C	100	-
Salmonella Thompson MCV1	Poultry	TSB 24h at 30°C	100	-
Serratia proteamaculans 42M	Meat	TSB 24h at 30°C	100	-
Staphylococcus 3S	Meat	TSB 24h at 30°C	102400	-

[a]TSB = Tryptone soya broth (Oxoid, Milan, Italy) supplemented with 0.5% yeast extract; [b]MRS = de Man Rogosa Sharp (Oxoid).

Table 2.3 Viable counts of different meat spoilage microbial groups in beef chops during storage at 1°C for 32 days.

Packaging	Storage time (days)	pH ± SD	†Log CFU g^{-1} ± SD				
			Total counts (PCA)	LAB (MRS agar)	*B. thermosphacta* (STAA)	*Carnobacterium* spp. (CTSI agar)	Enterobacteriaceae (VRBGA)
Non active (control)	0	5.78 ± 0.04	3.41 ± 0.01 [a]	2.31 ± 0.05 [a]	2.04 ± 0.01 [a]	<1.00 [a]	2.48 ± 0.06 [a]
	5	5.74 ± 0.01	4.58 ± 0.01 [a]	2.28 ± 0.01 [a]	3.52 ± 0.01 [a]	<1.00 [a]	2.31 ± 1.28 [a]
	11	5.74 ± 0.05	7.00 ± 0.05 [a]	3.00 ± 0.28 [a]	5.27 ± 0.51 [a]	2.48 ± 0.01 [a]	4.18 ± 0.13 [a]
	22	5.83 ± 0.01	7.13 ± 0.03 [a]	4.80 ± 0.13 [a]	5.42 ± 0.03 [a]	3.05 ± 0.01 [a]	7.16 ± 0.00 [a]
	32	5.71 ±0.01	8.54 ± 0.01 [a]	5.97 ± 0.99 [a]	5.31 ± 0.09 [a]	4.45 ± 0.45 [a]	6.10 ± 0.00 [a]
Nisin activated packaging	0*	5.69 ± 0.15	3.74 ± 0.01 [a]	1.78 ± 0.01 [b]	<1.00 [b]	<1.00 [a]	3.08 ± 0.01 [a]
	5	5.68 ± 0.01	3.88 ± 0.05 [b]	1.60 ± 0.18 [b]	<1.00 [b]	<1.00 [a]	3.70 ± 0.07 [b]
	11	5.81 ± 0.01	6.40 ± 1.94 [b]	1.75 ± 0.25 [b]	<1.00 [b]	<1.00 [b]	3.09 ± 0.08 [b]
	22	5.77 ± 0.01	5.06 ± 0.09 [b]	3.23 ± 0.17 [b]	<1.00 [b]	3.04 ± 0.22 [a]	4.79 ± 0.23 [b]
	32	5.60 ± 0.03	6.79 ± 0.39 [b]	2.52 ± 1.15 [b]	2.88 ± 0.07 [b]	2.67 ± 0.01 [b]	4.00 ± 0.18 [b]

*Sample analyzed after 1.5 h of contact with the activated plastic bag.

†Comparing control and activated packaging data, values with different superscripts in the same column and corresponding to the same time of storage, differ significantly (P<0.05).

Table 2.4 – Microbial species identification after sequencing of the variable V6-V8 of the 16S rRNA gene purified from PCR-DGGE profiles of meat samples.

Band[a]	Source[b]	Closest relative	Identity (%)	Closest relative Accession No.
1	Beef (C)	*Pseudomonas* spp.[c]	99	DQ405236
2	Beef (T)	*Carnobacterium spp.*	96	DQ405248
3	Beef (C)	*Carnobacterium divergens*	99	AY543037
4	Beef (C)	*Serratia grimesii*	98	EF491959
5	Beef (C)	*S. proteomaculans*	99	AB334771
6	Beef (C)	*Brochothrix thermosphacta*	97	AY543029
7	Beef (C)	*Photobacterium kishitaniiclade*	98	EF415487
8	Beef (C)	*Staphylococcus xylosus*	98	EU095643

[a] bands from number 1 to 8 are indicated in Figure 1.
[b] C, control sample; T, active packaging treated sample.
[c] when the sequence showed the same homology with more than 4 species of *Pseudomonas*, the result was reported as *Pseudomonas* spp.

Table 2.5 – Microbial species identification after sequencing of the variable V6-V8 of the 16S rRNA gene purified from PCR-DGGE profiles.

Band[a]	Source[b]	Closest relative	Identity (%)	Closest relative Accession No.
1	MRS (C)	*Carnobacterium divergens*	99	AY543037
2	MRS (T)	*Leuconostoc mesenteroides*	98	EF579730
3	MRS (C)	Lactobacillus *spp.*	98	DQ405252
4	MRS (C)	*Weissella* spp.	99	DQ405251
5	MRS (T)	*Staphylococcus* spp.	99	EF061904

[a] bands from number 1 to 5 are indicated in Figure 2.
[b] C, control sample; T, active packaging treated sample.

Table 2.6 – Microbial species identification after sequencing of the variable V6-V8 of the 16S rRNA gene purified from PCR-DGGE profiles.

Band[a]	Source[b]	Closest relative	Identity (%)	Closest relative Accession No.
1	VRBGA (T)	*Pseudomonas* spp.	99	DQ405232
2	VRBGA (C)	*Pseudomonas spp.*	100	DQ405241
3	VRBGA (T)	*Pseudomonas* spp.	99	DQ405236
4	VRBGA (C)	Pseudomonas *spp.*	99	DQ405241
5	VRBGA (T)	*Pseudomonas* spp.	99	DQ405232
6	VRBGA (C)	*Serratia grimesii*	100	EF491959
7	VRBGA (T)	*Rhanella* spp.	99	DQ405247
8	VRBGA (T)	*Rhanella* spp.	99	DQ405247

[a] bands from number 1 to 8 are indicated in Figure 3.
[b] C, control sample; T, active packaging treated sample.
[c] when the sequence showed the same homology with more than 4 species of *Pseudomonas*, the result was reported as *Pseudomonas* spp.

Table 2.7 Viable counts of different meat spoilage microbial groups in beef chops during storage at 1°C for 46 days.

Packaging	Storage time (days)	pH ± SD	†Log CFU cm^{-2} ± SD					
			Total counts (PCA)	*LAB* (MRS agar)	*B. thermosphacta* (STAA)	*Carnobacterium* spp. (CTSI agar)	Enterobacteriaceae (VRBGA)	*Pseudomonas* spp. (CFC)
Non active (control)	0	5,33 ± 0,23	1,87 ± 0,37[a]	<1,00 [a]	<1,00 [a]	<1,00 [a]	<1,00[a]	<1,00[a]
	9	5,45 ± 0,25	5,29 ± 0,70[a]	1,86 ± 0,82[a]	1,21 ± 0,95[a]	4,54 ± 0,63[a]	2,76 ± 0,10[a]	1,98 ± 0,29[a]
	20	5,43 ± 0,15	6,46 ± 0,07[a]	3,94 ± 0,19[a]	4,19 ± 1,25[a]	4,33 ± 0,31	1,58 ± 0,59[a]	3,56 ± 0,34[a]
	36	5,23 ± 0,09	6,73 ± 0,18[a]	4,53 ± 0,11[a]	4,39 ± 0,85[a]	5,85 ± 0,58	4,71 ± 0,37[a]	5,29 ± 0,17[a]
	46	5,58 ± 0,14	6,20 ± 0,10[a]	5,85 ± 0,38[a]	1,50 ± 0,15[a]	5,36 ± 0,13	4,24 ± 0,22[a]	4,86 ± 0,23[a]
Nisin activated packaging	0*	5,26 ± 0,08	1,94 ± 0,26[a]	<1,00[a]	<1,00[a]	<1,00[a]	1,00 ±0,17[a]	1,00 ± 0,23[a]
	9	5,38 ± 0,04	5,23 ± 0,31[a]	<1,00[a]	1,00 ± 1,40[a]	<1,00 ± 1,12[a]	1,58 ± 0,09[b]	2,94 ± 0,66[a]
	20	5,24 ± 0,08	4,90 ± 0,26[b]	1,10 ± 1,08[b]	1,00 ± 0,77[a]	1,98 ± 0,48[b]	3,33 ± 0,56[a]	3,25 ± 0,51[a]
	36	5,26 ± 0,01	6,01 ± 0,12[b]	1,00 ± 1,14[b]	<1,00[a]	2,80 ± 0,19[a]	4,08 ± 0,33[a]	3,55 ± 0,27[b]
	46	5,74 ± 0,03	6,42 ± 0,71[a]	3,67 ± 0,08[b]	1,00 ± 0,68[b]	4,82 ± 0,21[b]	4,54 ± 0,18[b]	4,97 ± 0,26[b]

*Sample analyzed after 1.5 h of contact with the activated plastic bag.
†Comparing control and activated packaging data, values with different superscripts in the same column and corresponding to the same time of storage, differ significantly (P<0.05).

Table 2.8– Microbial species identification after sequencing of the variable V6-V8 of the 16S rRNA gene purified from PCR-DGGE profiles of meat samples.

Band[a]	Source[b]	Closest relative	Identity (%)	Closest relative Accession No.
1	Beef (C)	*Pseudomonas* spp.	100	DQ405241
2	Beef (C)	*Pseudomonas* spp.	100	DQ405241
3	Beef (T)	*Carnobacterium* spp.	98	DQ405248
4	Beef (C)	*Carnobacterium divergens*	86	EU128490
5	Beef (T)	*Carnobacterium divergens*	99	AY543037
6	Beef (C)	*Rahnella aquatilis*	99	FJ811859
7	Beef (C)	Uncultured *Carnobacterium* spp.	98	EU826673
8	Beef (C)	*Carnobacterium* spp.	99	DQ405248

Table 2.9 Viable counts of different meat spoilage microbial groups in beef chops during storage at 4°C for 45 days

Packaging	Storage time (days)	Log CFU g^{-1} ± SD					
		Total counts (PCA)	*B. thermosphacta* (STAA)	LAB (MRS agar)	Enterobacteriaceae (VRBGA)	*Pseudomonas* spp. (CFC)	*Carnobacterium* spp. (CTSI agar)
Non active (control)	0	3,04 ± 0,19	<1,00	<1,00	<1,00	<1,00	1,00 ± 1,04
	7	3,35 ± 0,49	2,27 ± 0,70	1,30 ± 0,00	<1,00	<1,00	<1,00
	14	5,42 ± 0,35	4,00 ± 0,43	3,78 ± 0,34	<1,00	1 ± 0,87	4,70 ± 1,12
	21	6,05 ± 1,48	4,29 ± 0,76	6,88 ± 0,37	1,76 ± 1,08	2,49 ± 0,69	6,36 ± 0,34
	30	6,15 ± 0,11	4,64 ± 0,24	6,69 ± 0,28	2,33 ± 1,25	4,20 ± 1,58	5,84 ± 0,26
	35	7,41 ± 0,02	6,00 ± 0,00	6,57 ± 0,21	3,00 ± 0,00	2,97 ± 0,88	5,77 ± 0,01
	45	7,46 ± 0,14	5,74 ± 0,71	6,65 ± 0,34	1,40 ± 0,43	4,36 ± 0,42	7.07 ± 0,23
Nisin activated packaging	0	3,04 ± 0,19	<1,00	<1,00	<1,00	<1,00	1,00 ± 1,04
	7	2,00 ± 0,00	<1,00	1 ± 0,98	<1,00	<1,00	<1,00
	14	2,00 ± 0,00	<1,00	<1,00	<1,00	<1,00	<1,00
	21	4,06 ± 1,35	<1,00	<1,00	<1,00	1,69 ± 0,12	2,55 ± 0,11
	30	3,99 ± 0,26	<1,00	2,94 ± 1,12	<1,00	2,13 ± 0,49	2,56 ± 0,50
	35	6,13 ± 0,62	<1,00	2,12 ± 0,99	<1,00	2,00± 0,00	<1,00
	45	7,12 ± 0,35	5,02 ± 0,18	4,17 ± 0,46	<1,00	3,09 ± 0,62	3.91 ± 0,12

2.7 Figures

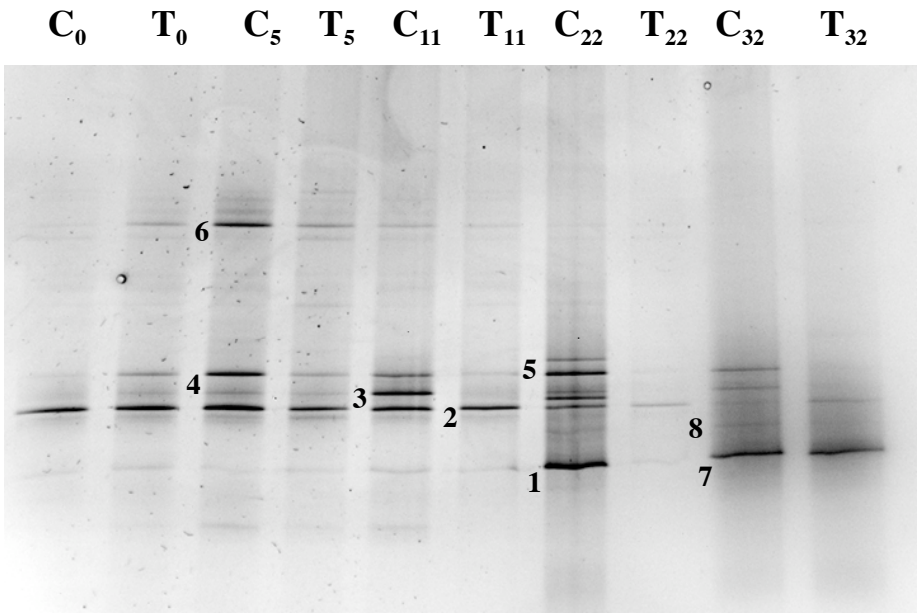

Figure 2.1 - PCR-DGGE profiles of the 16S V6-V8 amplicons from microbial DNA directly extracted from meat samples vacuum-packed in non active (C) and active (T) packaging and stored for 32 days; subscript numbers indicate the days of storage. Numbers placed at the bottom left of each band indicate the sequenced fragments reported in Table 2.4.

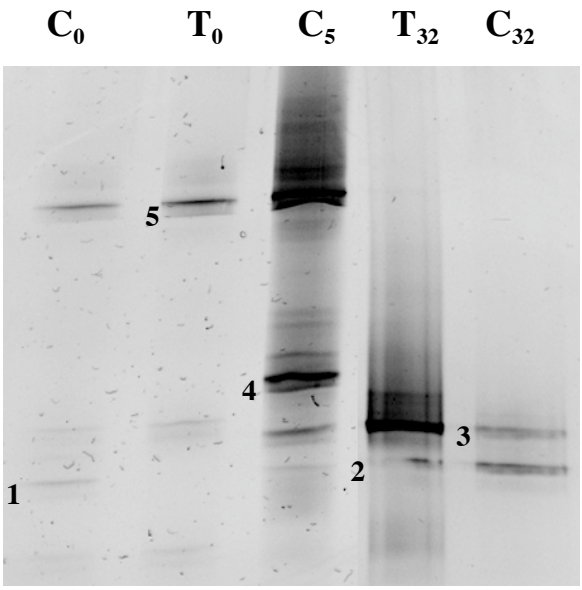

Figure 2.2 – Representative PCR-DGGE profiles of bulk cells of LAB population monitored on MRS Agar and isolated from meat samples vacuum-packed in non active (C) and active (T) packaging and stored for 32 days; subscript numbers indicate the days of storage. Numbers placed at the bottom left of each band indicate the sequenced fragments reported in Table 2.5.

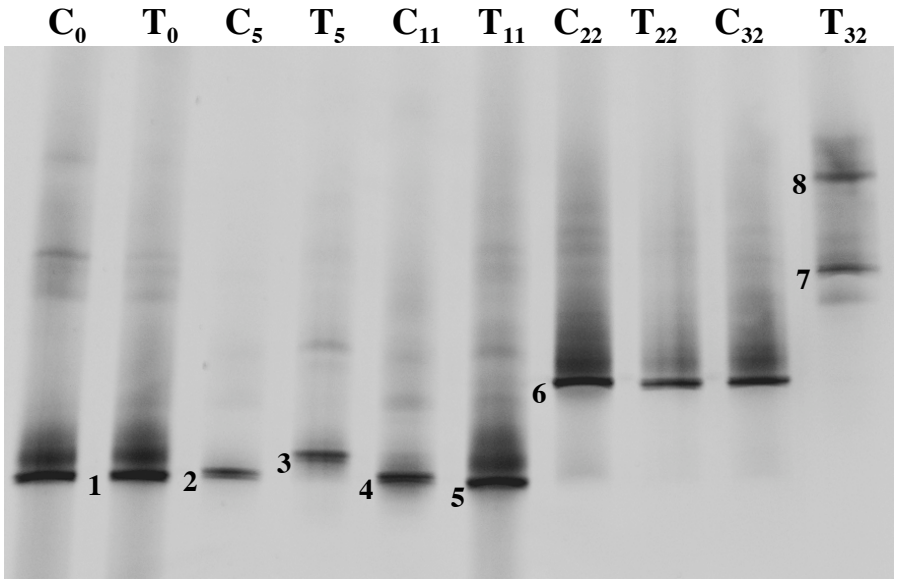

Figure 2.3 Representative PCR-DGGE profiles of bulk cells of *Enterobacteriaceae* population monitored on VRBGA and isolated from meat samples vacuum-packed in non active (C) and active (T) packaging and stored for 32 days; subscript numbers indicate the days of storage. Numbers placed at the bottom left of each band indicate the sequenced fragments reported in Table 2.6.

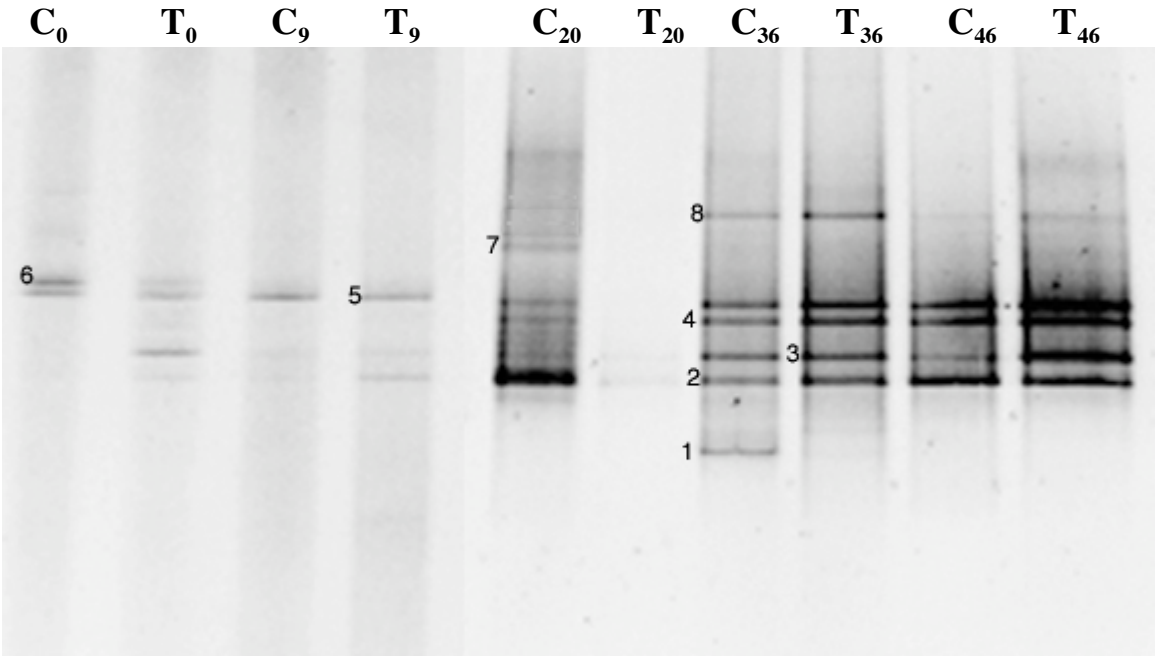

Figure 2.4 - PCR-DGGE profiles of the 16S V6-V8 amplicons from microbial DNA directly extracted from meat samples vacuum-packed in non active (C) and active (T) packaging and stored for 46 days; subscript numbers indicate the days of storage. Numbers placed at the bottom left of each band indicate the sequenced fragments reported in Table 2.8.

Chapter 3
Monitoring the common spoilage microbial populations of slices of beef by using modified atmosphere packaging and nisin activated packaging

3.1 Introduction

Modified atmosphere packaging (MAP) can be an effective method for meat shelf life extension (Brodi et al. 1996; Ercolini et al. 2006b). Substantial fractions of CO_2 are used to retard the growth of organisms responsible of aerobic spoilage, and a certain concentration of O_2 is employed for red meat MAP to preserve meat color (Gill 2003; Jeremiah 2001). This kind of packaging is normally associated with the use of packs made of materials that provide a barrier to the exchange of gases between the pack and the external atmosphere (Gill 2003). In addition, novel technologies of active packaging can inhibit microbial growth in meat products (Ercolini et al. 2006a; Mauriello et al. 2004; Skandamis et al. 2002; Skandamis et al. 2001).

A combination of MAP and active packaging was used for monitoring the common spoilage microbial populations during storage of refrigerated slices of beef.

3.2 Materials and Methods

3.2.1 Procedures for PET and PE antimicrobial films preparation by using NAS-1

NAS-1 (see paragraphs 2.2.1) was used for the activation of two plastic films polyethylene (PE EOEOPO74) and Polyethylentereftalato (PET).

One milliliter of antimicrobial solution was coated manually on one side of the film (10 x 20 cm^2) using a coating rod of 100μm (Lee et al. 2003). The films were then treated with warm air in order to let the solution dry and promoting a

homogenous distribution of the antimicrobial solution onto the surface of the plastic film.

3.2.2 Beef samples and storage conditions

Slices from the same meat muscle (chuck tender beef) about 100g each were singly covered on both faces with the antimicrobial films previously described. A series of samples were packed using activated PE, a series of samples were packed using activated PET, a series of samples without activation of the films and a series of samples without the application of the films that were used as control.

All the sample (3 slices for each condition) were placed in polystyrene trays whose interior was covered with a multilayer barrier film (volume, 750 ml) (CoopBox, Bologna, Italy), and a barrier polyethylene film (PO2 1.3 $cm^3/m^2/24$ h/atm at 23°C, 0% rH) was used as a sealing top. The samples were modified-atmosphere packaged using a packaging machine (TSM, 105 Minipack Torre; Cava dei Tirreni [SA], Italy). The ratio between the volume of gas and weight of food product (G/P ratio) was 3:1 (vol/wt). The samples were packed using 60% O_2 – 40% CO_2 and stored at 4°C. A series of samples for each condition were packed in aerobic conditions and used as a control. Samples for microbial analysis were taken after 0, 1 and 7 days of storage.

The populations monitored were *Enterobacteriaceae, Brochothrix thermosphacta,* LAB, and *Pseudomonas* spp. by using appropriate selective media (see paragraphs 2.2.2.1).

3.2.3 Procedures for HDPE antimicrobial films preparation by NAS-1 and NAS-2

NAS-1 and NAS-2 (see paragraphs 2.2.1) were used for the coating of high density polyethylene (HDPE) using the procedure described in paragraph 3.2.1.

Slices from the same meat muscle (chuck tender beef) about 100g each were singly covered on both faces with the antimicrobial films. Beef slices were packed using activated HDPE with NAS-1, or activated HDPE with NAS-2 or without activation of the films (control).

All the samples (3 slices for each conditions) were placed in polystyrene trays and packed in modified-atmosphere as above described. Samples for each condition were packed in aerobic condition and used as control. Samples for microbial analysis were taken after 0, 1, 7 and 12 days of storage at 4°C.

The populations monitored were: total viable counts at 20°C, *Enterobacteriaceae, Brochothrix thermosphacta,* LAB, and *Pseudomonas* spp.

3.3 Results

3.3.1 Efficacy of PE and PET coated with NAS-1 for storage of beef slices

The results of the viable counts on specific media during the storage of beef steaks in MAP and AIR conditions are shown in Table 3.1.

Brochothrix thermosphacta population in aerobic condition started to increase from 10^2 CFU g^{-1} and displayed a final load of 10^7 CFU g^{-1}, while in the treated samples the final load was 10^3 CFU g^{-1}.

There was no appreciable difference between the microbial loads by using PE or

PET activated with NAS-1 (both showed 4 log cycles reduction compared to the control). Similarly, in MAP there was no difference between PE or PET activated with NAS-1, both causing a 2 log cycles reduction compared to the control.

The viable counts of LAB in aerobic condition showed a reduction of about 3 log cycles compared to the control. The same reduction was registered using both MAP or AIR for the packaging. For *Enterobacteriaceae,* the presence of the antimicrobial films did not affect the final load but using MAP condition the populations showed a reduction of 2 log cycles compared to the control sample in air. For *Pseuodomonas* spp. only the MAP condition reduced the final load of this population. In control samples there was a reduction of 4 log cycles compared to the control.

3.3.1 Efficacy HDPE coated with NAS-2 for the storage of beef slices

The results of the viable counts on specific media during the storage of beef slices in antimicrobial and control plastic films in AIR and MAP conditions are shown in Table 3.2. All the targeted microbial populations showed to be affected by the use of MAP. The total viable counts showed a reduction of 2 log cycles by using MAP. In this condition there was a further reduction of more than 1 log cycle in treated samples compared to the control. The count of *Brochothrix thermosphacta* by using HDPE activated with NAS-2 in AIR, showed a reduction of 2 log cycles compared to the control in the same conditions. By using MAP the load in treated samples was uncountable for the first week of storage and displayed a final load lower than 10^3 CFU g^{-1}. However, in control samples the final load was about 10^5 CFU g^{-1}. In AIR, the load of LAB in control samples increased from 10^2 to about 10^5 CFU g^{-1} during the whole storage time, while in active packaging increased only to 10^4 CFU g^{-1} from the 7^{th} day of storage keeping constant until the end. The LAB population was dramatically reduced by the use of antimicrobial packaging in MAP condition, it was uncountable for the first week and the final load was 10^2 CFU g^{-1} at the end of the storage. Enterobacteria grew in control meat and in treated meat in AIR at the same level and displayed a final load of about 10^3 CFU g^{-1}. Combination of MAP and antimicrobial packaging resulted very efficient, the population was uncountable for all the storage time, while in the control it was 10^2 CFU g^{-1}. In aerobic conditions, *Pseudomonas* spp. reached values of 10^8 CFU g^{-1} after 1 week and were unaffected by the use of the antimicrobial film. In MAP condition the final

load of *Pseudomonas* spp. was 10^1CFU g^1 in control and treated samples.

3.3.1 Efficacy HDPE coated with NAS-1and NAS-2 for storage of beef slices

The results of the viable counts on specific media during the storage of beef slices in antimicrobial and control plastic films are shown in Table 3.3.

The total viable counts seemed to be initially unaffected by the use of the antimicrobial film. The final load in control and in treated samples in AIR and MAP showed a final load of about 10^7 CFU g^1 while in MAP the use of antimicrobial films reduced the final load of 2 log cycles compared to the control. *B. thermosphacta* was uncountable for the whole storage time with the use of antimicrobial film in AIR and MAP. However, in control samples the load reached 10^3 CFU g^{-1} and 10^6 CFU g^{-1} in air and MAP, respectively. The load of LAB in control samples in AIR and MAP increased from 10^2 to about 10^5 CFU g^{-1} during the whole storage time. While the LAB population was reduced in treated samples and display a final load of 3 log cycles lower than the control in MAP. Load of Enterobacteria during the storage time was lower than 10^1 CFU g^{-1} in all the conditions adopted. *Pseudomonas* spp. was affected only by MAP condition there was no difference between control and treated samples. By using MAP the final load showed a reduction of 3 log cycles compared to sample stored in aerobic conditions.

3.4 Discussion

Modified atmosphere packaging (MAP) is recognized as one of the most effective applications for shelf life extension of fresh meat products. Composition of the atmosphere determines to a large degree the extent and type of spoilage that can develop during storage. Efficacy of MAP is based on the antimicrobial activity of CO_2 present in the headspace of meat packages. The inhibitory effects of CO_2 have been attributed to alteration of the bacterial cells permeability, pH changes and enzymatic inhibition (King and Nagel, 1967). The inhibitory efficiency of CO_2 is increased at lower temperature, because of the solubility of the gases increase with decreasing temperature. This condition could inhibit aerobic Gram-negative bacteria such as *Pseudomonas* spp., *Enterobacteriaceae*, *Acinetobacter* spp. and *Moraxella* spp. (Church, 1994) and allow the growth of Gram-positive bacteria such as LAB and *B. thermosphacta* (Koutsoumanis et al. 2006). The results of the viable counts confirmed that *Pseudomonas* spp. and *Enterobacteriaceae* where inhibited only by using MAP. Presence of high level of CO_2 (40%) exert a bactericidal effect on the growth of both populations by a reduction of around 3 log cycle compared to the control in aerobic conditions. LAB populations in all experiments were not affected by using MAP, the results showed the same trends in control (air) and in MAP. *B. thermosphacta* in each condition showed a reduction of 3 log cycle by using MAP in all the cases studied. The use of antimicrobial films activated with NAS-1 and NAS-2 showed an antimicrobial power from the beginning by reduced the load of *B. thermosphacta* and LAB populations by approximately 2 to 3 logs in all the

conditions adopted. By using different antimicrobial conditions the best action was obtained by using HDPE activated with NAS-2. This solution allowed preserving meat color. Discoloration of beef is caused from oxidation state of myoglobin, controlling oxygen in MAP condition by controlling oxidation state of myoglobin can also preserve meat colour. Metmyoglobin is the oxidized pigment state of myoglobin, the dominant sarcoplasmatic pigment in muscle, and the Fe^{2+} results in a brown or grey meat color (McMillin, 2008). Metmyoglobin forms when pigments are exposed for long time to light, heat, $O_{2,}$ microbial growth or freezing. Ascorbic acid present in NAS-2 is known to preserve red meat colour and possess antioxidant properties (Lund et al. 2007), thus the addition of a chelator such as citric acid, could improve ascorbic acids's efficacy. (Mancini et al. 2007). Antimicrobial packaging is an extremely challenging technology that could extend shelf-life and improve food safety in meat products.

Combination of this application with MAP can provide improved methods of beef storage at retail allowing a prolonged shelf life of the products while preserving their quality.

3.5 References

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3.6 Tables

Table 3.1 Viable counts of different meat spoilage microbial groups in beef slices during storage at 4°C for 7 days in MAP and AIR condition

Packaging	Storage time (days)	Log CFU g^{-1} ± SD							
		B. thermosphacta (STAA)		*LAB* (MRS agar)		Enterobacteriaceae (VRBGA)		*Pseudomonas spp.* (CFC)	
		AIR	MAP	AIR	MAP	AIR	MAP	AIR	MAP
C	0	1,57 ± 0,56	1,57 ± 0,56	1± 0,85	1± 0,85	<1,00	<1,00	<1,00	<1,00
	1	2,52 ± 0,19	1,63 ± 0,31	<1,00	1,00 ± 0,75	<1,00	<1,00	1,63 ± 0,59	0,43 ± 0,75
	7	7,85 ± 0,13	5,03 ± 0,02	5,74 ± 0,03	4,07 ± 0,93	4,48 ± 0,43	2,00 ± 0,00	7,27 ± 0,25	3,00 ± 0,00
PE C	1	<1,00	1 ± 0,75	<1,00	1,26 ± 1,13	<1,00	<1,00	0,93 ± 0,81	1 ± 0,58
	7	5,80 ± 0,66	3,87 ± 0,89	4,92 ± 0,80	4,46 ± 0,67	3,78 ± 1,56	2,00 ± 0,00	7,11 ± 0,72	4,24 ± 2,15
PE T	1	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00
	7	3,00± 0,00	3,00± 0,00	3,00± 0,00	2,00± 0,00	2,79 ± 1,37	2,46 ± 0,80	6,69 ± 0,44	4,00 ± 1,73
PET C	1	0,33 ± 0,85	1 ± 0,81	1,14 ± 1,02	<1,00	<1,00	<1,00	<1,00	1,00
	7	4,46± 2,06	4,14± 1,32	5,16± 0,12	5,28± 0,04	2,51 ± 0,88	2,00 ± 0,00	4,24 ± 2,15	3,00 ± 0,00
PET T	1	<1,00	<1,00	<1,00	1 ± 0,58	<1,00	<1,00	1,14 ± 1,02	<1,00
	7	3,00± 0,00	3,00± 0,00	2,00± 0,00	2,00± 0,00	3,05± 1,81	2,00± 0,00	5,16± 1,23	3,00± 0,00

C: beef slices control
PE C : beef slices stored in polyethylene plastic films (PE EOEOPO74) without treatment
PE T: beef slices stored in polyethylene plastic films (PE EOEOPO74) activated by using NAS-1
PET C: beef slices stored in polyethylentereftalato plastic films without treatment
PET T: beef slices stored in polyethylentereftalato plastic films activated by using NAS-1

Table 3.2 Viable counts of different meat spoilage microbial groups in beef slices during storage at 4°C for 12 days in MAP and AIR condition

Packaging	Storage time (days)	Log CFU g^{-1} ± SD									
		Total counts (PCA)		*B. thermosphacta* (STAA)		LAB (MRS agar)		Enterobacteriaceae (VRBGA)		*Pseudomonas* spp. (CFC)	
		AIR	MAP	AIR	MAP	AIR	MAP	AIR	MAP	AIR	MAP
C	0	3,59 ± 0,27	3,59 ± 0,27	2,15 ± 0,23	2,15 ± 0,23	2,70± 0,35	2,70± 0,35	1,00 ± 0,58	1,00 ± 0,58	1,17 ± 1,05	1,17 ± 1,05
	1	2,26 ± 0,12	2,19 ± 0,17	<1,00	<1,00	2,20 ± 0,33	1,00 ± 1,17	<1,00	<1,00	1 ± 0,81	<1,00
	7	7,35± 0,19	3,27 ± 0,47	6,93 ± 0,38	4,02 ± 0,49	5,15 ± 0,40	4,14 ± 0,35	3,35 ± 0,51	<1,00	7,11 ± 0,03	1,00 ± 0,75
	12	8,92± 0,65	5,89± 0,75	7,75 ± 0,10	4,51 ± 0,88	5,15 ± 0,39	5,57 ± 0,67	3,67 ± 0,91	2,22 ± 0,19	8,88 ± 0,62	1 ± 0,34
HDPE	1	<1,00	2,23 ± 0,32	1± 0,75	1± 0,85	<1,00	1,00 ± 0,79	1,00 ± 0,75	1,00 ± 0,58	<1,00	1,00 ± 0,30
	7	7,37 ± 0,05	3,00 ± 0,00	6,81 ± 0,41	3,10 ± 0,17	4,42 ± 0,37	2,92 ± 0,81	2,38 ± 0,25	<1,00	6,66 ± 0,97	<1,00
	12	8,33 ± 0,93	5,96 ± 0,14	7,44 ± 0,41	5,99 ± 0,09	5,86 ± 0,16	4,34 ± 0,41	4,28 ± 0,49	1 ± 0,16	7,77 ± 0,67	4,28 ± 0,49
HDPE T	1	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00	1,00 ± 0,58	<1,00
	7	7,14 ± 1,38	3,00 ± 0,00	4,498 ± 0,88	<1,00	4,26 ± 0,25	<1,00	3,18 ± 1,68	<1,00	6,92 ± 0,52	<1,00
	12	6,53 ± 0,92	4,36 ± 0,30	3,43 ± 1,25	2,37 ± 0,64	4,26 ± 0,24	2,32 ± 0,24	3,43 ± 0,38	<1,00	7,19 ± 0,80	1 ± 0,50

C: beef slices control
HDPE : beef slices stored in HDPE without treatment
HDPE T: beef slices stored in HDPE activated by using NAS-2

Table 3.3 Viable counts of different meat spoilage microbial groups in beef slices during storage at 4°C for 12 days in MAP and AIR condition

Packaging	Storage time (days)	Log CFU g^{-1} ± SD									
		Total counts (PCA)		*B. thermosphacta* (STAA)		LAB (MRS agar)		Enterobacteriaceae (VRBGA)		*Pseudomonas* spp. (CFC)	
		AIR	MAP	AIR	MAP	AIR	MAP	AIR	MAP	AIR	MAP
HDPE	0	3,10 ± 0,45	3,10 ± 0,45	1,97 ± 0,35	1,97 ± 0,35	2,84± 0,42	2,84± 0,42	1,00 ± 0,85	1,00 ± 0,85	1,92 ± 0,56	1,92 ± 0,56
	1	2,79 ± 0,13	2,29 ± 0,57	<1,00	<1,00	2,48 ± 0,42	1,91 ± 0,86	<1,00	<1,00	1,40 ± 0,17	<1,00
	7	6,67± 0,55	6,37± 1,14	4,44 ± 1,60	3,73 ± 0,57	5,09 ± 0,58	5,23 ± 0,61	1 ± 0,51	<1,00	4,37 ± 0,81	2,25 ± 0,56
	12	7,04± 0,31	7,05± 0,13	3,33 ± 0,58	6,04 ± 0,51	5,01 ± 0,49	5,21 ± 0,16	1,60 ± 0,91	1,00 ± 0,91	6,60 ± 0,52	3,75 ± 1,39
HDPE 1	1	1,03 ± 0,89	1,00 ± 0,75	<1,00	<1,00	1,00 ± 0,85	1,00 ± 0,85	<1,00	<1,00	0,78 ± 1,35	<1,00
	7	5,43 ± 0,51	1,92 ± 0,41	1,37 ± 0,41	<1,00	3,94 ± 0,24	3,00 ± 0,00	1,00 ± 0,25	<1,00	5,55 ± 0,52	1,00 ± 0,90
	12	7,34 ± 0,93	5,20 ± 0,38	<1,00	<1,00	4,16 ± 1,02	2,39 ± 0,30	<1,00	1,00 ± 0,50	8,27 ± 0,93	2,44 ± 0,74
HDPE 2	1	<1,00	1,23 ± 0,40	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00	<1,00
	7	5,53 ± 0,21	3,75 ± 0,49	1,53 ± 0,88	<1,00	4,51 ± 0,25	3,20 ± 0,0,35	1,00 ± 0,68	<1,00	5,66 ± 0,22	1,00 ± 0,58
	12	8,22± 0,63	6,31± 0,22	<1,00	<1,00	4,14 ± 0,15	2,33 ± 0,16	<1,00	1,00 ± 0,68	7,44 ± 0,47	4,35 ± 0,39

HDPE : beef slices stored in HDPE without treatment
HDPE 1: beef slices stored in HDPE activated by using NAS-2
HDPE 2 beef slices stored in HDPE activated by using NAS-1

Chapter 4
Quorum sensing in *Pseudomonas fragi*

4.1 introduction

Bacteria are capable of modulating gene expression or other cellular functions in response to a variety of extracellular signals called autoinducers to regulate their behaviour according to population density. Several categories of signal molecules are involved in Quorum Sensing (QS) (Ammor et al. 2008; Gobetti et al. 2007; Shauder et al. 2001). N-acyl homoserine lactones (AHLs), generically called autoinducer-1 (AI-1) and furanosyl borate diesters, known as Autoinducer-2 (AI-2). *Pseudomonas* spp. are found in many ecosystems. Some species are plant (*P. syringae*) and human (*P. aeruginosa*) opportunistic pathogens, whereas others (*P. fluorescens, P. putida, P. chlororaphis*) promote plant growth and act as antagonists of plant pathogens through their ability to colonize the rhizosphere. In all the above species, quorum sensing (QS) has been found to regulate their activities in natural habitats.

The spoilage of meat during aerobic storage at chill temperature is associated with the presence of *Pseudomonas* spp., particularly *P. fluorescens, P. putida and P. fragi* (Arnault-Rollier et al. 1999, Ercolini et al. 2006; Kraft, 1992; Steidle et al. 2002). *P. fragi* is associated with the spoilage of several foods, but it has been recognized that meat may be its ecological niche for several reasons, including its need for iron and its unique system for release of proteolytic enzymes (Labadie, 1999). The aim of this study was to investigate the production of signaling molecules by *P. fragi* strains isolated from meat.

4.2 Materials and Methods

4.2.1 Bacterial strains.

Bacterial strains used in this study, their functions, and their antibiotic resistance markers are listed in Table 4.1. The *Pseudomonas fragi* strains isolated from fresh and spoiled meat were identified by multiplex PCR targeting the carA gene (Ercolini et al. 2007) and were shown to belong to different RAPD-PCR biotypes (unpublished data). Luria-Bertani (LB, Difco) broth or agar (supplemented with antibiotics when appropriate) was used for growth and maintenance of all strains unless otherwise stated. The cultures were ensured adequate aeration by shaking when needed. Antibiotics were used in the following concentrations when necessary: 100 µg/ml ampicillin (Ap), 60 µg/ml kanamycin (Km), 10 µg/ml tetracycline (Tc) and 50 µg/ml spectinomycin (Sp). Brain heart infusion broth (BHI, Difco) and skim milk were used as growth media in assessing the AHL production of *P. fragi*. ABT medium was used in bioassays with *A. tumefaciens* (Ravn et al. 2001), while ABM medium and LM medium were used when *V. harveyi* was employed as a biosensor (Hwang et al. 1994).

All pseudomonads were grown at 28°C, the biosensor strains *A. tumefaciens* A136, NTL4-2, *V. harveyi* BB170, *V. harveyi* BB152, *Chromobacterium violaceum* CV026 and *C. violaceum* ATCC31532 were grown at 30°C. *E. coli* JM109 (pSB401), *E. coli* JM109 (pSB536), *E. coli* JM109 (pSB1075) and *E. coli* DH5α were grown at 37°C.

4.2.2 AHL standards and other chemicals.

Appropriate AHL standards were used in all the AHL and biofilm formation assays as positive controls. N-butyryl-homoserine lactone (C4-HSL), N-hexanoyl-homoserine lactone (C6-HSL), N-octanoyl-homoserine lactone (C8-HSL), N-decanoyl-homoserine lactone (C10-HSL), N-dodecanoyl-homoserine lactone (C12-HSL), N-tetradecanoyl-homoserine lactone (C14-HSL), N-(beta-ketocaproyl)-homoserine lactone (3OC6-HSL) and 3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL) were purchased from Sigma-Aldrich Canada (Oakville, Ontario). Stock solutions of AHL standards were prepared in 5 mM acetonitrile and stored at – 20ºC. X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was dissolved in N, N-dimethylformamide to a concentration of 20 mg/ml, and stored at –20ºC, protected from light. The working solution used for detection of ß-galactosidase reporter gene activity was 45 µg/ml.

4.2.3 AI-1 production by P. fragi using spent culture supernatants.

All pseudomonads were grown in LB broth, incubated at 28 ºC with shaking until early stationary phase (about 17 h). The cultures were centrifuged (8,000 x g, 10 min, 4ºC). The supernatants were filter sterilized through a Millipore 0.22 µm filter and stored at –20 ºC for the following bioassay.

A Petri dish diffusion assay was carried out (Cha et al. 1998; Mclean et al. 1997) using *A. tumefaciens* A136 or *C. violaceum* CV026, grown in ABT medium or LB broth, respectively. Overnight cultures of A136 or CV026 were inoculated into fresh ABT medium or LB broth containing 0.7 % agar maintained at 45 ºC. For each Petri dish, 5 ml of the mixed culture were

overlaid onto 15 ml preset 1.5% agar (supplemented with 45 µg/ml X-gal if using A. tumefaciens A136). Wells were punched on the solidified overlaid agar using a sterile, glass Pasteur pipette (6mm diameter end). Samples of *P. fragi* supernatants (50 µl) were placed in the wells; the plates were incubated at 30 ºC for at least 24 h and subsequently examined for the induction of the biosensor. The induction diameters (mm) seen as either a blue circle due to induced ß-galactosidase activity or purple circle due to induced violacein formation were measured. All assays were done in duplicate. Positive and negative AHL producing strains (listed in Table 4.1), or appropriate AHL standards, were used to verify the assay using the same conditions described above.

4.2.4 AI-1 production by P. fragi using concentrated supernatants

The strains of *P. fragi* were grown in 250mL of three different media: LB, BHI and skim milk. Each early stationary phase broth culture of *P. fragi* was centrifuged (8,000 x g, 10 min, 4ºC). The supernatants were filter sterilized through a Millipore 0.22 µm filter and extracted twice in a separating funnel using an equal volume of ethyl acetate (containing 0.01% glacial acetic acid) each time. The extracts were combined and dried over anhydrous magnesium sulfate, then filtered through a Whatman No. 114 filter paper (Whatman Inc., Florham Park, NJ). The filtered extracts were evaporated overnight at room temperature to dryness. Residues were dissolved in 500µl of acetonitrile, and the appropriate amount was used for the bioassay previously described.

HPLC separations of extract were performed on an Alliance HPLC system (2690 separation module, Waters Canada, Mississauga, ON) using a VYDAC

C18 reverse phase column (5µm particle size, 4.6 × 250mm dimension, Mandel Scientific, Guelph, ON). The samples were eluted with a mobile phase of acetonitrile:water at a flow rate of 1ml min $^{-1}$ and monitored at 210nm. The following gradient of acetonitrile was used: 0-50% over 5 min, 50-100% over 11 min, and 100-50% over 4 min. A mixture of AHL standards, all at a concentration of 5mM, was run to compare the retention times.

4.2.5 Luminescence-based broth assays.

Luminescence-based broth assays for AI-1 production using *E. coli* JM109 (pSB536), *E. coli* JM109 (pSB401) and *E. coli* JM109 (pSB1075) were based on the method described by Winson et al. 1998. The bioluminescence assay for AI-2 detection was performed using *V. harveyi* BB170 as a biosensor strain (Surette et al. 1998). Briefly, 100 µl of sample (spent culture supernatants or concentrated supernatants from all the growth media described) were mixed with 100 µl (1:10 dilution in LB broth of an overnight culture in LB broth) of the *E. coli* biosensor strains in white 96 well microtitre plates with clear bottoms (Corning Costar); the plate was incubated at 37 ºC for 7 h. For the AI-2 production assay, 20 µl of sample (spent culture supernatants) to be tested were mixed with 180 µl (1:500 dilution in AB medium of an overnight culture in AB medium) of the *V. harveyi* BB170 biosensor strain (total volume of dilution: 0.2 ml) in white 96 well microtitre plates with clear bottoms (Corning Costar); the plate was incubated at 30 ºC for 5 h (25). Duplicates of each dilution were incubated in the same conditions (30 ºC for 5 h), then spread onto solid LM medium and incubated at 30°C for 16 h. Luminescence and turbidity (optical density at 450 nm) of the cultures

were measured using a Victor 1420 Multilabel Counter (Wallac, Turku, Finland). Induced bioluminescence was given in relative light units (RLUs) according to Malott et al. 2002 for each aliquot as follows:

$$RLU = \frac{lcs \times 1s \times 0.2ml}{CFU/ml}$$

Where: lcs is light count per second minus background

1s is the reading time

0,2 ml is the sample volume

Results in cpm cell-1 were averaged and the standard deviation of three independent sample measurements was calculated.

All assays were done in triplicate. AHL producing and non producing strains (listed in Table 1) or appropriate AHL standards were used to verify the AI-1 assay, while *V. harveyi* BB152 and *E. coli* DH5α served as positive and negative controls, respectively, to verify the AI-2 assay.

4.2.6 Biofilm formation assay

All pseudomonads were grown in 10 ml of BHI at 28°C. Overnight cultures were diluited 1:10 and 200 µl were transferred into eight PVC microtitre plates that were covered and incubated at 28°C for 48 h. The biofilm formation was assayed by using crystal violet staining method according to Djordjevic et al. 2002. After the incubation, the medium was removed and the wells were washed five times with sterile distilled water

Plates were air dried for 45 min and each well was stained with 150 µl of 1% crystal violet solution in water for 45 min. After staining, plates were washed with sterile distilled water for five times. The quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol to destain the

wells. An aliquot (100 µl) from each well was transferred to a new microtitre plate and the level (OD) of the crystal violet present in the destaining solution was measured at 595 nm. The assays were performed in triplicate. In order to investigate the possible enhancement of biofilm formation by AHLs, a parallel biofilm formation assay was performed following addition of AHL standards. All the cultures after dilution were mixed with an AHL standard mixture each at a concentration of 0.5 mM and 200 µl were transferred into eight PVC microtitre plates for the biofilm assay.

4.3 Results and Discussion

The frequent occurrence of *Pseudomonas* spp. in fresh and spoiled meat could be due to enhanced gene expression regulated by QS systems (Jay et al., 2003). The production of signal molecules could partly explain the advantage of this genus over several other spoilage-associated bacteria in spoiling meat.

AHL signaling molecules were detected in food extract during fresh meat storage when pseudomonads were present at populations of approximately 10^8-10^9 CFU/g and *Enterobacteriaceae* numbers were 10^3-10^4 CFU/g (Ammor et al.,2008). Moreover, AHL production has been detected concomitantly with and associated to proteolytic activity in milk (Liu et al., 2006). In addition, it has been suggested that *Pseudomonas* spp. use a QS system to produce slime at the surface of aerobically packed meat (Bruhn et al., 2004). Several *Pseudomonas* spp. produce different AHL signal molecules. In *P. aeruginosa* N-(3-oxododecanoyl) homoserine lactone, N-butyrylhomoserine lactone and N-(butanoyl)-L-homoserine lactone are involved in production of virulence

factors (Medina-Martinez et al., 2006; Venturi, 2006; Pearson et al. 1994). In *P. syringae*, synthesis of 3-oxo-hexanoyl-homoserine lactone is involved in cell survival (Quinones et al. 2004). *P. putida* uses N-(3-oxododecanoyl) homoserine and N-(3-oxodecanoyl) homoserine lactone to coordinate expression of phenotypic traits (Smith et al. 2004) while *P. fluorescens* produce N-(butanoyl)-L-homoserine lactone and N-(3-oxoectanoyl) homoserine lactone to partly regulate protease gene expression (Liu et al. 2007).

Our screening for AHL production revealed that none of the 72 *P. fragi* strains was able to produce AI-1 signal molecules using any of the biosensor strains. The same result was obtained even using the concentrated supernatant from different media. The chromatograms from HPLC analysis showed that none of the peaks found in the supernatant had the same retention time as the standard solution in all the samples (data not shown). Therefore, the *P. fragi* population were either unable to produce AHL or the production could be inhibited in the culture conditions used. Another possible explanation could be the degradation of AHLs after their production (Tryfinopoulou et al. 2002). It has recently been reported that *Rhodopseudomonas palustris* produces aryl homoserine lactones, which do not trigger the AI-1 biosensor strains (Schaefer et al. 2008). The presence of this class of signalling molecules in *Pseudomonas* spp. would also explain the results reported in this study. The genetic absence of the genes involved in the AHL production was not investigated. In fact, there is a lack of available sequence data for *P. fragi* that does not allow reliable primer design and PCR assay to verify the presence of AHL production associated genes. On the other hand, all the culture

supernatants from *P. fragi* were able to induce bioluminescence in *V. harveyi* BB170. The AI-2 detection in supernatants of a representative number of *P. fragi* strains is reported in Figure 4.1. Quantification of AI-2 was not possible because there was no linear relationship between induction value and AI-2 concentrations (Vilchez et al. 2006). The production of AI-2 molecules is generally linked to the expression of the luxS gene (Rezzonico and Duffy, 2008); however none of the *Pseudomonas* spp. studied so far has been shown to harbor a *luxS* gene (Duan et al. 2003). An alternative AI-2 production mechanism can involve ribulose-5-phosphate derived from several sugar phosphates by enzymatic conversion as a direct AI-2 precursor (Swift et al. 1997). In aqueous solution, this reactive molecule forms (S)-4,5-dihidroxy-2, 3-pentanedione (DPD) that can be spontaneously cyclized to form a range of furanosyl borate diester derivates by the addition of borate (Tavender et al. 2008).

P. fragi is known to produce biofilm (Jayaraman et al., 1998), and biofilm production is an important characteristic for a potential spoilage organism in meat and meat products. It was hypothesized that *Pseudomonas* spp. possess the capacity to form biofilms in fresh and spoiled meats and that QS is involved in the overall biofilm forming and functioning processes (Jay et al., 2003). In this study we evaluated the possible influence of exogenous AHL molecules on biofilm formation by strains of *P. fragi*. The results showed no significant difference between control and AHL-exposed strains, indicating that the presence of an AHL mixture did not enhance their capacity to form biofilm. Other food spoilage bacteria such as species of *Enterobacteriaceae*, are known to be able to produce significant amounts of AHL in meat during

refrigerated storage (Gram et al., 1999). Based on our evidence, the *P. fragi* strains will not be able to use those exogenous lactones produced by other spoilers to increase their ability to produce biofilm.

The link between food spoilage and QS is controversial. In some cases it was reported that QS can play an important role in the spoilage of food (Ammor et al. Jay et al. 2003; Shaw et al. 1997). On the other hand, the occurrence of AHL molecules from enterobacteria in spoiled meat was not considered as essential to spoilage development (Brohn et al. 2004; Gram et al. 1999). In the latter studies, a few unidentified species of *Pseudomonas* were found not to produce AHLs and it was concluded that they were not actively involved in QS mechanisms. However, other studies showed that biochemical activities related to spoilage such as proteolytic activity are controlled by an AHL mediated QS system in *Pseudomonas* fluorescens (Liu et al. 2006).

In conclusion, the efficient development of *P. fragi* in fresh meat is not apparently regulated by an AHL mediated QS system. The mechanism of AI-2 production and its possible role in spoilage dynamics remains to be further assessed.

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4.6 table

Table 4.1 List of strains

Strains	Description	Source
P. fragi (72 in total)	screening for AI-1 and AI-2 production; different RAPD-PCR profiles	Unpublished
A. tumefaciens A136	AI-1 sensor strain; contains traRG'::lacZ , ß-galactosidase reporter, SpR TcR; cognate signal: 3OC8-HSL	CRIFS[a] (McClean et al. 1997)
A. tumefaciens NT-2 (NTL4)	Bioassay negative control; negative AI-1 producer	CRIFS[a] (Shauder et al. 2001)
C. violaceum CV026	AI-1 sensor strain; mini Tn-5 mutant of CV31532, violacein reporter, KmR; cognate signal: C6-HSL	CRIFS[a] (McClean et al. 1997)
C. violaceum ATCC 31532	Bioassay positive control; positive AI-1 producer (C6-HSL)	CRIFS[a] (McClean et al. 1997)
E. coli JM109 (pSB536)	AI-1 sensor strain; contains rhlRI'::luxCDABE , bioluminescent reporter, ApR; cognate signal: C4-HSL	CRIFS[a] (Surette et al. 1999)
E. coli JM109 (pSB401)	AI-1 sensor strain; contains luxRI'::luxCDABE, bioluminescent reporter, TcR; cognate signal: 3OC6-HSL	CRIFS[a] (Surette et al. 1999)
E. coli JM109(pSB1075)	AI-1 sensor strain; contains lasRI'::luxCDABE, bioluminescent reporter, ApR; cognate signal: 3OC12-HSL	CRIFS[a] (Surette et al. 1999)
P. aeruginosa PAO1	Bioassay positive control; positive AI-1 producer (C4-HSL; 3OC12-HSL)	CRIFS[a] (Holloway et al. 1979)
Vibrio harveyi BB170	AI-2 sensor strain; contains luxS, bioluminescent reporter	CRIFS[a] (Surette et al. 1998)
Vibrio harveyi BB152	Bioassay positive control; positive AI-2 producer	CRIFS[a] (Surette et al. 1998)
E.coli DH5α	Bioassay negative control; negative AI-2 producer	CRIFS[a] (Surette et al. 1998)

[a]CRIFS, Canadian Research Institute for Food Safety

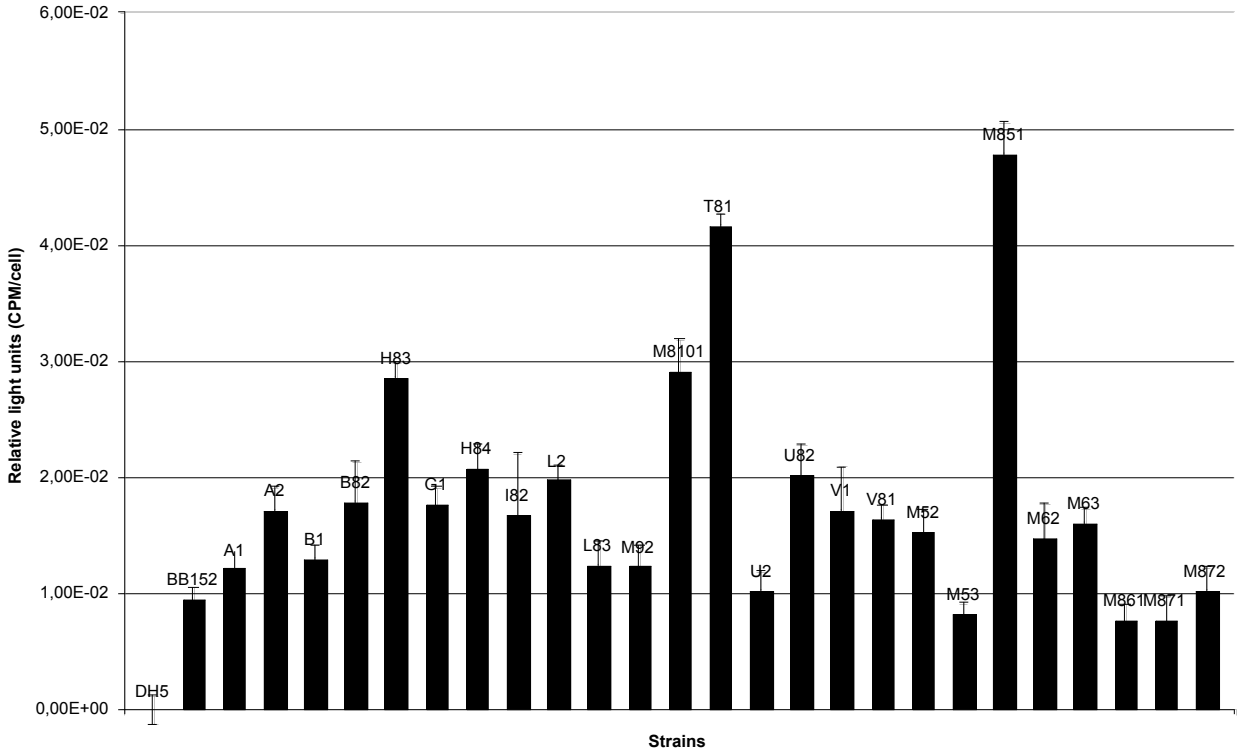

Figure 4.1 Bioluminescence produced by *V. harveyi* BB170 in response to the addition of culture supernatants from representative *P. fragi* strains. *V. harveyi* BB152 and *E. coli* DH5α served as positive and negative controls, respectively to verify the AI-2 assay. RLU were calculated according to Malott et al. 2002. Error bars indicate standard deviation of three independent sample measurements.